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Original Article

Peginterferon-alpha-2b plus ribavirin therapy in patients with chronic hepatitis C as assessed by a multi-institutional questionnaire in Japan

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Background and aim: There has so far been no questionnaire report on patients who were treated with peginterferon plus ribavirin (PEG IFN+RBV) therapy. The purpose of this study was to investigate the problems of this therapy by a questionnaire survey.

Patients and methods: A survey of 681 patients with chronic hepatitis C who received treatment with PEG IFN+RBV was conducted in the Kyushu region of Japan. Using an original questionnaire, the survey was conducted prior to the treatment, during the third month of treatment, at the completion of treatment or the discontinuation of treatment, and at 6 months after the completion of treatment.

Results: It was indicated that the patients had a high level of comprehension and understanding of chronic hepatitis C and PEG IFN+RBV treatment. However, the results also indicated that patients had a high level of anxiety. Side effects were adequately dealt with by physicians. However, dermatological symptoms were not adequately explained to the patients, although they were the second most severe side-effect. It was also revealed that side-effects were most distressing during the first and second months after the start of treatment.

Conclusion: The questionnaire survey provided new information that has never been reported. It is believed that understanding this information is important for future treatment.

Key words: hepatitis C virus, peginterferon, questionnaire, ribavirin

INTRODUCTION

INTERFERON THERAPY FOR chronic hepatitis C has greatly advanced in recent years, and combination therapy using peginterferon and ribavirin (PEG-IFN+RBV) has become a standard treatment method.[1–6] In Japan, PEG-IFN+RBV was approved in December 2004. However, there are many problems, such as side-effects, costs of treatment, and duration of the treatment. It is not known whether these problems are adequately being explained and managed by health care providers.

In order to clarify this issue, it is believed that information from patients is important, and understanding these problems will be beneficial for future treatment. Although there have been reports on patients with hepatitis C and their quality of life (QOL) while undergoing IFN treatment,[7–13] there have been no reports in which information regarding the side-effects and state of mind of patients that have been collected from the patients. Therefore, we conducted a questionnaire survey of patients who have been treated with PEG-IFN+RBV.

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PATIENTS AND METHODS

THIS STUDY WAS carried out from January 2005 to June 2008. Six-hundred eighty-one patients with a compensated chronic HCV genotype 1 infection who started PEG-IFN+RBV treatment at 63 hospitals in the Kyushu region were included. The eligible patients tested positive for HCV-RNA by a quantitative reverse-transcription polymerase chain reaction (PCR; Amplicor Monitor HCV vs. 2.0 using the 10-fold dilution method; Roche Diagnostics, Tokyo, Japan; lower limit of detection 5 KIU/mL) with a concentration >100 KIU/mL. Patients with an HCV genotype other than 1 infection, hepatitis B surface antigen, autoimmune hepatitis, primary biliary cirrhosis, sclerosing cholangitis, or decompensated cirrhosis were excluded. Patients with platelet counts of $8 \times 10^4/mm^3$ or less, leukocyte counts of $2500/mm^3$ or less, or hemoglobin levels of 12 g/dL or less were also excluded from the study. An HCV subtype was classified by either the method of Okamoto *et al.*[14] or that of Tanaka *et al.*[15] Genotypes 1a and 1b corresponded to serological group 1 according to the classification of Simmonds *et al.*[16] Chronic hepatitis was diagnosed based on the histological scoring system of Desmet *et al.*[17]

All patients received both 1.5 μg/kg/week s.c peginterferon α-2b (Pegintron; Schering-Plough, Kenilworth, NJ, USA) and oral ribavirin (Rebetol; Schering-Plough) at 600 mg/day (body weight <60 kg), 800 mg/day (body weight between 60 kg and 80 kg), or 1000 mg/day (body weight >80 kg) according to the manufacturer's drug information for ribavirin. All patients were monitored every 4 weeks. Serum HCV RNA was determined using the standardized automated qualitative PCR (Cobas Amplicor Hepatitis C Virus Test, version 2.0; Roche Diagnostics, Tokyo, Japan; detection limit: 50 IU/mL). Dose modification followed standard procedures in principle. Therefore, according to the intensity of the an adverse event or when laboratory results showed hemoglobin of <10 g/dL in subjects with no cardiac disease, the dose of ribavirin was decreased by 200 mg/day. When the neutrophil count was $<750/mm^3$ or the platelet count was $<80\,000/mm^3$, the dose of peginterferon was reduced by 50%. When the hemoglobin was <8.5 g/dL, the neutrophil count was $<500/mm^3$ or the platelet count was $<50\,000/mm^3$, then both drugs were discontinued. After the end of the treatment, the patients were followed-up for a further additional 24 weeks. The sustained viral response (SVR) was defined as undetectable serum HCV-RNA levels by qualitative PCR at the end of the 24-week post-treatment follow-up period. The others were classified as non-SVR.

All patients provided written informed consent and the study protocol was approved by the ethics committee of all participating hospitals.

Questionnaire

The questionnaires were distributed by the physicians in charge after informed consent was obtained, and were completed anonymously. As for the method of collection, the questionnaires were mailed to the office (Department of Digestive and Life-style Related Disease, Kagoshima University) by the patients due to the concern that it might be difficult for them to respond truthfully if their physicians were to collected the questionnaires directly from the patients. The survey was conducted four times in total: prior to the treatment, during the course of treatment (in the third month), at the end of treatment or at the discontinuation of treatment, and at 6 months after the completion of treatment. The details of the questionnaire are shown in Table 1. There were a total of 89 questions in 24 categories.

RESULTS

THE FOLLOWING NUMBER of questionnaires was collected: prior to the treatment: 681 cases; during the course of treatment: 458 cases; at the discontinuation of treatment: 59 cases; at the completion of treatment: 245 cases; and at 6 months after the completion of treatment: 188 cases. The characteristics of 437 patients were collected at baseline (Table 2). Excerpts from the questionnaire will be shown herein as it is not possible to refer to the results of all of the questions.

Questionnaire before therapy (681 patients)

In response to the question of whether they knew that chronic hepatitis C is a disease that can potentially progress to cirrhosis, 672 cases (98.7%) responded Yes and seven cases (1.3%) responded No (two were left blank). In response to the question of whether they knew that chronic hepatitis C is a disease that progresses to hepatocellular carcinoma, 672 cases (98.7%) responded Yes and 9 cases (1.3%) responded No. The reasons why they consulted the hepatologists were: Referral from a family doctor (437 cases, 64.2%); Referral from a friend (84, 12.3%); By coincidence (32, 4.7%); Newspapers and Internet (13, 1.9%); and None of the above/blank (115, 16.9%). The levels of satisfaction or anxiety regarding the treatment method and

Table 1 Details of questionnaire (Number of sections: 24)

Prior to the treatment (6 sections, 33 questions)
[Section 1] Treatment history (1 question)
[Section 2] Knowledge/understanding of chronic hepatitis (5 questions)
[Section 3] Explanations regarding treatment from physician (14 questions)
[Section 4] Level of expectation for treatment (4 questions)
[Section 5] Implementation of tests prior to the start of treatment (3 questions)
[Section 6] Survey of patient's attitudes (6 questions)
During the course of treatment (7 sections, 23 questions)
[Section 1] Practice regarding patient follow-up during the treatment (9 questions)
[Section 2] Implementation of tests during administration (2 questions)
[Section 3] Patient's perceptions of treatment (2 questions)
[Section 4] Care during treatment (2 questions)
[Section 5] Impact of lifestyle habits on treatment (4 questions)
[Section 6] Level of understanding of explanations from physician (2 questions)
[Section 7] Patient factors (2 questions)
At Discontinuation/Completion (7 sections, 23 questions)
[Section 1] Course of treatment (1 questions)
[Section 2] Practice regarding patient follow-up during the treatment (6 questions)
[Section 2] Implementation of tests during administration (4 questions)
[Section 4] Care during treatment (2 questions)
[Section 5] Impact of lifestyle habits on treatment (4 questions)
[Section 6] Level of understanding of explanations from physician (2 questions)
[Section 7] Patient factors (4 questions)
At 6 months after the end of treatment (4 sections, 10 questions)
[Section 1] Level of satisfaction with treatment (2 questions)
[Section 2] Explanations from physician regarding treatment effects (3 questions)
[Section 3] Impressions of the treatment (3 questions)
[Section 4] Patient's plans to visit medical institution (2 questions)

side-effects are shown in Figure 1. The patients were satisfied with the explanations regarding the treatment but were also feeling anxious. The causes of anxiety (multiple answers were allowed) were: Worried about side-effects (258 cases); Presence/absence of treatment efficacy (173); The duration of treatment is long (150); and Cost of treatment is high (109).

Table 2 Characteristics of patients at baseline

Age (years)	56.3 ± 10.2	(n = 473)
Sex (M : F)	232:241	
Body mass index (kg/m^2)	23.1 ± 2.9	(n = 451)
ALT (IU/L)	74.7 ± 60.1	(n = 471)
Platelet count (×10^4/mm^3)	15.6 ± 5.0	(n = 472)
Hemoglobin (g/dL)	14.0 ± 1.4	(n = 473)
Grade of activity		
0–1	126	
2–3	218	
Fibrosis stage		
0–2	273	
3–4	71	
HCV RNA level (KIU/mL)	1932 ± 1400	(n = 469)

Continuous variables are presented as mean ± SDs.

Questionnaire at three months into therapy

In response to the question asking to what extent they were satisfied with the course of treatment, Extremely satisfied and Very satisfied comprised 203 cases (44.3%) and 103 cases (22.5%), respectively, followed by Satisfied (136 cases, 29.7%) and Completely dissatisfied/Not satisfied (14, 3.1%) (two were left blank). In response to the question of whether the grade of side-effects was stronger in the explanations provided by their physicians or in their actual experience, the responses were: Same as explained (216 cases, 47.2%), Much stronger or Slightly stronger than what was explained (116, 25.3%), and Much lighter or Slightly lighter than what was explained (100, 21.8%) (26 were left blank). In addition, we investigated the grade of side-effect based on the interferon therapy history. Of 42 patients who had experienced interferon therapy, 10 cases (23.8%) responded Lighter than what was explained, 18 cases (42.9%) responded Same as explained, and 14 (33.3%) responded Stronger than what was explained. On the other hand, of 101 patients who had no experience of interferon therapy, 24 cases (23.8%) responded Lighter than what was explained, 50 (49.5%) responded Same as explained, and 27 (26.7%) responded Stronger than what was explained. There was no significant difference in grade of side-effects between those who had experienced and who had not experienced interferon treatment. Furthermore, we investigated the grade of side-effect based on sex and age. Of 56 female patients 60 years or more, 13 cases (23.2%) responded Lighter than what was explained, 31 cases (55.4%) responded Same as explained, and 12 (21.4%) responded Stronger than what was explained. Other results are given below as follows: sex, age, total number, number (%)

