

Attenuation of Responsiveness to Interferon- α Treatment by Preceded Overactivation of Interferon-mediated Pathway in Patients Chronically Infected by Hepatitis C Virus

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The Interferon (IFN) receptor-mediated signal transduction pathways involve the two novel DNA-binding factors, interferon regulatory factor-1 (IRF-1) and IRF-2. Both recognize the same DNA sequence, in which the expression ratio of IRF-1 to IRF-2 is relevant to induction of IFN-inducible genes, because IRF-1 acts as a transcriptional activator and IRF-2 as a counterpart. In the present study, 54 patients with chronic hepatitis C and the age- and sex-matched 7 subjects with fatty liver as a control were subjected to analysis of the expression ratio of IRF-1 to IRF-2 mRNA in the liver tissue obtained at the time of liver biopsy by reverse transcription-polymerase chain reaction in combination with a restriction fragment length polymorphism assay. The expression ratio of IRF-1 to IRF-2 mRNA in the liver tissue in patients chronically infected by hepatitis C virus (HCV) was significantly higher than that in control, although the values did not correlate with the serum levels of HCV-RNA. Of 54 patients, 28 received IFN treatment, resulting in complete response in 8 patients. With respect to responsiveness to IFN treatment, patients who had complete response had the relatively lower ratios of IRF-1 to IRF-2 mRNA in the liver tissue, compared with those who did not. These results indicate that the IFN-mediated pathway is spontaneously activated in patients with chronic hepatitis C, and that its preceded overactivation counteracts on the efficacy of IFN treatment in these patients.

Key Words: interferon treatment, chronic hepatitis C, interferon regulatory factor-1, interferon regulatory factor-2, hepatitis C virus

Introduction

Interferon (IFN) treatment succeeds in viral eradication in 20-30% of patients chronically infected by hepatitis C virus (HCV) ¹⁻⁴⁾. Its efficacy is closely associated with low levels of viremia or mutations in the HCV-RNA sequence, especially in the NS5A region ^{5,6)}. However, the relationship between antiviral states against HCV and response to IFN is still unclear. Recent advances in molecular biology lead to a great deal of progress in host-defense mechanisms against viral infections. In the IFN system, both interferon regulatory factor-1 (IRF-1) and IRF-2 genes are induced by viral infections or by IFNs or, to some degrees, other cytokines ⁷⁻¹⁰⁾. Both gene products act as the transcriptional factors which recognize the same DNA sequence. However, since IRF-1 functions as a transcriptional activator and IRF-2 as a counterpart, the expression ratio of IRF-1 to IRF-2 is relevant to induction of IFN-inducible gene expression ⁷⁻⁹⁾. In the present study, the expression ratios of IRF-1 to IRF-2 mRNA in the liver tissue were analyzed in patients with chronic hepatitis C.

Patients and Methods

Patients population

The sample population included 54 patients chronically infected by HCV who were admitted to our hospital

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between 1994 and 1995; 39 were men and 15 were women, and they were 24 to 75 years of age (mean [\pm SD], 50 \pm 13). The 54 patients were positive for HCV-RNA by reverse transcription-polymerase chain reaction (RT-PCR) and antibody to HCV, but negative for hepatitis B surface antigen. Informed consent was obtained from all patients. Liver biopsies were performed, and the diagnosis of chronic hepatitis was made on the basis of the histological findings in liver tissue in all patients. The serum levels of HCV-RNA were determined by a branched DNA (bDNA) assay. We also studied the age- and sex-matched 7 subjects with fatty liver who were admitted to our hospital during the same period and gave informed consent. There were 5 men and 2 women, aged 36 to 61 years (mean [\pm SD], 50 \pm 10). The 7 control subjects were negative for any viral markers tested or autoantibodies to nuclear antigens or mitochondrial antigens. Any of alcohol or drug abusers were excluded from the study.

