Interferon Alpha Receptor 2 Expression by Peripheral Blood Monocytes in Patients with a High Viral Load of Hepatitis C Virus Genotype 1 Showing Substitution of Amino Acid 70 in the Core Region

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Key Words

Chronic hepatitis C • Amino acid substitution • Core region • Interferon- α receptor 2 • Peripheral blood leukocyte

Abstract

Background/Aim: When patients with chronic hepatitis C (CHC) are treated with interferon (IFN)-based therapy, achieving serum HCV-RNA negativity by week 12 (early viral response, EVR) is an important predictor of a sustained virologic response. The aim of this study was to clarify whether changes in IFN- α receptor 2 (IFNAR-2) expression by peripheral blood monocytes (Mo) and the EVR rate differed between patients with genotype 1b and a high viral load showing substitution of amino acid 70 in the core region of HCV (mutant, $n = 20$) and patients without this substitution (wild, $n = 23$). **Patients and Methods:** Forty-three CHC patients were studied, and received pegylated IFN plus ribavirin. IFNAR-2 expression by Mo was determined using flow cytometry to measure the mean fluorescence intensity (MFI) before and up to 28 days after starting therapy. **Results:** The EVR rate of the mutant group was significantly lower than that of the wild group (35 vs. 70%). The MFI of Mo was significantly higher in the wild group than in the mutant group

before and also 3, 7, and 28 days after starting therapy. **Conclusions:** Mutation of HCV was related to lower IFNAR-2 expression by Mo before and after starting therapy.

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Introduction

Interferon (IFN) is an essential component of current treatment for chronic hepatitis C (CHC). The most effective therapy available at present is the combination of pegylated (PEG)-IFN and ribavirin (RBV) [1], and achieving serum hepatitis C virus (HCV)-RNA negativity by week 12 of treatment (early virologic response: EVR) is an important predictor of a sustained virologic response in CHC patients with genotype 1b and a high viral load [2, 3]. HCV with substitutions of amino acid (aa) 70 in the core region has been reported to be more difficult to treat than HCV without aa 70 substitution [4, 5].

The HCV core protein has been reported to interfere with intracellular IFN signaling [6, 7]. When IFN initiates its intracellular signal transduction pathway, Jak1 and STAT2 kinases are activated and in turn phosphorylate STAT1 and STAT2. Association of Jak1 with STAT1

Table 1. Background of patients with wild and mutant aa 70

	Wild	Mutant	p value
Number of patients	23	20	
Age, years	56 (43–67)	55 (32–66)	0.06
Sex, M/F	12/11	7/13	0.26
Serum			
aa 91 (mutant/wild)	4/19	3/17	0.85
HCV-RNA, log copies/ml	6.6 (6.0–6.7)	6.4 (5.8–6.6)	0.06
AST, IU/ml	55 (29–89)	56 (27–136)	0.27
ALT, IU/ml	55 (30–125)	68 (35–188)	0.13
Peripheral blood			
WBC, / μ l	5,600 (4,120–7,160)	4,500 (3,650–6,740)	0.02
RBC, $\times 10^4$ / μ l	459 (410–505)	445 (404–503)	0.18
PLT, $\times 10^4$ / μ l	14.7 (13.3–15.9)	14.2 (12.3–15.8)	0.36
Pathological findings			
Activity 1/2/3/none	6/15/1/1	9/10/0/1	0.49
Fibrosis 1/2/3/4/none	7/5/8/2/1	10/3/3/3/1	0.51
Previous IFN therapy			
No/yes	15/8	12/8	0.72

Unless otherwise indicated the values are medians with ranges in parentheses.
Ranges represent the 10–90th percentile.

initiates phosphorylation of STAT1, after which phosphorylated STAT1 forms a heterotrimeric complex together with phosphorylated STAT2 and IFN regulatory factor 9. This complex is known as IFN-stimulated gene (ISG) factor 3 (ISGF3), and it undergoes translocation to the nucleus where it binds to IFN-stimulated response element (ISRE) and upregulates a large number of ISGs including 2',5'oligoadenylate synthetase (2-5AS) [8–10].