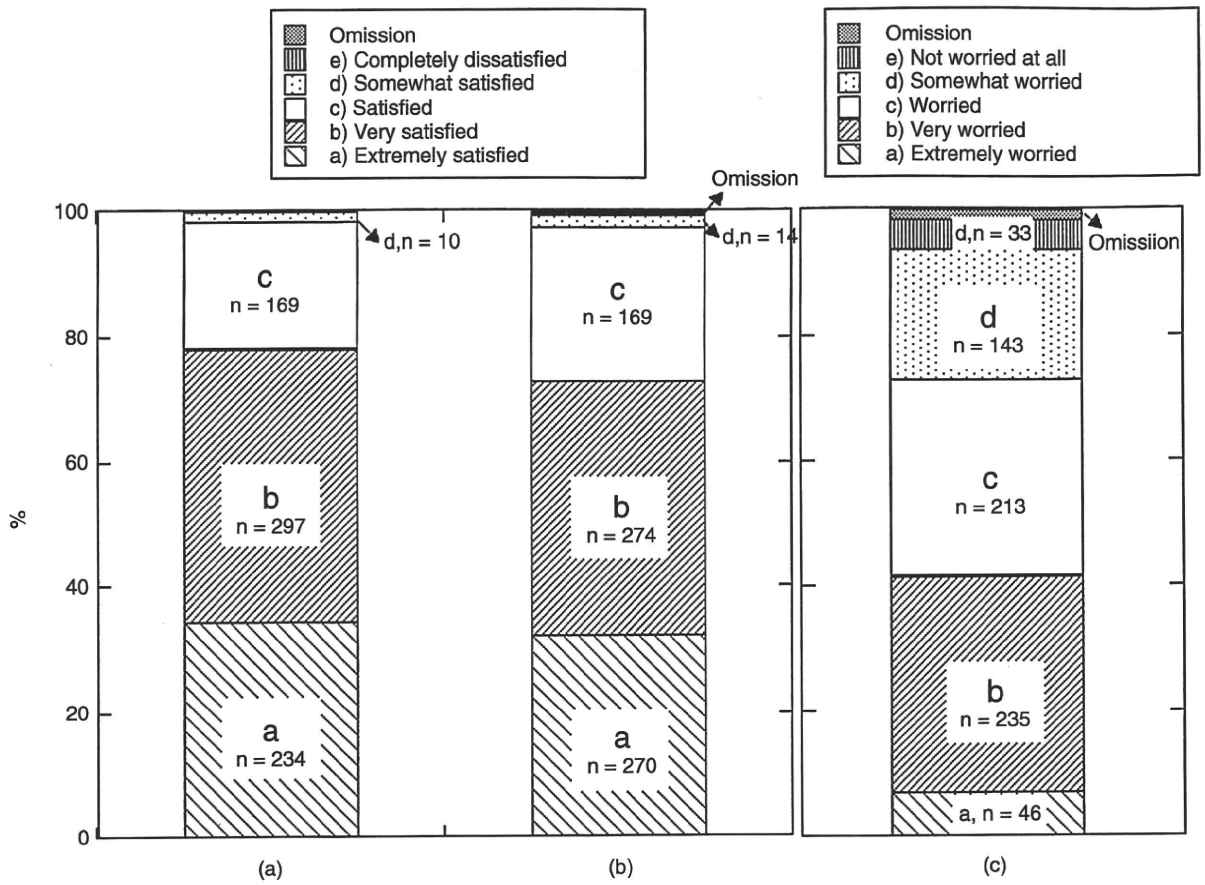

Figure 1 (a) To what extent are you satisfied with the explanations regarding the treatment method? (b) To what extent are you satisfied with the explanations regarding the side-effects? a. Extremely satisfied, b. Very satisfied, c. Satisfied, d. Somewhat satisfied, e. Completely dissatisfied C. To what extent are you worried about the therapy? a. Not worried at all, b. Somewhat worried, c. Worried, d. Very worried, e. Extremely worried.

responded Lighter than what was explained, number (%) responded Same as explained, number (%) responded Stronger than what was explained. Female, less than 60 years, 63, 15 (23.8%), 27 (42.9%), 21 (33.3%). Male, 60 years or more, 45, 8 (17.8%), 25 (55.6%), 12 (26.7%). Male, less than 60 years, 72,18 (25.0%), 36 (50.0%), 18 (25.0%). There were no significant differences between high age and low age, female and male.

In response to the question asking to what extent they were satisfied with the way their physicians dealt with the side-effects, Extremely satisfied and Very satisfied comprised 66 cases (14.4%) and 179 cases (39.1%), respectively, followed by satisfied (173 cases, 37.8%), and Slightly or Completely dissatisfied (31, 6.8%) (9 were left blank). In response to the question asking what they were most concerned about during the course of treatment (multiple answers were allowed), the responses were: Treatment efficacy (337 cases, 73.6%), Side effects (295, 64.4%), Costs of treatment (177, 38.6%), and Duration of treatment (144, 31.4%).

The most severe side-effects are shown in Figure 2. The most common side-effect was general malaise, and dermatological symptoms ranked second. In addition, wishes to discontinue the treatment and the reasons thereof are shown in Figure 3.

Questionnaire at either the end of therapy or at the discontinuation of therapy

Among the 59 cases of discontinuation, 34 cases were discontinued due to side-effects, 23 cases were discontinued because no disappearance of the hepatitis C virus in the sera was observed, and 2 cases were for other reasons. Among the 34 cases of discontinuation due to

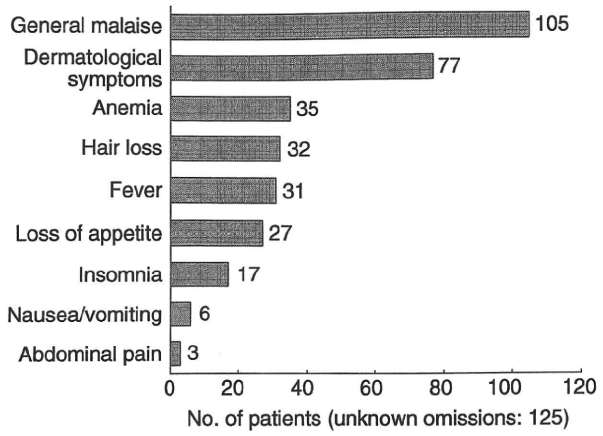

Figure 2 What was the most severe side-effect you experienced?

side-effects, five cases (14.7%) responded that they were not satisfied with the way their physicians dealt with the side-effects. In response to the question of whether the grade of side-effects was stronger in the explanations provided by their physicians or in their actual experience, in the completed treatment group, 55 cases (22.4%) responded Lighter than what was explained, 110 cases (44.9%) responded Same as explained, and 74 cases (30.2%) responded Stronger than what was explained (six were left blank). On the other hand, among the cases of discontinuation due to side-effects, there were 0 cases of Lighter than what was explained, 11 cases (32.4%) of Same as what was explained, and 22 cases (64.7%) of Stronger than what was explained (one case was left blank).

The number of cases that developed side-effects is shown in Figure 4. Among the side-effects for which no explanation was provided by the physicians, dermatological symptoms were the most common.

Questionnaire after 6 months of therapy

Regarding satisfaction with the treatment efficacy, the responses were: Extremely dissatisfied and Slightly dissatisfied (47 cases, 25.0%), followed by Satisfied (31, 16.5%) and Very satisfied or Extremely satisfied (110, 58.5%), indicating a high level of satisfaction. In response to the question asking whether they would recommend this treatment to other patients, the responses were: Definitely not or Probably not together accounted for 23 cases (12.2%), followed by Not sure (54 cases, 28.7%), and Probably and Definitely (111, 59.0%). As reasons for the recommendations, 99/111 cases (89.1%) responded that they achieved SVR. The reasons for not recommending the treatment were: Expected efficacy was not achieved (10/23 cases, 43.5%) and Side effects were severe (6/23, 26.1%).

The periods during which the patients experienced the most distressing side-effects are shown in Figure 5. The largest numbers were observed during the first and second months.

As the most distressing side-effect was general malaise and the period of that was during the first and second months, the association between general malaise and hemoglobin levels was investigated. The hemoglobin levels decreased by approximately 3 g/dL during the second month and then became flat. However, the decrease in hemoglobin levels was almost the same between patients whose most distressing side-effects

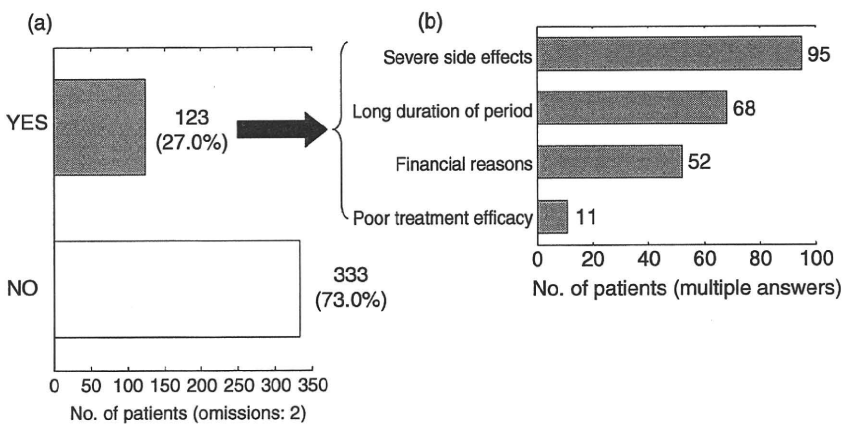

Figure 3 (a) Have you ever thought about discontinuing the treatment? (b) What was the reason why you intended to discontinue the treatment?