IFN treatment

IFN treatment was performed in 28 of 54 patients with chronic hepatitis C. The 28 patients received IFN- α for 6 months in a dose of 6 million to 9 million units intramuscularly everyday for 14 days and then three times a week for 22 wk (total dose, 480 million to 720 million units). All patients were followed, and those who persisted to become negative for HCV-RNA by RT-PCR together with normalization of liver function tests for more than 1 year after cessation of IFN treatment were defined as complete responders as described previously⁶. According to the definition, 8 of 28 patients had complete response, whereas 20 had no response because they persisted or returned to be positive for HCV-RNA during the follow-up period.

Analysis of expression ratio of IRF-1 to IRF-2 mRNA in liver tissue

The liver tissue specimens obtained at the time of liver biopsy were frozen at -70°C immediately after the time of liver biopsy for later analysis of the expression ratio of IRF-1 to IRF-2 mRNA in the liver tissue by RT-PCR in combination with a restriction fragment length polymorphism assay^{11,12}. Total cellular RNA was isolated from the frozen samples by the guanidium isothiocyanate method. Two μ g of total RNA in a volume of 10 μ l was heated at 68°C for 3 min, chilled on ice, then added in a reaction mixture containing 50 μ M Tris-HCl (pH8.3), 75mM KCl, 3mM MgCl₂, 200 μ M each of dNTPs, 100pmol random hexamer, 8U of RNase inhibitor, and 200U of M-MLV reverse transcriptase to a

volume of 20 μ l. The RT reaction mixture was incubated at 37°C for 90 min, heated at 95°C for 5min, and chilled on ice. The two pairs of oligonucleotide primers, by which IRF-1 and IRF-2 DNA sequences having a relatively higher homology⁹ (nucleotide 302-508 and nucleotide 162-368, respectively) were amplified in the same length (207bp) and only the IRF-2 sequence was digested by a restriction enzyme, Nsi-I (which recognizes ATGC A/T), resulting in 155bp and 52bp fragments, were used in this study as follows: for IRF-1, 5'-CCGGGGCTCATCTGGATTAA-3' and 5'-ATCTGGCAGG-GAGTTCATGG-3', and for IRF-2, 5'-CCGGGGCTCAAG-TGGCTTAA-3' and 5-ATCAGGCAAGGAATTCATGG-3'. Briefly, the PCR was performed in a reaction volume of 50 μ l containing 2 μ l of the RT reaction product, 50mM KCl, 10mM Tris (pH9.0), 1.5mM MgCl₂, 0.1% Triton X-100, 200 μ M each of dNTPs, 25pmol each of PCR primers and 2.5IU of Taq DNA polymerase. The amplification was performed by 25 cycles in a programmable DNA thermal cycler. Each reaction cycle involved denaturation at 92°C for 30 sec, primer annealing at 60°C for 30 sec and primer extension at 72°C for 1 min. In the last cycle, the reaction at 72°C continued for 10 min to ensure complete DNA extension. The PCR product was extracted with phenol-chloroform and precipitated with ethanol. The precipitated product was digested by 5IU of Nsi-I at 37°C for 2hr, and the digested product was separated by electrophoresis on a 2% agarose gel. The separated bands corresponding to IRF-1 and IRF-2 DNA fragments were visualized with ethidium bromide staining. The intensity of each band was quantified by densitometry with a image program from Macintosh¹³. When IRF-1 cDNA and IRF-2 cDNA (generous gifts from professor T.Taniguchi, Tokyo University, Japan) were mixed to yield different ratios and the mixed samples were subjected to the same analysis, the ratio of IRF-1 to IRF-2 determined by this method closely correlated with that in the mixed sample prepared before analysis (Figure 1). Accordingly, each assay always contained the prepared DNA samples with the different ratios of IRF-1 to IRF-2 to make a standard curve, and the expression ratio of IRF-1 to IRF-2 in each liver tissue sample was calculated from the standard curve.

The statistical analyses were performed with Student's t-test and the chi-square test. All P values were two-tailed, and P values of less than 0.05 were considered to indicate statistical significance.