The effect of aa substitutions in the core region of HCV on intracellular signal transduction induced by IFN has not been investigated. Since signaling is initiated following the binding of IFN to receptors on the plasma membrane of target cells, the role of IFN receptors should also be worth investigating. The type I IFN receptor consists of at least two subunits, which are referred to as IFNAR-1 (IFN- α receptor) and IFNAR-2 (IFN- α / β receptor) [11, 12]. Recent studies have demonstrated a significant correlation between hepatic expression of this receptor and the response of CHC patients to IFN [13–16]. It has also been reported that expression of messenger RNA (mRNA) for IFNAR-2 by peripheral blood mononuclear cells is correlated with the hepatic expression of this mRNA [17]. However, determining the level of expression of functional IFN receptors would be more useful, if this was possible.

Recently, a rapid flow cytometry method for measuring IFNAR-2 in small samples of whole blood has been established [18], and it can also be used to evaluate IFNAR-2 protein expression by leukocyte subsets [19]. The aim of the present study was to clarify whether changes in IFNAR-2 expression by peripheral blood Mo up to day 28 of IFN treatment differed between patients with substitution of aa 70 in the core region of HCV and those without this substitution.

Patients and Methods

The subjects were 43 patients with genotype 1b CHC, including 19 men and 24 women ranging (10–90th percentiles) from 41 to 67 (median 56) years of age (table 1). Written informed consent for the study was obtained from each patient. All patients were positive for anti-HCV antibody by a third-generation enzyme-linked immunosorbent assay (ELISA) and for serum HCV-RNA quantified by Taqman PCR method (Roche Diagnostics, Tokyo, Japan; detection limit 15 IU/ml). Serum levels of HCV-RNA in the subjects ranged (10–90th percentiles) from 5.8 to 6.7 (median 6.5) log kilocopies/ml. Patients known to be homosexual or intravenous drug users, those positive for hepatitis B surface antigen (HBs-Ag) or antinuclear antibody, and those with metabolic liver dysfunction, diabetes mellitus, and renal dysfunction were excluded.

Detection of aa substitutions in the HCV core region was performed using the previously reported method [4, 5], and the patients were divided into 2 groups on the basis of the findings. Pa-

tients with substitution of aa 70 in the core region of HCV were assigned to the mutant group and patients without this substitution were assigned to the wild group.

Of the 43 patients, 41 had undergone liver biopsy before combination therapy, and the tissue specimens thus obtained were graded according to the histology activity index (HAI) of Knodell et al. [20], after which the specimens were divided into 3 groups according to Desmet et al. [21]: grade 1 for HAI scores of 1–3; grade 2 for HAI scores of 4–8, and grade 3 for HAI scores of 9 or more. The specimens were also divided into 4 groups from stage 1 to stage 4 based on the fibrosis score of Desmet et al. [21].

IFN Regimens

All patients received IFN therapy after giving informed consent. PEG-IFN- α -2b (Pegintron®, Schering Plough Pharmaceutical Co. Ltd., Tokyo, Japan) was given intramuscularly for 40 weeks in a weekly dose adjusted for body weight according to the manufacturer's instructions (<45 kg = 60 μ g/dose, 46–60 kg = 80 μ g/dose, 61–75 kg = 100 μ g/dose, 76–90 kg = 120 μ g/dose, and >91 kg = 120 μ g/dose). RBV (Rebetol®, Schering Plough Pharmaceutical Co. Ltd) was given orally for 48 weeks in daily doses adjusted for body weight according to manufacturer's instructions (<60 kg = 600 mg/day, 61–80 kg = 800 mg/day, and >81 kg = 1,000 mg/day).

If serum HCV-RNA quantified by PCR was negative in week 12, this was defined as an EVR. If serum HCV-RNA quantified by PCR was positive in week 12, this was defined as a non-EVR.

Assays

Blood samples were collected from all patients before and 3, 7, 14, and 28 days after the start of IFN therapy. Then IFNAR-2 expression by peripheral leukocytes was determined using flow cytometry to measure the mean fluorescence intensity (MFI) after staining with anti-IFNAR-2 antibody, as reported previously [18].

Serum 2-5AS activity was assessed before and 3, 7, 14, and 28 days after the start of therapy with an RIA (Eiken Immunochemical Laboratory, Tokyo, Japan), that had a detection limit of 10 pmol/dl.