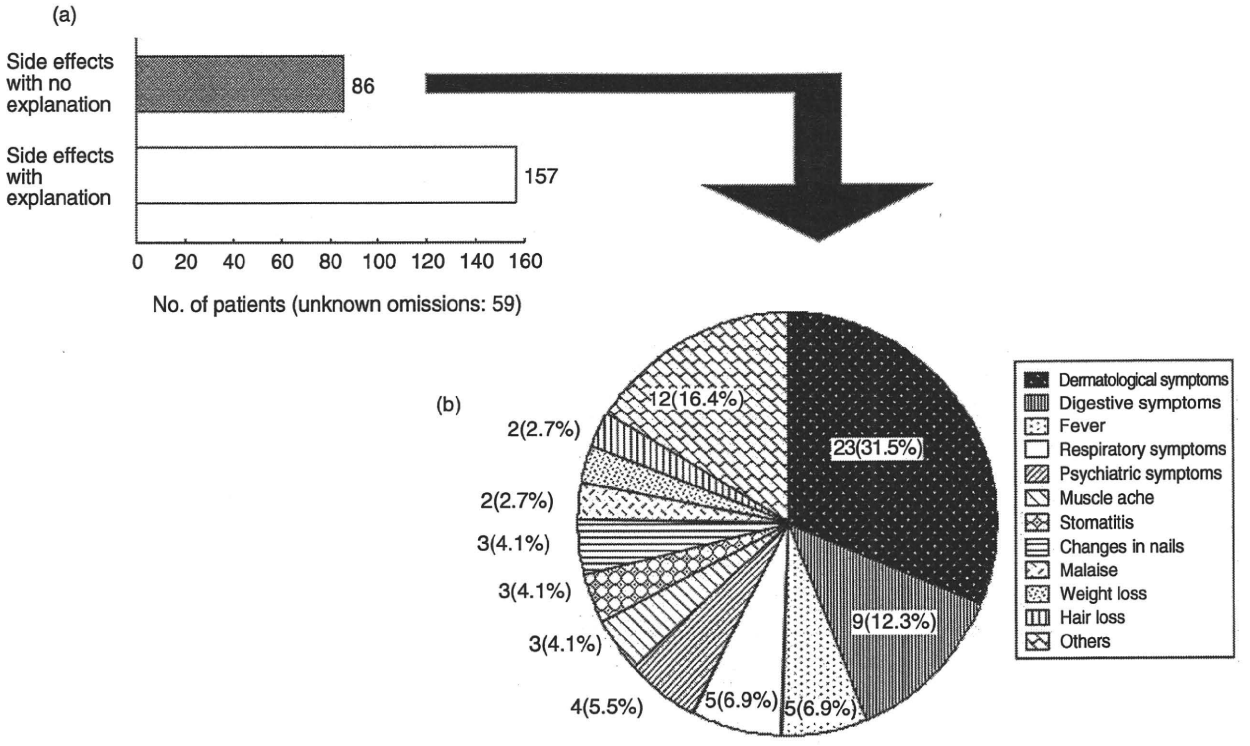

Figure 4 (a) Have you experienced side-effects that were not included in the explanations of your physician? (b) What kind of side-effects did you experience with no explanation?

were general malaise and patients for whom that was not the case (Fig. 6).

Efficacy of PEG•RBV therapy

Treatment efficacy was assessed among 439 patients who received follow-up for 24 weeks after completion of the therapy. Undetectable HCV RNA were observed in 235/411 cases (57.2%) by the 12th week after the start of treatment. At the end of treatment, of the 439 cases, 314 cases (71.5%) had undetectable HCV RNA. In the final results, SVR was observed in 206 cases (46.9%), and non-SVR was observed in 233 (53.1%).

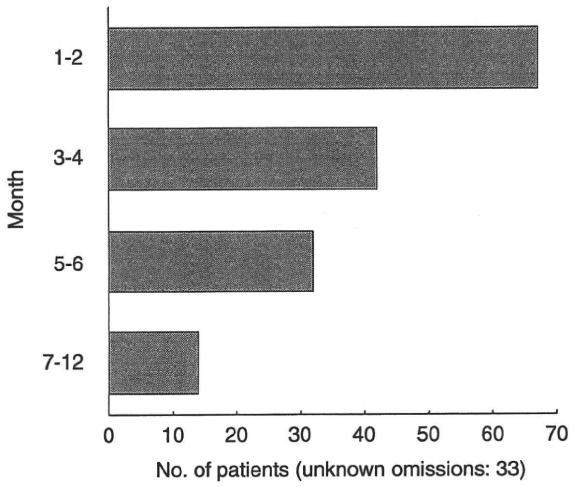

Figure 5 When did you experience the most distressing side-effects?

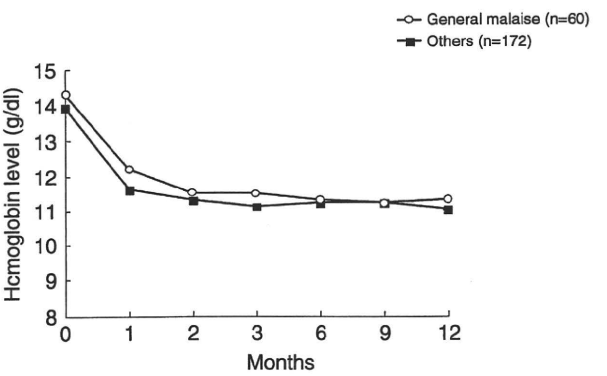

Figure 6 Changes of hemoglobin levels in patients whose most distressing side-effects were general malaise and patients for whom that was not the case.

DISCUSSION

AS NO REPORTS on questionnaire surveys similar to the one we conducted at this time were found, it is believed that this study is valuable.

Important information was obtained from the questionnaire survey. First, prior to the treatment, most of the patients understood the pathology and natural history of chronic hepatitis C, as well as the treatment method and its side-effects. However, it was revealed that they had a high level of anxiety.

Both the explanations regarding the course of treatment and the way in which health care providers managed the side-effects were adequate. It is believed that this is because patients were being seen by hepatologists mostly at hospitals where the survey was conducted.

This survey also revealed that dermatological symptoms were the second most severe side-effect. According to the data from a Phase 3 clinical trial in Japan, flu-like symptoms (96.7%) have been the most commonly observed side-effects, followed by loss of appetite (83.9%), hair loss (68.5%), and insomnia (66.9%). In addition, dermatological symptoms were injection site erythema (41.7%) and pruritus (61.0%). In this survey, however, nearly twice as many patients found dermatological symptoms to be severe, compared to anemia, hair loss, fever, loss of appetite, and insomnia. In addition, dermatological symptoms were the most commonly observed side-effects among the side-effects that were not explained by the physician. Based on these results, it is inferred that no adequate explanations are being provided prior to treatment and, moreover, management of the dermatological symptoms is not adequate.

Regarding the cases of discontinuation due to side-effects, only 14.7% of the patients were not satisfied with the way the side-effects were dealt with by their physicians, and it is believed that the physicians dealt with the side-effects relatively properly. While patients who discontinued the treatment due to side-effects felt that the side-effects were stronger than what was explained to them by the physicians, this perception was less common in the cases of completed treatment, and it is therefore suggested that it is not a lack of explanation by the physicians Of the patients, 27.0% wished to discontinue treatment, and side-effects were the most common reason; it is therefore important to manage side-effects. Regarding treatment efficacy, the SVR rate was 46.9%, which was equivalent to the 47.6% (121/254) from the Phase 3 trial that was carried out in Japan.

The level of satisfaction with treatment efficacy was 58.5%, and the level of satisfaction was higher in the cases in which SVR was achieved. The most distressing period was during the first and second months of treatment. It appears that this is because during this period, fever and malaise occur due to interferon, and anemia develops due to ribavirin, but after 3 months the patients subsequently were accustomed to these side-effects. It is believed that by letting the patients know in advance that the side-effects of the first and second months will not last until the end of the therapy, they can endure these side-effects.

In this survey, it was not possible to obtain responses from all of the cases. The reasons for this are: patients responded to the questionnaire on a voluntary basis; mere lack of explanation by the physicians; and the patients forgot to submit the questionnaire. In summary, explanations regarding dermatological symptoms were not adequately provided to the patients and, moreover, these symptoms were the second most severe side-effect. Also, it was revealed that the period when the side-effects were most distressing was during the first and second months after the start of treatment. Based on these results, it is necessary to provide explanations to the patients in order to alleviate their anxiety, etc.

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REFERENCES

1 National Institutes of Health. National Institutes of Health Consensus Development Conference Statement: management of hepatitis C: 2002. *Hepatology* 2002; **36**: S3–20.
2 Alberti A, Benvegnú L. Management of hepatitis C. *J Hepatol* 2003; **38**: S104–18.
3 Manns MP, McHutchison JG, Gordon SC *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958–65.
4 Hadziyannis S, Sette H Jr Morgan TR *et al.* Peginterferon-alfa-2a plus ribavirin combination therapy in chronic hepatitis C. A randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; **40**: 346–55.
5 Zeuzem S, Hultcrantz R, Bourliere M *et al.* Peginterferon alfa-2b plus ribavirin for treatment of chronic hepatitis C in previously untreated patients infected with HCV genotypes 2 or 3. *J Hepatol* 2004; **40**: 993–99.
6 Zeuzem S. Heterogeneous virologic response rates to interferon-based therapy in patients with chronic hepatitis C: who responds less well? *Ann Intern Med* 2004; **140**: 370–81.
7 Taliani G, Rucci P, Biliotti E. Therapy expectations and physical comorbidity affect quality of life in chronic hepatitis C virus infection. *J Viral Hepat* 2007; **14**: 875–82.
8 Kallman J, Margaret O'Neil M, Larive B. Fatigue and health-related quality of life (HRQL) in chronic hepatitis C virus infection. *Dig Dis Sci* 2007; **52**: 2531–9.
9 Svirtlih N, Pavic S, Terzic D. Reduced quality of life in patients with chronic viral liver disease as assessed by SF12 questionnaire. *J Gastrointestin Liver Dis* 2008; **17**: 405–9.
10 McHutchison JG, Ware JE Jr Bayliss MS *et al.* Hepatitis Interventional Therapy Group. The effects of Interferon alpha-2b in combination with ribavirin on health related qualityof life and work productivity. *J Hepatol* 2001; **34**: 140–7.
11 Bernstein D, Kleinman L, Barker CM, Revicki DA, Green J. Relationship of health-related quality of life to treatment adherence and sustained response in chronic hepatitis C patients. *Hepatology* 2002; **35**: 704–8.
12 Hassanein T, Cooksley G, Sulkowski M *et al.* The impact of peginterferon alfa-2a plus ribavirin combination therapy onhealth-related quality of life in chronic hepatitis C. *J Hepatol* 2004; **40**: 675–81.
13 Arora S, O'Brien C, Zeuzem S *et al.* Treatment of chronic hepatitis C patients with persistently normal alanine aminotransferase levels with the combination of peginterferon alpha-2a (40 kDa) plus ribavirin: impact on health-related quality of life. *J Gastroenterol Hepatol* 2006; **21**: 406–12.

14 Okamoto H, Sugiyama Y, Okada S *et al.* Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. *J Gen Virol* 1992; **73**: 673–79.
15 Tanaka T, Tsukiyama-Kohara K, Yamaguchi K *et al.* Significance of specific antibody assay for genotyping of hepatitis C virus. *Hepatology* 1994; **19**: 1347–53.
16 Simmonds P, Alberti A, Alter HJ *et al.* A proposed system for the nomenclature of hepatitis C viral genotypes. *Hepatology* 1994; **19**: 1321–4.
17 Desmet VJ, Gerber M, Hoofnagle JH, Manna M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994; **19**: 1513–20.

Research Article

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Altered interferon-α-signaling in natural killer cells from patients with chronic hepatitis C virus infection☆

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Background & Aims: Natural killer (NK) cells play an important role in the immune response against virus infection. Interferon (IFN)-α, an essential component in therapy against hepatitis C virus (HCV) infection, regulates NK cell function. However, it remains obscure how chronic HCV infection (CHC) modifies intracellular IFN-α signaling in NK cells. We investigated IFN-α signaling in NK cells in patients with CHC.

Methods: Peripheral blood mononuclear cells were obtained from patients with CHC and healthy subjects (HS) as controls.