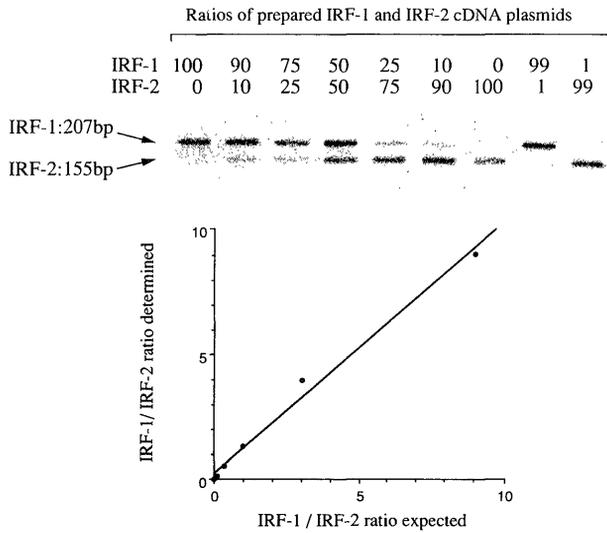


Figure 1. Correlation between the ratios of IRF-1 to IRF-2 determined by our method and those expected before analysis. IRF-1 cDNA and IRF-2 cDNA were mixed to obtain the indicated ratios. The prepared samples were subjected to analysis as described in Methods.

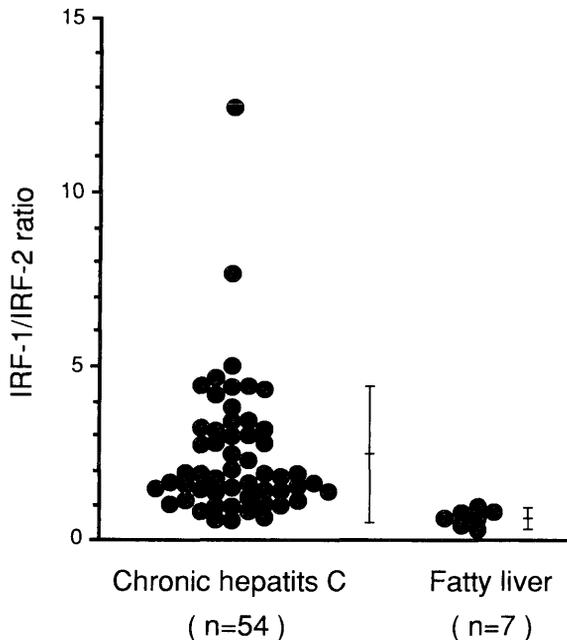


Figure 2. Hepatic expression ratios of IRF-1 to IRF-2 mRNA in patients with chronic hepatitis C and those with fatty liver. The expression ratios were determined in 54 patients with chronic hepatitis C and 7 patients with fatty liver as a control as described in Methods. Bars are means \pm SD.

Results

Elevated expression ratio of IRF-1 to IRF-2 mRNA in the liver tissue in patients with chronic hepatitis C

The expression ratio of IRF-1 to IRF-2 mRNA in the liver tissue was significantly higher in patients with

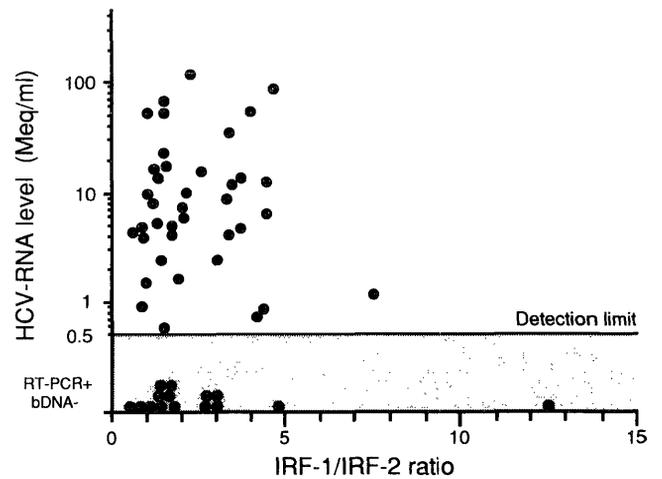


Figure 3. Correlation between the expression ratio of IRF-1 to IRF-2 mRNA in the liver tissue and the serum level of HCV-RNA in patients with chronic hepatitis C. The HCV-RNA level was assayed using a branched DNA assay, and its detection limit was 0.5 megaequivalent per milliliter (Meq/ml).