Comparisons Performed

We investigated the following points: (1) comparison of the EVR rate between the wild and the mutant groups; (2) comparison of IFNAR-2 expression by peripheral blood Mo and serum 2-5AS levels between the wild and mutant groups before therapy and also 3, 7, 14, and 28 days after the start of therapy, and (3) comparison of IFNAR-2 expression by peripheral blood Mo and serum 2-5AS levels between the EVR and non-EVR patients from the wild and mutant groups before therapy, and also 3, 7, 14, and 28 days after the start of therapy.

Statistical Analysis

Results in table 1 are presented as the median and the 10–90th percentiles. The χ^2 test was used to compare characteristics between the groups.

Data shown in the figures 1–3 are expressed as the mean $\pm$ SD. Comparisons between 2 groups were done by the nonparametric Mann-Whitney test or the χ^2 test. A probability value of <0.05 was considered to indicate statistical significance.

Results

EVR Rates

The EVR rate of the wild group was significantly higher than that of the mutant group (16/23, 70%, vs. 7/20, 35%; $p < 0.05$ by the χ^2 test).

Changes in IFNAR-2 Expression and Serum 2-5AS

The MFI of Mo was always higher in the wild than in the mutant group during the study period. The MFI of Mo before therapy and also 3, 7, and 28 days after the start of therapy was significantly higher in the wild than in the mutant group ($p < 0.05$ by the Mann-Whitney test; fig. 1a).

There were no significant differences in the serum level of 2-5AS between the wild and mutant groups throughout the study period (fig. 1b).

Changes in IFNAR-2 and Serum 2-5AS in EVR or Non-EVR Patients from the Wild Group

The MFI of Mo was always higher in the EVR patients than in the non-EVR patients during the study period, although the difference did not reach statistical significance (fig. 2a).

Serum levels of 2-5AS were always higher in the EVR than in the non-EVR patients during the study period. Serum levels of 2-5AS were significantly higher in the EVR than in the non-EVR patients on days 3, 7, 14, and 28 of treatment ($p < 0.05$ by the Mann-Whitney test; fig. 2b).

Changes in IFNAR-2 and Serum 2-5AS in EVR or Non-EVR Patients from the Mutant Group

The MFI of Mo did not differ between the EVR and non-EVR patients throughout the study period (fig. 3a).

Serum levels of 2-5AS were significantly higher in the EVR than in the non-EVR patients only on day 3 of treatment ($p < 0.05$ by the Mann-Whitney test; fig. 3b).

Discussion

It has been reported that HCV core protein influences IFN signaling in target cells such as hepatocytes and peripheral blood mononuclear cells. However, substitution of aa 70 in the core region of HCV is not known to affect intracellular signal transduction by IFN. The present study demonstrated that IFNAR-2 expression by Mo in patients with aa 70 substitution in the core region of HCV (mutant) was lower than that in patients without aa 70

Fig. 1. a IFNAR-2 expression by Mo in all patients. The solid line shows the mean IFNAR-2 expression by peripheral blood Mo (+SD) in patients without aa 70 substitution in the HCV core region (wild). The dotted line shows mean IFNAR-2 expression by Mo (-SD) in patients with aa 70 substitution in the HCV core region (mutant). * Significant difference ($p < 0.05$) between the wild

and mutant groups. **b** Serum 2-5AS in all patients. The solid line shows the mean serum 2-5AS level (+SD) in the wild group. The dotted line shows the mean serum 2-5AS level (-SD) in the mutant group. No significant difference ($p > 0.05$) between the wild and mutant groups.

Fig. 2. a IFNAR-2 expression by Mo in the wild group. The solid line shows the mean IFNAR-2 expression by peripheral blood Mo (+SD) in the patients from the wild group who obtained an early virologic response (EVR). The dotted line shows the mean IFNAR-2 expression by Mo (-SD) in patients from the wild group who did not obtain EVR (non-EVR). No significant difference ($p > 0.05$) between the EVR and non-EVR patients from the wild

group. **b** Serum 2-5AS activity in the wild group. The solid line shows mean serum 2-5AS level (+SD) in EVR patients from the wild group. The dotted line shows mean serum 2-5AS level (-SD) in non-EVR patients from the wild group. * Significant difference ($p < 0.05$) between the EVR and non-EVR patients from the wild group.