Results: The expression level of signal transducer and activator of transcription (STAT) 1, a key molecule of IFN-α signaling, was clearly higher in NK cells from the CHC patients than in those from HS. The phosphorylation level of STAT1 with IFN-α stimulation was significantly greater in NK cells from the CHC patients than in those from the HS, while that of STAT4 was significantly less. These phosphorylation levels of STAT1 and STAT4 positively and negatively correlated with the STAT1 level in NK cells, respectively. The IFN-α induced messenger RNA level of the suppressor of cytokine signaling 1, which is a downstream gene of phosphorylated-STAT1, was clearly greater in NK cells from the CHC patients than in those from the HS, while that of IFN-γ, which is a downstream gene of phosphorylated-STAT4, was clearly lower.

Conclusions: These results indicate altered IFN-α signaling in NK cells in CHC patients, suggesting that this alteration is associated with the persistence of HCV infection and resistance to IFN-α therapy.

Keywords: Natural killer cells; Interferon; Hepatitis C virus; Signal transducer and activator of transcription.

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Abbreviations: NK, natural killer; IFN, interferon; STAT, signal transducer and activator of transcription; HCV, hepatitis C virus; CHC, chronic hepatitis C virus infection; HS, healthy subject; SOCS, suppressor of cytokine signaling; mRNA, messenger RNA; PBMC, peripheral blood mononuclear cell; IL, interleukin; pSTAT, phosphorylated-signal transducer and activator of transcription; RT-PCR, reverse transcription polymerase chain reaction; NKT, natural killer T; ISG, interferon stimulated gene.

Introduction

Natural killer (NK) cells play an important role in innate immune responses against a variety of viral infections by directly killing infected cells with cytotoxic molecules such as perforin and granzyme [1]. The cells also have a great ability to secrete a key cytokine, interferon (IFN)-γ, which activates subsequent adaptive immune responses as well as inhibits viral replication [1,2]. Another major component in innate immune responses during viral infections is IFN-α, which is the most abundant cytokine released during viral infections [3]. In addition to its anti-viral effects, IFN-α activates NK cells to induce IFN-γ production via activation of the signal transducer and activator of transcription (STAT) 4, as well as its cytotoxic ability via activation of STAT1 [4–7].

Hepatitis C virus (HCV) causes persistent infection in more than 70% of infected patients. Whereas some of the patients show a carrier-like state, most develop chronic liver diseases, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, which is why HCV infection is a worldwide health problem [8]. The administration of IFN-α is a well-established anti-viral therapy for HCV infection. More than 90% of patients with acute HCV infection respond to IFN-α based therapy, while only around 50% of patients with chronic HCV infection (CHC) do [9–12], suggesting a mechanism by which persistent HCV infection leads to resistance to IFN-α based therapy. NK cell number has been demonstrated to decrease in patients with CHC, while it is controversial whether NK cell functions are impaired in patients with CHC [13–15]. It thus remains unclear whether perturbation of NK cells is involved in the persistence of CHC as well as resistance to the therapy [13–15].

In the present study, we investigated how chronic HCV infection modifies intracellular IFN-α signaling in NK cells. The expression level of total STAT1, a key molecule of IFN-α signaling, was clearly higher in NK cells from patients with CHC than in those from healthy subjects (HS). Phosphorylation of STAT1, resulting in induction of the suppressor of cytokine signaling (SOCS) 1 messenger RNA (mRNA) expression in response to IFN-α was clearly greater in NK cells from the CHC patients than

in those from the HS. On the other hand, the phosphorylation of STAT4, resulting in induction of IFN-γ mRNA expression in response to IFN-α, was clearly less in NK cells from the CHC patients than in those from the HS. NK cell degranulation was significantly enhanced in response to IFN-α in the HS, but not in the CHC patients. These findings suggest altered IFN-α signaling in NK cells in patients with CHC. The alteration of IFN-α signaling might be associated with the persistence of chronic HCV infection and resistance to IFN-α therapy.

Materials and methods

Subjects

Twenty-six patients with CHC (HCV RNA genotype 1) and 26 healthy volunteers were enrolled in this study. The profile of these subjects is shown in the Supplementary data and Supplementary Table 1. The study was approved by the ethical committee of Osaka University Hospital.

Isolation of peripheral blood mononuclear cell (PBMC) populations

PBMCs were isolated from fresh heparinized peripheral blood by Ficoll-Hypaque density gradient centrifugation as described [16].

In vitro stimulation of cells

Prepared cells were unstimulated or stimulated with either natural human IFN-α, recombinant human IFN-γ or recombinant human interleukin (IL)-12. The details are provided in the Supplementary data.

Cell lysates and Western blot analysis

Prepared cells were lysed as described [17]. A 25 μg sample of protein was separated on 10% SDS polyacrylamide gels and transferred onto PVDF membrane. Monoclonal anti-STAT1 antibody (1/Stat1) was purchased from BD Biosciences (San Jose, CA, USA). Polyclonal anti-β-actin antibody from Abcam (Cambridge, MA, USA) was used as the loading control. Detection of immunolabeled proteins was performed as described [17].

Flow cytometric analysis

The staining of prepared cells was performed as described [16,18]. Briefly, cells were stained with fluorescein isothiocyanate-conjugated anti-CD3 (UCHT1) and biotin-conjugated anti-CD56 antibody (B159), fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) and cold pure methanol, and then stained with phycoerythrin-conjugated anti-phosphorylated-STAT (pSTAT) 4 (pY693) (38/p-Stat4) and Alexa Fluor® 647-conjugated anti-pSTAT1 (pY701) antibody (4a), or phycoerythrin-conjugated anti-STAT1 (1/Stat1) antibody alone, or the corresponding isotype control, followed by staining with peridinin chlorophyll protein-conjugated streptavidin (BD Biosciences). All antibodies were purchased from BD Biosciences. The stained cells were analyzed with a FACScan (Becton Dickinson, Mountain View, CA, USA), and the data were processed using the FlowJo program (Tree Star Inc., Ashland, OR, USA).

NK cell enrichment from PBMCs

To obtain pure populations of $CD56^+$ $CD3^-$ NK cells from PBMCs, NK cells were negatively isolated by magnetic cell sorting with a human NK cell isolation kit (Miltenyi Biotec, Gladbach, Germany). The purity of the isolated population was confirmed using FACS analysis and was more than 90%.

RNA isolation and analysis

Total RNA isolation and the real-time reverse transcription polymerase chain reaction (RT-PCR) analysis are presented in detail in the Supplementary data, Supplementary Fig. 1, and Supplementary Table 2.

NK cell degranulation assay

NK cell degranulation was assessed as described [19], with minor modifications. Details and representative data are provided in the Supplementary data and Supplementary Fig. 2.

Statistical analysis

The statistical significance of differences between the patient and control groups or that of changes due to IFN-α stimulation in the NK cell degranulation assay was determined by applying unpaired or paired Student's *t*-test, respectively. Correlations were assessed using the Pearson product–moment correlation coefficient. The statistical significance was defined as $p < 0.05$.

Results

NK cells from CHC patients showed a higher level of STAT1 expression than those from HS

Murine NK cells have been reported to exhibit a lower level of STAT1 expression than non-NK cells [17,18]. Also, the increase of STAT1 expression level in murine NK cells has been observed in viral infection [18]. We examined whether similar findings could be observed for human NK cells. Western blot analyses revealed that human NK cells from representative HS have a clearly lower level of STAT1 expression than non-NK cells (Fig. 1A). We then examined whether chronic HCV infection affected the STAT1 expression level in NK cells. It was clearly higher in NK cells from the CHC patients than in those from the HS (Fig. 1B).

We next examined STAT1 expression level within individual cells by flow cytometry. Consistent with the results of Western blot analyses, flow cytometric analyses demonstrated that NK cells from a representative healthy subject had a lower level of STAT1 expression than non-NK cells such as T cells or natural killer T (NKT) cells (Fig. 2A). NK cells from a representative CHC patient displayed a clearly higher level of STAT1 expression than

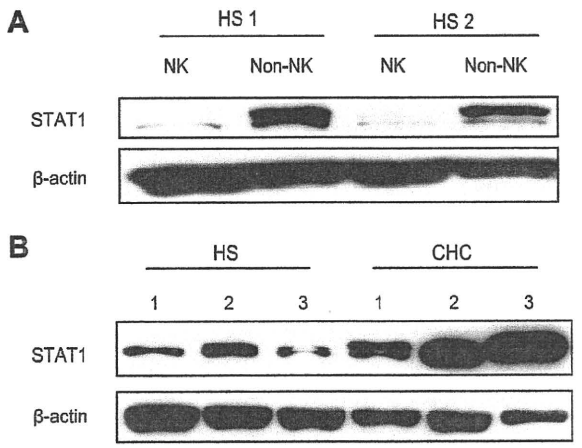

Fig. 1. STAT1 expression in NK cells from patients with chronic HCV infection. STAT1 protein levels were evaluated by Western blot analyses with β-actin measurement as a loading control. PBMCs were obtained from patients with chronic HCV infection (CHC) and healthy subjects (HS). NK cells and non-NK cells were purified from those cells. (A) STAT1 protein levels in NK cells and non-NK cells from two representative HS are shown. (B) STAT1 protein levels in NK cells from three other representative HS and three representative patients are shown.

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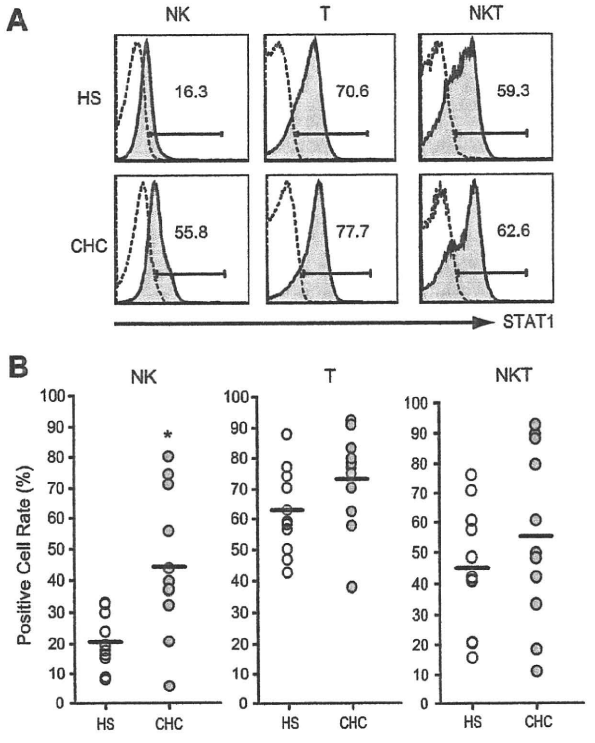

Fig. 2. STAT1 expression level in NK, T or NKT cells from patients with chronic HCV infection. STAT1 protein level was evaluated by flow cytometry, electronically gating on $CD56^+$ $CD3^-$ NK cells, $CD56^-$ $CD3^+$ T cells, and $CD56^+$ $CD3^+$ NKT cells. PBMCs were derived from patients with chronic HCV infection (CHC) and healthy subjects (HS). (A) Representative histograms from a patient and an HS are shown. Dotted lines show staining with the isotype control. Solid lines with shaded areas show staining with the antibody. Numbers are percentages of positive cells (positive cell rate) determined based on the isotype control staining. (B) Comparison of STAT1 expression level between the patients with CHC ($n = 11$) and HS ($n = 11$) are shown as positive cell rates. Each circle represents data for an individual. Horizontal bars represent means. *$p < 0.005$ vs. HS.

those from the healthy subject. Fig. 2B summarizes the profile of the intracellular STAT1 expression level of NK, T, and NKT cells. The intracellular STAT1 expression level of NK cells from the CHC patients was significantly higher than that from the HS, while that of T cells or NKT cells did not show any significant difference.

