

Effect of *in vitro* interferon-beta administration on hepatitis C virus in peripheral blood mononuclear cells as a predictive marker of clinical response to interferon treatment for chronic hepatitis C

Kaori Mochizuki, Tatehiro Kagawa, Shinji Takashimizu, Kazuya Kawazoe, Sei-Ichiro Kojima, Naruhiko Nagata, Atsushi Nakano, Yasuhiro Nishizaki, Koichi Shiraishi, Masaru Itakura, Norihito Watanabe, Tetsuya Mine, Shohei Matsuzaki

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Correspondence to: Dr. Tatehiro Kagawa, Department of Internal Medicine, Division of Gastroenterology, Tokai University School of Medicine, Bohseidai, Isehara 259-1193, Japan. kagawa@is.icc.u-tokai.ac.jp

Telephone: +81-463-93-1121

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Abstract

AIM: To test whether *in vitro* incubation of peripheral blood mononuclear cells (PBMC) with interferon (IFN) could efficiently decrease hepatitis C virus-RNA (HCV-RNA) amount and to analyze whether this effect was associated with clinical response to IFN.

METHODS: Twenty-seven patients with histologically proven chronic hepatitis C were given intravenous administration of 6 million units (MU) IFN- β daily for 6 weeks followed by three times weekly for 20 weeks. PBMC collected before IFN therapy were incubated with IFN- β and HCV-RNA in PMBC was semi-quantitatively determined.

RESULTS: Twenty-five patients completed IFN therapy. Eight patients (32%) had sustained loss of serum HCV-RNA with normal serum ALT levels after IFN therapy (complete responders). HCV-RNA in PBMC was detected in all patients, whereas it was not detected in PBMC from healthy subjects. *In vitro* administration of IFN- β decreased the amount of HCV-RNA in PMBC in 18 patients (72%). Eight of these patients obtained complete response. On the other hand, none of the patients whose HCV-RNA in PBMC did not decrease by IFN- β was complete responders. Multiple logistic regression analysis revealed that the decrease of HCV-RNA amount in PBMC by IFN- β was the only independent predictor for complete response ($P < 0.05$).

CONCLUSION: The effect of *in vitro* IFN- β on HCV in PBMC reflects clinical response and would be taken into account as a predictive marker of IFN therapy for chronic hepatitis C.

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INTRODUCTION

Interferon (IFN) is effective in the treatment of chronic hepatitis C. However, the efficacy of IFN is limited and approximately 20% of patients obtain sustained virological response^[1,2]. Even the combination therapy with IFN and ribavirin induces it in 30% to 40% of patients^[3-5]. Because IFN is expensive and also has potentially severe adverse effects, it is important to discriminate which patient is responsive to IFN before starting therapy. The low amount of serum hepatitis C virus (HCV)-RNA, viral genotypes other than 1b and the absence of cirrhosis are associated with favorable response^[1]. However, accurate prediction is still difficult before IFN treatment and more sensitive markers related to good response are required.

HCV is a hepatotropic virus, but was reported to exist in peripheral blood mononuclear cells (PBMC)^[6-8]. The existence of minus strand HCV-RNA^[7,9] suggests the proliferation of HCV in PBMC. Both *in vivo* and *in vitro* administration of IFN induce antiviral enzymes such as 2',5'-oligoadenylate synthetase (2-5AS) in PBMC as well as in liver^[10,11]. We hypothesized that *in vitro* administration of IFN might exert antiviral effect on HCV proliferating in PBMC. We tested whether *in vitro* incubation of PBMC with IFN could efficiently decrease HCV-RNA amount and also analyzed whether this effect was associated with clinical response to IFN.

MATERIALS AND METHODS

Patients and study design

Twenty-seven patients with chronic hepatitis C were enrolled into this study. Criteria for enrollment were elevation of serum ALT levels, positivity for anti-HCV antibody and serum HCV-RNA, presence of histologically proven chronic hepatitis, and absence of other chronic liver diseases such as autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, Wilson's disease. Liver histology was obtained from all patients before IFN treatment and evaluated for staging of fibrosis and grade of activity according to the METAVIR scoring system^[12,13]. They were given intravenous administration of 6 million units (MU) IFN- β (Feron, Toray Pharmaceuticals, Tokyo, Japan) daily for 6 weeks followed by three times weekly for 20 weeks. Informed consent was obtained from all patients.

The effect of IFN was defined as complete response (CR): sustained loss of serum HCV-RNA and normal ALT levels, partial response (PR): disappearance of serum HCV-RNA at the end of treatment and reappearance after treatment, and no response (NR): positive serum HCV-RNA at the end of treatment.

Serum viral load was defined by multicyclic reverse transcription (RT)-polymerase chain reaction (PCR) method^[14,15] and patients were categorized into 2 groups, namely high: $\geq 10^8$ copies/mL, and low: $< 10^8$ copies/mL. HCV genotyping was performed with type-specific primers^[16].