Fig. 3. a IFNAR-2 expression by Mo in the mutant group. The solid line shows the mean IFNAR-2 expression by peripheral blood Mo (+SD) in patients from the mutant group who obtained an early virologic response (EVR). The dotted line shows the mean IFNAR-2 expression by Mo (-SD) in patients from the mutant group who did not obtain EVR (non-EVR). No significant difference ($p > 0.05$) between the EVR and non-EVR patients

from the mutant group. **b** Serum 2-5AS activity in the mutant group. The solid line shows the mean serum 2-5AS level (+SD) in EVR patients from the mutant group. The dotted line shows the mean serum 2-5AS level (-SD) in non-EVR patients from the mutant group. * Significant difference ($p < 0.05$) between the EVR and non-EVR patients from the mutant group.

substitution in the core region (wild). Therefore, aa 70 substitution in the HCV core region was suggested to be associated with reduced IFNAR-2 expression by Mo and, to our knowledge, this is the first report of such a finding.

It is impossible to observe serial changes in IFNAR-2 expression in the liver after combination therapy with PEG-IFN and RBV in CHC patients. Since hepatic expression of IFNAR-2 and its expression by peripheral blood mononuclear cells are reported to be correlated [17], it could be valuable to observe serial changes in receptor expression on peripheral blood Mo. Further, it is thought that assessing expression of the functional IFN receptor is more useful than determining expression at the mRNA level. IFN receptor protein located on the cell membrane is functional, while intracellular receptor protein is not, and flow cytometry makes it possible to determine the amount of a particular receptor protein expressed on the surface of peripheral blood Mo in CHC patients.

In the present study, IFNAR-2 expression by peripheral blood Mo was consistently higher in patients without aa 70 substitution than in those with aa 70 substitution after the start of combination therapy for HCV, because

a significant difference was obtained except on day 14. In contrast, serial changes in serum 2-5AS levels were not different between the wild and mutant groups throughout the observation period. Therefore, it is possible that aa 70 substitution in the HCV core region influences IFNAR-2 expression by target cells before and during combination therapy, but not intracellular IFN signaling, in CHC patients with genotype 1b and a high viral load.

In the wild group, the EVR rate was a high 70%, and serial changes in IFNAR-2 expression by Mo were not significantly different between the EVR and non-EVR patients up to day 28. However, the serum level of 2-5AS, which is an antiviral molecule, was higher in the EVR patients. These findings suggested that EVR was easier to obtain if signal transduction by IFN proceeded smoothly in the target cells. Therefore, IFNAR-2 expression and IFN signaling in the target cells might be factors related to EVR in the wild group.

In the mutant group, the EVR rate was a low 35%, and there were no significant differences in the changes in IFNAR-2 expression by Mo and serum 2-5AS levels between the EVR and non-EVR patients except on day 3. These findings suggested that not only IFNAR-2 expression but also IFN signaling was downregulated and dis-

turbed in the target cells of the mutant group. Thus, EVR might be related to factors other than IFNAR-2 expression and IFN signaling in the target cells of the mutant group. Although EVR is not easy to obtain in the mutant group, other factors such as IFN-mediated innate and adaptive immune responses, may play a role in EVR patients from this group.

In the present study, we only evaluated IFNAR-2 expression and 2-5AS, which is one of final products of intracellular IFN signaling. However, the intracellular IFN signaling pathway has several molecules, including Jak1, STAT1, 2, ISGF3, and ISRE, while there are numerous products of ISGs [8, 9]. Accordingly, these factors should

be evaluated in CHC patients with aa 70 substitution in the HCV core region during combination therapy with PEG-IFN and RBV.

In conclusion, flow cytometry showed that aa 70 substitution in the core region of HCV downregulated baseline IFNAR-2 expression by Mo. After initiating combination therapy, this substitution might interfere with the induction of IFNAR-2 expression by Mo and with intracellular signal transduction by IFN. Further studies may lead to a better understanding of the pathophysiological role of IFNAR-2 in CHC patients with genotype 1b and a high viral load receiving combination therapy with PEG-IFN and RBV.

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