Altered activation of STAT1/4 occurred in response to IFN-α in NK cells from CHC patients

Activation of STAT1/4 in response to IFN-α in murine NK cells has been reported to shift from pSTAT4 dominant to pSTAT1 dominant as the intracellular STAT1 expression level increases [18]. This led us to examine the activation of STAT1/4 in response to IFN-α in human NK cells using samples from CHC patients and HS. Although IFN-α can phosphorylate both STAT1 and STAT4, IFN-γ can phosphorylate STAT1 and IL-12 can phosphorylate STAT4 in NK cells [1,7]. We examined the phosphorylation level of STAT1/4 in response to IFN-α, compared with IFN-γ or IL-12, in NK cells from the subjects by flow cytometry. It was found that IFN-α phosphorylated STAT1 more strongly in NK cells from the representative CHC patient than in those from the representative HS, while IFN-γ did not (Fig. 3A). On the other hand, IFN-α phosphorylated STAT4 more weakly in NK cells from the CHC patient than in those from the HS, while IL-12 did not. Fig. 3B summarizes the profile of STAT1/4 phosphorylation level in NK cells in response to IFN-α, IFN-γ or IL-12. IFN-α, but not IFN-γ, phosphorylated STAT1 significantly more strongly in NK cells from the CHC patients than in those from the HS. IFN-α, but not IL-12, phosphorylated STAT4 significantly more weakly in NK cells from the CHC patients than in those from the HS. These results suggested altered signaling of IFN-α, but not of IFN-γ or of IL-12, in NK cells of patients with CHC.

We then examined the relationship between STAT1 expression level and STAT1/4 phosphorylation level in response to IFN-α in NK cells from the CHC patients. The phosphorylation level of STAT1 in response to IFN-α correlated significantly and positively with the STAT1 expression level in NK cells ($R^2 = 0.67$, $p < 0.003$), while that of STAT4 correlated significantly and negatively ($R^2 = 0.49$, $p < 0.02$) (Fig. 4). These results suggested that, as in murine NK cells, the activation of STAT1/4 in response to IFN-α in human NK cells shifts from a preference for pSTAT4 to one for pSTAT1 as intracellular STAT1 expression level increases.

Altered induction of interferon stimulated gene (ISG) expression occurred in response to IFN-α in NK cells

We examined how the altered activation of STAT1/4 in response to IFN-α in NK cells affected the induction of downstream gene expression. Real-time RT-PCR analyses revealed that the induction level of SOCS1 mRNA expression with IFN-α stimulation, which is a downstream gene of pSTAT1, was clearly greater in NK cells isolated from the CHC patients than in those from the HS, and that the induction level of IFN-γ mRNA expression with IFN-α stimulation, which is a downstream gene of pSTAT4, was clearly lower (Fig. 5). On the other hand, the mRNA induction level of perforin or granzyme B, which is a cytotoxic molecule induced by IFN-α via pSTAT1, was not greater but modestly lower, suggesting negative regulation by the large induction of SOCS1, which is a negative regulator of the pSTAT1 pathway [20].

Altered activation of NK cells occurred in response to IFN-α

To examine how the altered IFN-α signaling in NK cells affected the activation of NK cells in response to IFN-α, we evaluated the NK cell degranulation ability in response to IFN-α. NK cell degranulation assay showed that CD107a expression, as a marker of degranulation, in the presence of K562 cells was significantly up-regulated in response to IFN-α in NK cells from HS, but not in those from CHC patients (Fig. 6), suggesting altered NK cell activation in response to IFN-α in CHC patients.

Discussion

Both IFN-α and IFN-γ have been observed in sera from patients with CHC [21,22]. Their production may be induced by a host response to HCV within the liver, which would make these IFNs detectable in the systemic circulation of patients with CHC. The present study has shown that NK cells from patients with CHC display higher levels of STAT1 expression compared to those from HS (Fig. 1B and Fig. 2). STAT1 itself is one of the ISGs, whose

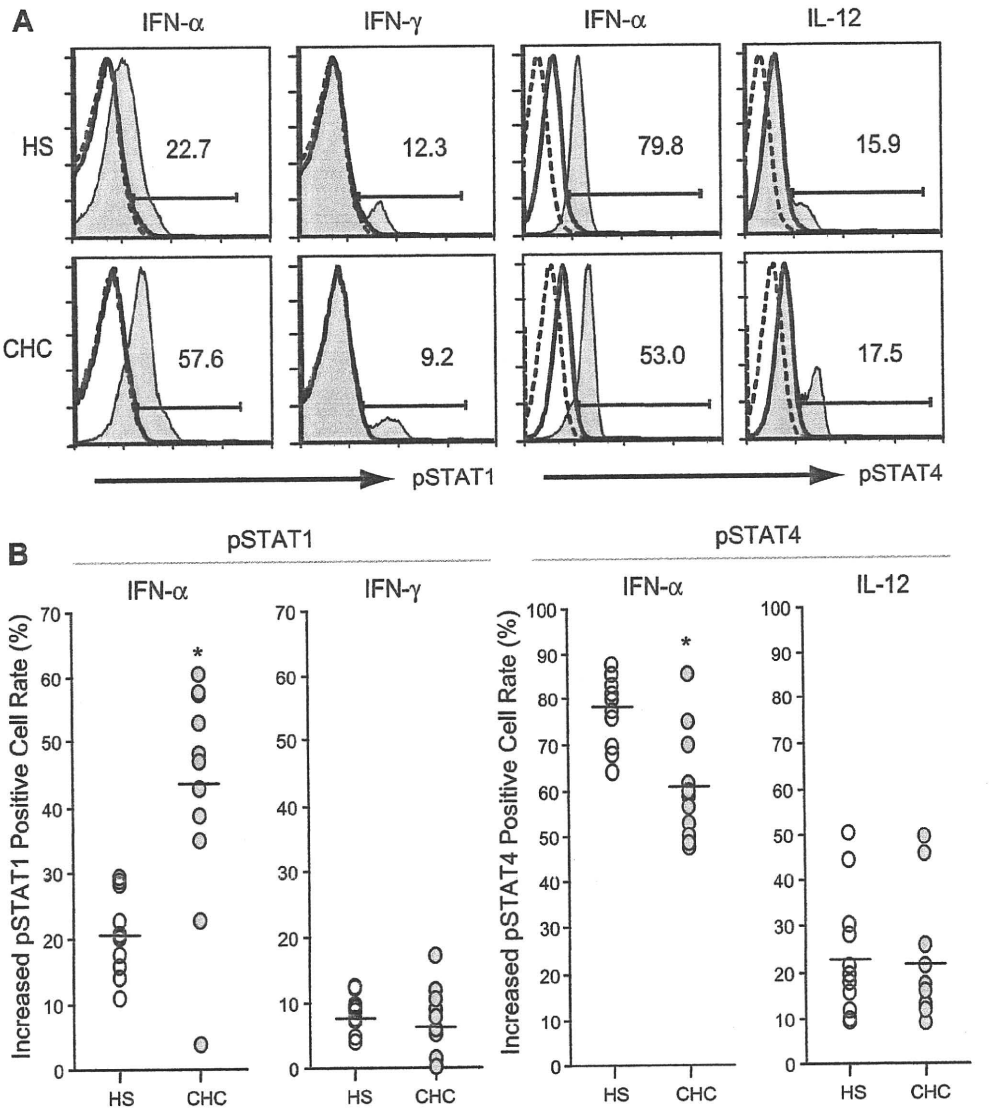

Fig. 3. Altered activation of STAT1/4 in response to IFN-α, but not to IFN-γ or IL-12 in NK cells from patients with chronic HCV infection. pSTAT1 and pSTAT4 protein levels were evaluated by flow cytometry with isotype control staining. PBMCs were derived from patients with chronic HCV infection (CHC) and healthy subjects (HS). (A) Prepared PBMCs were unstimulated or stimulated with natural IFN-α, IFN-γ or IL-12 for 90 min *in vitro*, and then collected. pSTAT1 and pSTAT4 protein levels were evaluated by flow cytometry, electronically gating on $CD56^+$ $CD3^-$ NK cells. Representative histograms of a patient and an HS are shown. Dotted lines show staining of stimulated cells with isotype control. Thick lines show staining of unstimulated cells with the antibody. Thin lines with shaded areas show staining of stimulated cells with the antibody. Positive cell rates were determined based on the staining with isotype controls. Numbers are increased positive cell rates which were determined by subtracting the positive cell rate of unstimulated cells from those of stimulated cells. (B) Comparison of pSTAT1/4 level in response to IFN-α, IFN-γ or IL-12 between the patients with CHC ($n = 11$) and the HS ($n = 11$) are shown as increased pSTAT1/4 positive cell rate. Each circle represents individual data. Horizontal bars represent means. *$p < 0.001$ vs. HS.

expression is up-regulated by IFN-α or IFN-γ [23,24]. It is thus possible that the higher level of STAT1 in NK cells from the CHC patients was up-regulated by IFN-α and/or IFN-γ induced by a chronic host response to HCV.