Effect of IFN- β on PBMC

Twenty ml of peripheral blood was drawn before IFN

treatment. PBMC were separated by gradient centrifugation and washed with phosphate-buffered saline (PBS) seven times. After this procedure the supernatants were negative for HCV-RNA even after two-step PCR as described below. PBMC were suspended in RPMI-1640 medium supplemented with fetal bovine serum (IWAKI, Tokyo, Japan) at a concentration of 2×10^6 cells/mL, plated on a 24-well plate (IWAKI), and cultured at 37 °C in a humidified atmosphere with 50 mL/L CO₂ in the absence or presence of IFN- β at a concentration of 100 IU/L. After incubation for 72 h PBMC were washed five times with PBS followed by RNA extraction with guanidium thiocyanate. Total RNA was reversely transcribed into cDNA in a 40 μ L reaction mixture containing 100 pmoles antisense primer (5' -AACACTACTCGGCTAGCAGT-3') and 200 units of SuperScript II reverse transcriptase (Invitrogen Japan, Tokyo, Japan). The amplification of cDNA was performed for 35 cycles in four temperature steps (at 94 °C for 2 min, 37 °C for 2 min, 55 °C for 2 min and 72 °C for 3 min) in a 100 μ L reaction mixture containing the template, 1 unit of Taq polymerase (Perkin Elmer, Norwalk, CT), 20 pmol of each outer primer, 2.5 nmol of each deoxyribonucleotide triphosphates, 10 mmol/L Tris HCl, pH 8.3, 50 mmol/L KCl and 0.15 mmol/L MgCl₂. After the first PCR, 10 μ L of the PCR product was subjected to a second PCR amplification using the inner primer pair under the same conditions as described for the first PCR. Primers were designed from the sequence of 5' non-coding region, an outer primer pair of 242-base span: sense (5' -ACTCCACCATAGATCATCCC-3') and antisense (5' -AACACTACTCGGCTAGCAGT-3') and an inner primer pair of 145-base span: sense (5' -TTCACGCAG-AAAAGCGTCTAG-3') and antisense (5' -GTTGATCCAA-GAAAGGACCC-3'). PCR products were analyzed by gel-electrophoresis. The amount of HCV-RNA in PBMC was determined semi-quantitatively as 2+: positive after first PCR; 1+: negative after first PCR and positive after second PCR; and -: negative after first and second PCR. PBMC from 5 healthy subjects were also examined for the presence of HCV-RNA.

Statistical analysis

Categorical variables were analyzed by the chi-square test. Continuous numeric variables were examined by the Student's *t*-test (two-tail). Multiple logistic regression analysis was preformed by SPSS for Macintosh to identify independent predictors for CR.

RESULTS

Response to IFN treatment (Table 1)

Twenty-five of 27 patients completed IFN treatment. One discontinued IFN because of retinal hemorrhage, the other could not visit our hospital due to moving. All patients had fever, general fatigue or myalgia. However, no severe adverse effects were seen. Eight patients (32%) maintained normal serum ALT levels with negative serum HCV-RNA after IFN treatment, resulting in CR. Ten patients (40%) were negative for HCV-RNA at the end of treatment but relapsed after the discontinuation of IFN (PR). The other 7 patients (28%) were still positive for HCV-RNA at the end of IFN treatment (NR). The distribution of gender, age and serum ALT levels was not significantly different among three groups. Neither grade of activity nor staging of fibrosis in liver histology was different. Low serum viral load and genotypes other than 1b were associated with favorable response ($P < 0.05$).

HCV-RNA in PBMC (Table 2)

We analyzed the presence of HCV-RNA in PBMC by two-step RT-PCR. All patients were positive for HCV-RNA in PBMC, whereas none of the healthy subjects was positive.

Three patients (12%) revealed 1+; 2 with low serum viral load and 1 with high viral load. The other 22 patients (88%) resulted in 2+. The amount of HCV-RNA in PBMC before the addition of IFN- β was not related to clinical response to IFN.

Table 1 Demographic characteristics and clinical response

Variable	NR ^a (n=7)	PR (n=10)	CR (n=8)	P value
Sex (F:M)	4:3	8:2	6:2	NS
Age (mean \pm SD)	57.2 \pm 6.7	51.4 \pm 7.2	44.4 \pm 15.7	NS
Serum ALT levels (mean \pm SD)	68.5 \pm 39.5	95.4 \pm 67.9	90.9 \pm 40.1	NS
Liver histology				
Activity index				
A1	4	3	1	NS
A2	2	6	7	
A3	1	1	0	
Fibrosis index				
F1	3	5	5	NS
F2	3	3	2	
F3	1	2	1	
Serum viral load ^b				
Low	1	6	6	<0.05
High	6	4	2	
Genotype				
1b	6	7	2	<0.05
Others	1	3	6	

^aComplete response (CR): sustained loss of serum HCV-RNA and normal ALT levels, partial response (PR): disappearance of serum HCV-RNA at the end of treatment and reappearance after treatment, and no response (NR): positive serum HCV-RNA at the end of treatment. ^bhigh: $\geq 10^8$ copies/mL, low: $< 10^8$ copies/mL.