Our real-time RT-PCR analyses showed that the induction level of SOCS1 mRNA in response to IFN-α was significantly greater in NK cells from the CHC patients than in those from the HS (Fig. 5). This finding is consistent with the observation that the phosphorylation level of STAT1 in response to IFN-α was significantly stronger in NK cells from the CHC patients than in those from the HS (Fig. 3), because SOCS1 is a down-stream gene of pSTAT1 [20]. On the other hand, SOCS1 is an inducible negative regulator which inhibits further activation of the pSTAT1 pathway [20]. It is therefore possible that this greater up-regulation of SOCS1 owing to the greater level of STAT1 phosphorylation in response to IFN-α finally results in a weaker response to IFN-α, that is, a weaker induction of ISGs via the pSTAT1 pathway. Indeed, an increase of the STAT1 level,

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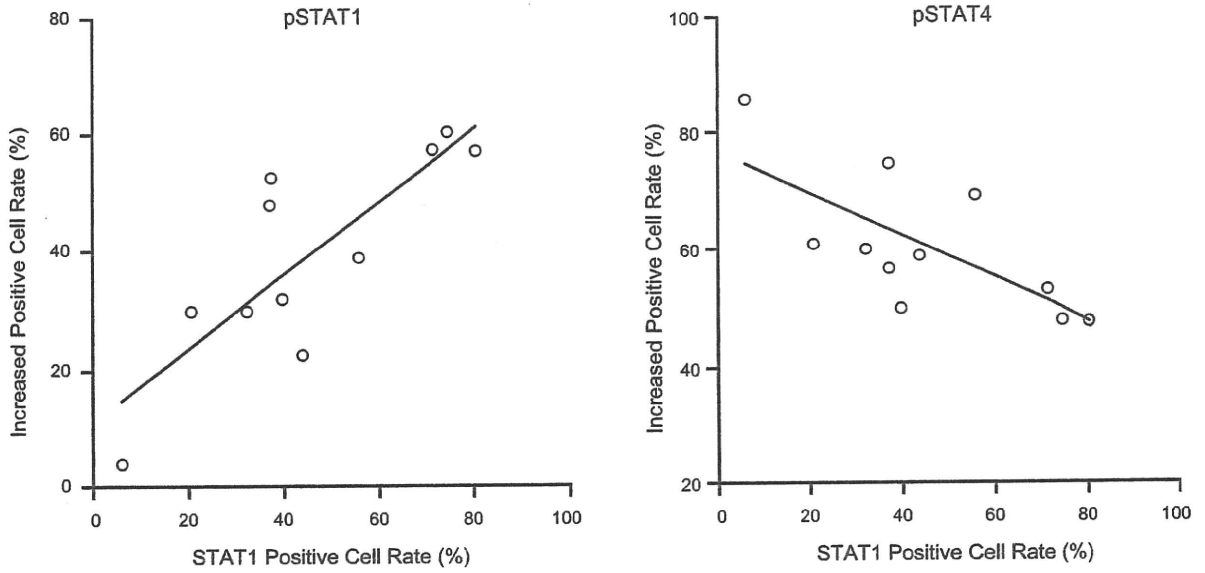

Fig. 4. Correlation between STAT1 level and pSTAT1/4 level in response to IFN-α in NK cells from patients with chronic HCV infection. The relationship was statistically analyzed between intracellular STAT1 level in NK cells and increased pSTAT1/4 level in response to IFN-α in NK cells from patients with chronic HCV infection. Each circle represents individual data. The lines represent regression lines.

which is itself a downstream gene of pSTAT1, by IFN-α based therapy, correlated negatively in vivo with the basal STAT1 expression level before therapy (Miyagi et al. unpublished data). The higher basal STAT1 expression causes a greater level of STAT1 phosphorylation in response to IFN-α, which is followed by a greater level of SOCS1 induction and then might result in a lower increase of STAT1 level. Since perforin and granzyme B are also ISGs from the pSTAT1 pathway [6,25], it is reasonable that the mRNA induction of perforin and granzyme B in response to IFN-α in NK cells from the CHC patients was not significantly greater but modestly lower than those from the HS (Fig. 5). Indeed, the enhancement of degranulation by IFN-α, that is, the increase of CD107a expression, in the presence of K562 cells in response to IFN-α, was significant in NK cells from the HS, but not in those from the CHC patients (Fig. 6).

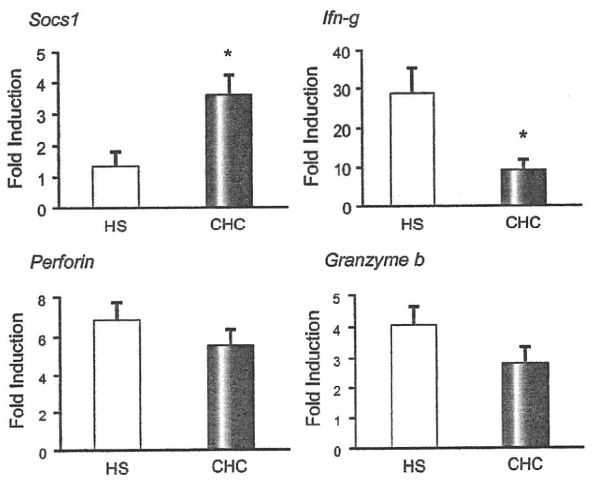

Fig. 5. Induction of ISGs in NK cells in response to IFN-α *in vitro*. NK cells were purified from PBMCs derived from patients with chronic HCV infection (CHC) and healthy subjects (HS). Isolated NK cells were untreated or treated with natural IFN-α *in vitro* for 3 h, and then collected. The collected cell RNA level of the indicated genes and β-actin as a control were analyzed by real-time RT-PCR. Data are shown as the fold increase of treated cells compared with untreated cells, with means ± standard error of the mean from five subjects in each group. *p <0.05 vs. HS.

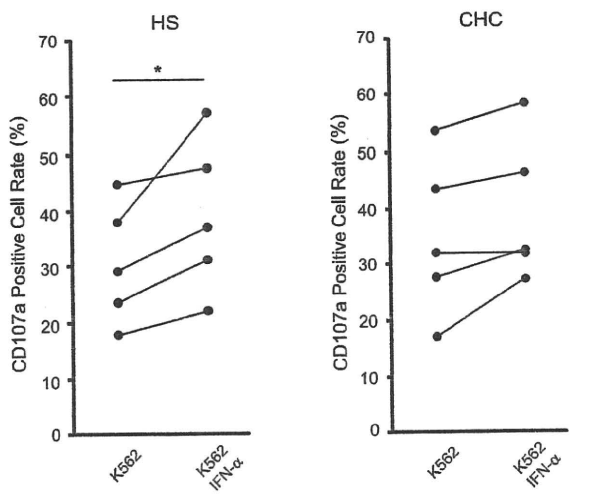

Fig. 6. Increase of NK cell degranulation by IFN-α stimulation. PBMCs derived from patients with chronic HCV infection (CHC) and healthy subjects (HS) were treated with or without IFN-α in the presence of K562 cells. The CD107a expression of NK cells were evaluated by flow cytometry, electronically gating on $CD56^+$ $CD3^-$ NK cells. Data are shown as the frequency of CD107a-positive NK cells treated with or without IFN-α. Each circle represents individual data. *p <0.05.

Patients who were clear of HCV due to IFN-α based therapy exhibited a significant increase in NK cell numbers and activity in the peripheral blood as well as in the liver compared to those who were not able to become clear of the virus [26–28]. IFN-γ produced from NK cells have been reported to suppress HCV replication in vitro [29]. IFN-γ from NK cells is also considered to activate the subsequent adaptive immune response during virus infection as well as inhibit viral replication [1,2,7]. These findings suggest the involvement of NK cells with IFN-γ in the response to IFN-α based therapy. The present study showed that the level of STAT4 phosphorylation in response to IFN-α in NK cells correlated negatively with the intracellular STAT1 level in NK cells (Fig. 4), and that IFN-γ induction, which is one of the downstream genes of pSTAT4, was clearly weaker in NK cells from the patients with CHC than in those from the HS (Fig. 5). The lower activation in CHC patients of the pSTAT4-to-IFN-γ pathway in response to IFN-α in NK cells compared to those from the HS might be one of the mechanisms which make CHC patients resistant to IFN-α based therapy.

Ahlenstiel et al. have recently reported that chronic exposure to HCV-induced IFN-α caused NK cells to become functionally polarized towards a cytotoxic phenotype, but that lacked an increase in IFN-γ production [30]. Moreover, Oliviero et al. showed that NK cells from CHC patients were of a predominantly activating phenotype, and that these phenotypic changes were associated with enhanced cytotoxic activity and defective IFN-γ production [31]. These reports would be associated with our finding that NK cells from the CHC patients displayed a high level of STAT1 expression. Cytotoxic molecules such as perforin and granzyme, as well as STAT1, belong to the ISGs [6,25]. A high level of STAT1 in NK cells in the CHC patients might correspond to a high level of cytotoxic molecules in NK cells resulting in enhanced cytotoxic activity at the basal level. Indeed, the CD107a expression at basal level in NK cells from the CHC patients seemed to be modestly higher than that in NK cells from the HS (Fig. 6), although NK cells from the CHC patients did not significantly increase the CD107a expression in response to IFN-α. On the other hand, our present study showed that STAT1 expression level in NK cells correlated negatively with the activation of STAT4 to produce IFN-γ in response to IFN-α in NK cells. A high level of STAT1 in NK cells would also cause defective IFN-γ production in the NK cells of patients with CHC.

Recent studies have revealed that the higher level of ISGs in hepatocytes as well as in PBMCs before IFN-α based therapy is associated with resistance to this therapy [32–33]. The present study demonstrated that NK cells from CHC patients displayed higher levels of STAT1, which is one of the ISGs, compared with those from HS (Fig. 2). Those who had a higher STAT1 level in NK cells showed less STAT4 phosphorylation and more STAT1 phosphorylation in response to IFN-α, resulting in less IFN-γ induction and more SOCS1 induction. Thus, it is possible that those who have a higher STAT1 level in NK cells would display less response to IFN-α based therapy. Our preliminary data with a small number of patients treated with IFN-α based therapy revealed a tendency for those who had a higher STAT1 level in NK cells to not respond well to the therapy in the context of HCV clearance by week 8 after initiation of therapy (Supplementary Fig. 3).

In the present study, we found that the expression level of total STAT1, a key molecule of IFN-α signaling, was clearly higher in NK cells from the patients with CHC than in those from the HS. The phosphorylation levels of STAT1 and STAT4 with IFN-α stimulation were altered in NK cells from the CHC patients, compared with those from the HS. The induction of IFN-γ mRNA expression with IFN-α stimulation, which is one of the downstream genes of pSTAT4, was clearly weaker in isolated NK cells from the CHC patients than in those from the HS. The induction of SOCS1 mRNA expression, which is one of the downstream genes of pSTAT1, was clearly stronger. The enhancement of NK cell degranulation, the increase of CD107a expression, in response to IFN-α was significant in the HS, but not in the CHC patients. These results indicate that IFN-α signaling in NK cells is altered in CHC patients, suggesting that this alteration of IFN-α signaling is associated with the persistence of chronic HCV infection and resistance to IFN-α therapy. The basal total STAT1 level in NK cells might enable the prediction of the outcome of IFN-α therapy against HCV infection, and thus could serve as a molecular target for more effective IFN-α based therapy.

Conflict of interests

The authors who have taken part in this study declare that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2010.03.018.

References

[1] Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu Rev Immunol 1999;17:189–220.
[2] Farrar MA, Schreiber RD. The molecular cell biology of interferon-gamma and its receptor. Annu Rev Immunol 1993;11:571–611.
[3] García-Sastre A, Biron CA. Type 1 interferons and the virus-host relationship: a lesson in détente. Science 2006;312:879–882.
[4] Cho SS, Bacon CM, Sudarshan C, Rees RC, Finbloom D, Pine R, et al. Activation of STAT4 by IL-12 and IFN-alpha: evidence for the involvement of ligand-induced tyrosine and serine phosphorylation. J Immunol 1996;157:4781–4789.
[5] Matikainen S, Paananen A, Miettinen M, Kurimoto M, Timonen T, Julkunen I, et al. IFN-alpha and IL-18 synergistically enhance IFN-gamma production in human NK cells: differential regulation of Stat4 activation and IFN-gamma gene expression by IFN-alpha and IL-12. Eur J Immunol 2001;31:2236–2245.
[6] Liang S, Wei H, Sun R, Tian Z. IFNalpha regulates NK cell cytotoxicity through STAT1 pathway. Cytokine 2003;23:190–199.
[7] Lee SH, Miyagi T, Biron CA. Keeping NK cells in highly regulated antiviral warfare. Trends Immunol 2007;28:252–259.
[8] Liang TJ, Rehermann B, Seeff LB, Hoofnagle JH. Pathogenesis, natural history, treatment, and prevention of hepatitis C. Ann Intern Med 2000;132:296–305.

Research Article

[9] Kamal SM, Fouly AE, Kamel RR, Hockenjos B, Al Tawil A, Khalifa KE, et al. Peginterferon alfa-2b therapy in acute hepatitis C: impact of onset of therapy on sustained virologic response. Gastroenterology 2006;130:632–638.

[10] Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 2001;358:958–965.

[11] Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Gonçales FL, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 2002;347:975–982.

[12] Santantonio T, Fasano M, Sinisi E, Guastadisegni A, Casalino C, Mazzola M, et al. Efficacy of a 24-week course of PEG-interferon alpha-2b monotherapy in patients with acute hepatitis C after failure of spontaneous clearance. J Hepatol 2005;42:329–333.

[13] Takehara T, Hayashi N. Natural killer cells in hepatitis C virus infection: from innate immunity to adaptive immunity. Clin Gastroenterol Hepatol 2005;3:S78–S81.

[14] Golden-Mason L, Rosen HR. Natural killer cells: primary target for hepatitis C virus immune evasion strategies? Liver Transpl 2006;12:363–372.

[15] Szabo G, Chang S, Dolganiuc A. Altered innate immunity in chronic hepatitis C infection: cause or effect? Hepatology 2007;46:1279–1290.

[16] Jinushi M, Takehara T, Tatsumi T, Kanto T, Miyagi T, Suzuki T, et al. Negative regulation of NK cell activities by inhibitory receptor CD94/NKG2A leads to altered NK cell-induced modulation of dendritic cell functions in chronic hepatitis C virus infection. J Immunol 2004;173:6072–6081.

[17] Takehara T, Uemura A, Tatsumi T, Suzuki T, Kimura R, Shiotani A, et al. Natural killer cell-mediated ablation of metastatic liver tumors by hydrodynamic injection of IFNalpha gene to mice. Int J Cancer 2007;120:1252–1260.

[18] Miyagi T, Gil MP, Wang X, Louten J, Chu WM, Biron CA. High basal STAT4 balanced by STAT1 induction to control type 1 interferon effects in natural killer cells. J Exp Med 2007;204:2383–2396.

[19] Alter G, Malenfant JM, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. J Immunol Methods 2004;294:15–22.

[20] Alexander WS, Hilton DJ. The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immune response. Annu Rev Immunol 2004;22:503–529.

[21] Pfeffer LM, Madey MA, Riely CA, Fleckenstein JF. The induction of type I interferon production in hepatitis C-infected patients. J Interferon Cytokine Res 2009;29:299–306.

[22] Dolganiuc A, Norkina O, Kodys K, Catalano D, Bakis G, Marshall C, et al. Viral and host factors induce macrophage activation and loss of toll-like receptor tolerance in chronic HCV infection. Gastroenterology 2007;133:1627–1636.

[23] Der SD, Zhou A, Williams BR, Silverman RH. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proc Natl Acad Sci USA 1998;95:15623–15628.

[24] Ji X, Cheung R, Cooper S, Li Q, Greenberg HB, He XS. Interferon alfa regulated gene expression in patients initiating interferon treatment for chronic hepatitis C. Hepatology 2003;37:610–621.

[25] Zimmerer JM, Lesinski GB, Kondadasula SV, Karpa VI, Lehman A, Raychaudhury A, et al. IFN-alpha-induced signal transduction, gene expression, and antitumor activity of immune effector cells are negatively regulated by suppressor of cytokine signaling proteins. J Immunol 2007;178:4832–4845.

[26] Bonavita MS, Franco A, Paroli M, Santilio I, Benvenuto R, De Petrillo G, et al. Normalization of depressed natural killer activity after interferon-alpha therapy is associated with a low frequency of relapse in patients with chronic hepatitis C. Int J Tissue React 1993;15:11–16.

[27] Appasamy R, Bryant J, Hassanein T, Van Thiel DH, Whiteside TL. Effects of therapy with interferon-alpha on peripheral blood lymphocyte subsets and NK activity in patients with chronic heparitis C. Clin Immunol Immunopathol 1994;73:350–357.

[28] Yamagiwa S, Matsuda Y, Ichida T, Honda Y, Takamura M, Sugahara S, et al. Sustained response to interferon-alpha plus ribavirin therapy for chronic hepatitis C is closely associated with increased dynamism of intrahepatic natural killer and natural killer T cells. Hepatol Res 2008;38:664–672.

[29] Wang SH, Huang CX, Ye L, Wang X, Song L, Wang YJ, et al. Natural killer cells suppress full cycle HCV infection of human hepatocytes. J Viral Hepat 2008;15:855–864.

[30] Ahlenstiel G, Titerence RH, Koh C, Edlich B, Feld JJ, Rotman Y, et al. Natural killer cells are polarized toward cytotoxicity in chronic hepatitis C in an interferon-alfa-dependent manner. Gastroenterology 2010;138:325–335.

[31] Oliviero B, Varchetta S, Paudice E, Michelone G, Zaramella M, Mavilio D, et al. Natural killer cell functional dichotomy in chronic hepatitis B and chronic hepatitis C virus infections. Gastroenterology 2009;137:1151–1160.

[32] Feld JJ, Nanda S, Huang Y, Chen W, Cam M, Pusek SN, et al. Hepatic gene expression during treatment with peginterferon and ribavirin: identifying molecular pathways for treatment response. Hepatology 2007;46:1548–1563.

[33] Sarasin-Filipowicz M, Oakeley EJ, Duong FH, Christen V, Terracciano L, Filipowicz W, et al. Interferon signaling and treatment outcome in chronic hepatitis C. Proc Natl Acad Sci USA 2008;105:7034–7039.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Clinical significance of alanine aminotransferase levels and the effect of ursodeoxycholic acid in hemodialysis patients with chronic hepatitis C

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Abstract
Background The natural history of hepatitis C virus (HCV) carriers and the effect of ursodeoxycholic acid (UDCA) have not been fully elucidated among hemodialysis (HD) patients.
Methods Eighty-four anti-HCV antibody- and HCV RNA-positive and 154 anti-HCV antibody-negative HD patients who were retrospectively observed for at least 3 years were analyzed. We investigated the factors associated with thrombocytopenia ($< 1.3 \times 10^5/\mu L$) and decreased platelet count (PLT) (more than 20% decrease during the follow-up period), which were considered to be indicators of hepatic fibrosis. In addition, another 16 HD patients with HCV who received 300 mg/day UDCA orally for at least 6 months were investigated. Changes in alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT) and PLT were assessed.
Results After the 60.3-months mean follow-up period, HCV infection was independently associated with both thrombocytopenia [odds ratio (OR) 2.589] and decreased PLT (OR 2.339) in 238 HD patients. In 84 HD patients with HCV, the average ALT levels ($\geq$ 15 IU/L) during the follow-up period was associated with thrombocytopenia (OR 3.882) and decreased PLT (OR 4.470). In addition, ALT, AST and GGT significantly decreased at 6 months after starting UDCA, but PLT did not change in 16 HD patients with HCV.
Conclusions These results indicate that HCV infection is a risk for thrombocytopenia which should be associated with hepatic fibrosis in HD patients. In addition, the clinical course of ALT levels predicts the progression of thrombocytopenia, and UDCA may effectively lower ALT levels in HD patients with HCV.

Keywords Hemodialysis · HCV · Thrombocytopenia · ALT · Ursodeoxycholic acid

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Introduction

Chronic kidney disease (CKD) patients who are on hemodialysis (HD) continue to have a higher prevalence of hepatitis C virus (HCV) infection than the general population [1–4]. The prevalence of anti-HCV seropositivity among patients undergoing regular dialysis in developed countries ranges between 7 and 40% [5–8].

HCV infection in HD patients is usually recognized as asymptomatic and cirrhosis is infrequent in this population [9]. One of the reasons for these findings is that the clinical course of chronic hepatitis C extends over decades and dialysis patients generally have higher morbidity and mortality rates than the general population, making the long-term consequences of HCV infection with HD difficult to establish [6]. However, more recently, the prognosis of HD patients has been improving, so addressing HCV infection in these patients is becoming more important [10].

The strong association between serum alanine aminotransferase (ALT) levels and the fibrosis progression rate or occurrence of hepatocellular carcinoma has been well documented in HCV carriers without HD [11–13]. HD

patients with persistent HCV infection also have higher ALT levels than those patients without HCV, and ALT values may predict the outcome of HCV infection in patients with HD [14]. In contrast, ALT values are still typically within the normal range in HCV carriers with HD and ALT values are lower in HCV carriers with HD than those without HD. Recently, the risk of liver disease-related deaths is higher in chronic hepatitis C patients with ALT levels closer to the upper limit of the normal range (ULN) (20–29 IU/L) compared to patients with lower ALT levels (< 20 IU/L) [15, 16]. In addition, it has been proposed that the cut-off for serum ALT levels should be reduced by half to screen for hepatic damage in HCV carriers with HD [17]. However, the association between serum ALT levels in those patients with HCV and fibrosis progression has not been fully elucidated.

Platelet count (PLT) is a simple biomarker of hepatic fibrosis in HCV carriers [18]. PLT is also lower in HCV RNA-positive HD patients than in HD patients with HCV RNA-negative serum [16]. In addition, severe hepatic fibrosis is independently associated with thrombocytopenia ($< 1.3 \times 10^5/\mu L$) in HCV carriers with end-stage renal disease [19]. This study evaluated the association of ALT status over a long period and changes in PLT, which was considered an indicator of hepatic fibrosis, in HD patients.

Several trials have examined the efficacy of interferon monotherapy or interferon plus ribavirin combination therapy in HD patients with HCV, and some of these patients obtained a sustained virological response [20]. However, the virological response was limited and side effects may occur more frequently in patients with HD than in those without HD [21, 22]. Therefore, other therapies should be considered for these patients. For chronic hepatitis C patients with or without HD, ursodeoxycholic acid (UDCA) has already been used up to 150 mg/day as routine care in Japan. In addition, the effect of UDCA up to 900 mg/day in HCV carriers who are not undergoing HD was investigated [23], and the use of UDCA up to 900 mg/day was approved for use by chronic hepatitis C patients after April 2007 in Japan. However, the effect of UDCA was not fully elucidated in HCV carriers with HD. Therefore, in this retrospective study we investigated the clinical significance of biochemical markers in the natural course of disease with particular emphasis on PLT and assessed the effect of oral UDCA on serum biomarkers in those patients with HCV.