Table 2 Viral load in PBMC and clinical response

Variable	NR ^a (n=7)	PR (n=10)	CR (n=8)	P value
Viral load ^b (pretreatment)				
-	0	0	0	NS
1+	0	2	1	
2+	7	8	7	
Decrease of HCV-RNA by IFN- β ^c				
Yes	1	9	8	<0.05
No	6	1	0	

^aComplete response (CR): sustained loss of serum HCV-RNA and normal ALT levels, partial response (PR): disappearance of serum HCV-RNA at the end of treatment and reappearance after treatment, and no response (NR): positive serum HCV-RNA at the end of treatment. ^bThe amount of HCV-RNA in PBMC was determined semi-quantitatively as follows; 2+: positive after first PCR, 1+: negative after first PCR and positive after second PCR, and -: negative after first and second PCR. ^cHCV-RNA amount in PBMC was semi-quantitatively determined before and after incubation with IFN- β (100 IU/L).

We studied the effect of *in vitro* administration of IFN- β on HCV in PBMC. We used 100 IU/L as a concentration of IFN- β because preliminary experiments revealed that 10 IU/L of IFN- β was insufficient to decrease HCV-RNA in PBMC and that 1 000 IU/L of IFN- β did not have more effects than 100 IU/L of IFN- β (data not shown). After incubation with IFN- β the HCV-RNA amount in PBMC decreased in 18 patients (72%), 7 patients: 2+ to -; 8 patients: 2+ to 1+; and 3 patients: 1+ to -. Whereas no decrease was observed in the other 7 patients (28%). Incubation without IFN- β did not lead

to decrease in the amount of HCV-RNA (data not shown), suggesting that this decrease was attributable to the antiviral effect of IFN- β .

We analyzed the relationship between the effect of IFN- β on HCV in PBMC and clinical response. Patients with HCV-RNA decrease were significantly associated with better clinical response, 8 (44%) and 9 patients (50%) obtained CR and PR, respectively. On the other hand, 6 of 7 patients (86%) whose HCV-RNA amount did not decrease by IFN resulted in NR. Multiple logistic regression analysis revealed that the decrease in HCV-RNA amount in PBMC was the only independent predictor for CR ($P < 0.05$).

DISCUSSION

In our study 8 patients (32%) obtained CR. IFN- β therapy was as effective as IFN- α in the treatment of chronic hepatitis C as already reported^[17-19]. In one patient (4%) IFN treatment was discontinued due to retinal hemorrhage, which was the only major adverse effect observed in this study. Retinopathy was one of the common adverse effects of IFN^[20,21]. Low serum viral load and viral genotypes other than 1b were associated with good clinical response in consistent with other studies using IFN- α ^[22-25].

We could detect HCV-RNA in PBMC before IFN therapy in all cases. HCV-RNA was not found in PBMC from healthy subjects, suggesting that the technique we used was specific for HCV. Several studies demonstrated the presence of HCV-RNA in PBMC^[26-30], although the positive rate was variable from 25% to 100%. The *in vitro* administration of IFN- β decreased HCV-RNA in PBMC in 72% patients. Because incubation with medium alone did not affect HCV-RNA amount, this decrease would be attributable to IFN's antiviral effect. In fact Pawlotsky *et al* reported that 2-5AS activity in PBMC from patients with chronic hepatitis C was augmented by incubation with IFN^[10]. Furthermore a preliminary experiment showed that *in vitro* addition of IFN at a concentration of 1 000 IU/mL reduced the HCV-RNA amount in PBMC^[30]. In our study 7 patients (28%) maintained the same amount of HCV-RNA in PBMC even after incubation with IFN. It should be noted that none of these patients was complete responders. The reason why IFN could not decrease HCV-RNA in these patients is unclear. One possibility is that IFN could not induce antiviral enzymes such as 2-5AS. In this case the breakdown of IFN-induced antiviral system might be responsible. A more likely explanation is that HCV strains in these patients were resistant to antiviral enzymes. Some strains such as those with mutations in NS5A region^[31] are refractory to IFN.

Clinical outcome was significantly different between those with decreased HCV-RNA amount in PBMC and those without. Multivariate analysis demonstrated the decrease of HCV-RNA amount in PBMC by IFN as the only independent predictive factor for CR. These data suggest that the effect of *in vitro* IFN- β on HCV-RNA in PBMC reflects clinical response and would be taken into account as a predictive marker of IFN therapy for chronic hepatitis C.

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