Materials and methods

Study population

The patients in this study were retrospectively recruited. This study was approved by the Kagoshima University Graduate School of Medical and Dental Sciences. The study population consisted of patients who were on HD in August 2008 and whose data were obtained at least 3 years before August 2008 at 17 HD facilities in Kagoshima, Japan. Their alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), total cholesterol (TC) and PLT were monitored once or twice each month. In 2539 patients, 243 patients were positive for anti-HCV, 143 patients were excluded because they were positive for hepatitis B virus surface (HBs) antigen, they were positive for anti-HCV antibody (anti-HCV) but were not examined for HCV RNA, they had received antiviral treatment or they had hepatocellular carcinoma (HCC). The final population enrolled in this study consisted of 100 patients. Among this cohort of 100 HD patients who were both anti-HCV- and HCV RNA-positive, 84 subjects had not received UDCA and were enrolled in study 1 (HD + HCV Group) and 16 subjects had already received 300 mg/day UDCA for at least 3 months after April 2007 when UDCA up to 900 mg/day was approved for use by chronic hepatitis C patients in Japan and were enrolled in study 2 (UDCA Group). The control subjects in study 1 were 154 HD patients who were anti-HCV-negative (HD Group), and the controls in study 2 were the 84 HD patients among the study 1 population who were both anti-HCV- and HCV RNA-positive but had not received previous treatments including UDCA and were observed until August 2008 (non-UDCA Group). Of the 84 HD patients who were controls in study 2, 2 patients died before November 2008. Blood samples were obtained before routine HD procedures and then were used to assay for ALT, AST, GGT, TC and PLT. The relationship of these markers to PLT and the percent change in PLT were examined, and the percent change in PLT was calculated according to the formula: $\Delta\%PLT = [PLT\ (\text{at the end of study}) - PLT\ (\text{at enrollment})]/PLT\ (\text{at enrollment}) \times 100$.

Serum HCV markers

Serum anti-HCV and HBsAg were determined using a commercially available third-generation enzyme-linked immunosorbent assay and anti-HBs assay, respectively. For anti-HCV antibody-positive patients, HCV RNA was quantified using the COBAS TaqMan HCV kit (COBAS AmpliPrep/COBAS TaqMan HCV assay, Roche Diagnostics, Tokyo, Japan) during the follow-up period. The serologically defined HCV genotype (HCV serotype) was also determined with a serological genotyping assay kit (Immunocheck F-HCV Grouping, International Reagents Co., Tokyo, Japan). In some patients, the HCV genotype was examined (HCV Core Genotype, SRL, Tokyo, Japan). HCV genotype 1b was included with serotype I, and genotypes 2a and 2b with serotype II. No other HCV genotype was detected in this study population.

Study 1

The HD + HCV Group, which contained 84 HD patients with HCV, was compared to the HD Group, which contained 154 HD anti-HCV-negative patients. We compared the basal characteristics at enrollment and the changes in PLT during the follow-up period between the two groups. In addition, we divided the HD + HCV patients into the following four groups according to the average ALT level of all available ALT levels during the follow-up period: Group A, ALT < 15; Group B, 15 ≤ ALT < 20; Group C, 20 ≤ ALT < 30; and Group D, 30 ≤ ALT. Clinical characteristics at baseline or average ALT levels and change in PLT during the follow-up period were compared between these four groups.

Study 2

Sixteen patients with HD and HCV had been treated with 300 mg/day UDCA orally for at least 3 months after April 2007, when UDCA up to 900 mg/day was approved for chronic hepatitis C patients, until August 2008 (UDCA Group). These patients were observed every month for at least 6 months before the administration of UDCA and then monitored for the efficacy of UDCA for more than 3 months until August 2008. Then, these patients were observed for a total of at least 6 months until November 2008. We compared the basal characteristics between the UDCA Group just before UDCA treatment and the non-UDCA Group in May 2008. In addition, the changes in ALT, AST, GGT and PLT during the follow-up period were compared between the two groups. For example, the percent of ALT was calculated according to the formula: %ALT = [ALT (−6, 0, 1, 2, 3 or 6 M]/ALT[0 M] × 100).

Statistical analysis

When appropriate, χ^2 test, Fisher's exact test, Student's t test and Mann–Whitney U test were used to compare the frequencies or means. Logistic regression models were used for calculating the odds ratios (ORs), 95% confidential intervals (CIs) and P values. Statistical analyses were performed using STATVIEW (version 5.0; Abacus Concepts, Berkeley, CA), or SPSS (SPSS Inc., Chicago, IL) software programs. A P value less than 0.05 was considered statistically significant.

Results

Demographic characteristics of study 1 subjects

As shown in Table 1, 84 HD patients among the anti-HCV-positive patients were HCV carriers (positive for HCV RNA). One hundred fifty-four HD patients were anti-HCV-negative. On average, the frequency of males, levels of AST, ALT and GGT were higher and TC and PLT were lower at baseline in patients with HCV than those in patients without HCV. The follow-up period was also shorter in patients with HCV than those in patients without HCV. In contrast, there were no significant differences between the two groups with respect to age and duration of dialysis.

Table 1 Baseline characteristics of hemodialysis patients

	HCV (+)[a]	HCV (−)[b]	P value
Number	84	154	
Age (year)	64.4 ± 10.3	62.2 ± 12.5	0.165
Sex (male/female)	54/30	77/77	0.034
Duration of HD (years)	13.5 ± 9.6	11.8 ± 7.4	0.669
Follow-up period (months)	56.8 ± 15.8	62.2 ± 7.9	0.039
HCV RNA (Log IU/mL)[c]	4.9 ± 1.4	–	
Serotype (I/II/undetermined)[c]	59/21/4	–	
AST (IU/L)	19.7 ± 8.5	14.9 ± 6.7	<0.001
ALT (IU/L)	18.5 ± 9.3	13.2 ± 7.1	<0.001
GGT (IU/L)	41.5 ± 43.0	30.1 ± 42.1	0.002
TC (mg/dl)	153.7 ± 41.0	167.1 ± 35.0	0.003
PLT (× 10^5/μl)	1.59 ± 0.53	1.93 ± 0.73	<0.001

Unless otherwise indicated, data are given as the mean ± SD or number of patients

HD hemodialysis, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *GGT* gamma-glutamyl transpeptidase, *TC* total cholesterol, *PLT* platelet count

[a] HCV (+), both anti-HCV antibody and HCV RNA positive

[b] HCV (−); anti-HCV antibody negative

[c] HCV RNA and serotype were examined during follow-up period

Predictors of thrombocytopenia in HD patients

Table 2 summarizes the results of a univariate analysis of factors associated with thrombocytopenia (PLT < 1.3 × 10^5/μl) at the end of study 1 (August 2008) using 9 baseline characteristics in all HD patients with or without HCV. Older age, HCV viremia, elevated AST, ALT, and GGT levels were significantly associated with thrombocytopenia. In addition, a multivariate analysis revealed that HCV viremia was independently associated with thrombocytopenia (Table 2). Furthermore, after the 60.3-month mean follow-up period (mean of HD + HCV Group, 56.7 months; HD Group, 62.2 months), PLT in the HD + HCV Group had decreased (from 1.59 × 10^5/μL to 1.22 × 10^5/μL) significantly compared to that in the HD Group (from 1.93 × 10^5/μL to 1.77 × 10^5/μL) (average Δ%PLT in each patient: −22.4 vs. −5.3%, $P < 0.001$). Variables that were statistically significant by a univariate analysis were further analyzed to identify variables that

Table 2 Univariate and multivariate analyses of variables associated with thrombocytopenia (< 1.3 × 10^5/μl) in HD patients

Variables	Odds ratio	95% CI	*P* value
Univariate analysis			
Age (years)			
<60	1.0		
≥60	1.994	1.141–3.484	0.015
Sex			
Female	1.0		
Male	1.494	0.868–2.571	0.147
Duration of dialysis (years)			
<10	1.0		
≥10	1.065	0.624–1.818	0.816
Follow-up period (months)			
<55	1.0		
≥55	0.727	0.4–1.321	0.296
HCV			
(−)	1.0		
(+)	4.533	2.555–8.043	<0.0001
AST (IU/L)			
<30	1.0		
≥30	7.741	2.095–28.603	0.002
ALT (IU/L)			
<20	1.0		
≥20	3.793	2.017–7.133	<0.0001
GGT (IU/L)			
<50	1.0		
≥50	2.836	1.396–5.758	0.004
TC (mg/dl)			
<150	1.0		
≥150	0.58	0.296–1.135	0.112
Multivariate analysis			
Age (years)			
<60	1.0		
≥60	1.783	0.937–3.394	0.078
HCV			
(−)	1.0		
(+)	2.589	1.317–5.091	0.006
AST (IU/L)			
<30	1.0		
≥30	5.123	0.996–26.339	0.050
ALT (IU/L)			
<20	1.0		
≥20	1.75	0.786–3.896	0.171
GGT (IU/L)			
<50	1.0		
≥50	1.743	0.783–3.88	0.174

Abbreviations as in Table 1

were independently associated with a more than 20% decrease in PLT. As a result, male sex (OR 2.375; 95% CI, 1.319–4.278; $P = 0.004$) and HCV viremia (OR 2.339; 95% CI, 1.295–4.224; $P = 0.005$) were factors that were independently associated with more than a 20% decrease in PLT.

Predictors of thrombocytopenia in HD patients with HCV

Table 3 summarizes the results of a univariate analysis of factors associated with thrombocytopenia (PLT < 1.3 × 10^5/μL) at the end of study 1 (August 2008) using 10 baseline characteristics in HD patients with HCV. The patients with HCV and thrombocytopenia had significantly higher frequencies of elevated ALT and GGT levels at baseline. However, age, sex, duration of HD, follow-up period, history of diabetes mellitus (DM), and elevated AST and TC levels were not significantly different between patients with and without thrombocytopenia. In addition, elevated ALT and GGT levels at baseline were not significantly associated with thrombocytopenia in patients with HCV by a multivariate analysis.

On the other hand, a univariate analysis that compared a decrease in PLT of more than 20% with a decrease less than 20% revealed that male sex and elevated ALT levels at baseline were associated with decreased PLT in patients with HCV. A multivariate analysis of two variables that were statistically significant by a univariate analysis also revealed that high ALT levels (ALT ≥ 20 IU/L) at baseline were independently associated with decreased PLT in patients with HCV (OR 3.318; 95% CI, 1.256-8.764; $P = 0.016$).

Furthermore, we divided patients with HCV into four groups according to average ALT levels during the follow-up period. As Table 4 shows, 30, 19, 18 and 17 patients were in Groups A, B, C and D, respectively. Age, duration of dialysis, follow-up period, HCV RNA levels, distribution of HCV serotype, frequency of diabetes mellitus, TC levels and PLT were not significantly different between the four groups. However, serum AST levels and ALT levels at baseline were significantly different, and these levels gradually increased from Group A to D. The distribution of sex was also significantly different and the frequency of males was higher in Groups B, C and D than in Group A. The decreasing rate of change in PLT was significantly higher in Groups B, C, and D compared to Group A (Fig. 1). In addition, the average ALT levels (≥ 15 IU/L) during the follow-up period were independently associated with thrombocytopenia (OR 3.882; 95% CI, 1.257–11.987;