chronic hepatitis C than in control (mean [\pm SD], 2.48 ± 1.97 vs. 0.63 ± 0.34 , $P < 0.01$), although the ratios in patients ranged widely from 0.57 to 12.3 (Figure 2). The serum levels of HCV-RNA were determined by a branched DNA (bDNA) assay. Among 54 patients, the values were less than 0.5 Meq/ml (a detection limit) in 15 patients and ranged from 0.6 to 126 Meq/ml in 39 patients. When the expression ratios of IRF-1 to IRF-2 mRNA in the liver tissue were compared with the serum levels of HCV-RNA in the 54 patients, no correlation was observed (Figure 3).

Analysis of factors involved in response to IFN in patients with chronic hepatitis C

Twenty-eight of 54 patients received IFN treatment. Of 28 patients, 8 had complete response, because these patients persisted to become negative for HCV-RNA together with normalization of liver function tests for more than 1 year after cessation of IFN treatment. The age or sex did not correlate with outcome of IFN treatment (Table 1). However, sustained response to IFN was observed more frequently in patients who had relatively low levels of HCV-RNA with less than 1 Meq/ml at baseline, compared with those who did not ($P < 0.01$). To evaluate the correlation between the expression ratio of IRF-1 to IRF-2 mRNA in the liver tissue and response to IFN, the cutoff value for the ratio was set at 2.5, which was the mean value in the 54 patients chronically infected by HCV. Seven (47%) of 15 patients who had the ratios of less than 2.5 resulted in complete response, whereas only 1 (8%) of 13 patients who had the ratios of 2.5 or more did. The difference was

statistically significant ($P < 0.05$). Moreover, when the ratio was combined with the baseline serum level of HCV-RNA for an index of response to IFN, 6 (86%) of 7 patients who had both the ratio of less than 2.5 and the aseline HCV-RNA level of less than 1Meq/ml resulted in complete response, whereas only 2 (10%) of 21 patients who did not had complete response ($P < 0.001$).

Table 1 Factors involved in responses to interferon in 28 patients

Variable	No. of patients	No. of complete responders (%)	χ^2
Age			
<50yr.	19	5 (26%)	$p=0.7011$
≥ 50 yr.	9	3 (33%)	
Sex			
Male	19	6 (32%)	$p=0.6088$
Female	9	2 (22%)	
Baseline HCV level			
< 1 Meq/ml	13	7 (54%)	$p=0.0059$
≥ 1 Meq/ml	15	1 (7%)	
IRF-1/IRF-2 ratio			
< 2.5	15	7 (47%)	$p=0.0228$
≥ 2.5	13	1 (8%)	
Combination of HCV level and IRF-1/IRF-2 ratio			
Both HCV < 1 Meq/ml and ratio < 2.5	7	6 (86%)	$p=0.0001$
Others	21	2 (10%)	

The definition of complete response was mentioned in Methods. The statistical analysis was carried out using the chi-square test.

Discussion

In this study, the expression ratio of IRF-1 to IRF-2 mRNA in the liver tissue in patients with chronic hepatitis C was significantly higher than that in control. In the IFN system, IRF-1 and IRF-2 regulate both IFN genes and IFN-inducible genes coordinately⁷⁾. IFNs bind to their receptors and stimulate IRF-1 gene expression. IRF-1 activates expression of IFN genes, resulting in further induction of IRF-1. However, since the 5'-upstream regulatory sequence of the IRF-2 gene contains the IRF response element, IRF-1 overexpression subsequently induces IRF-2 expression which represses IRF-1 function by competing in the same cis-acting element. In addition, the half life of IRF-1 mRNA is much shorter than that of IRF-2 mRNA⁷⁾. Accordingly, IRF-2 expression is always predominant over IRF-1 expression in quiescent cells without stimulation, while ongoing stimulation leads to the increased expression ratio of IRF-1 to IRF-2. Thus, our result seems to indicate that the IFN-mediated pathway in the liver tissue is steadily activated in patients chronically infected by HCV. The expression ratios of IRF-1 to IRF-2 mRNA in

the liver tissue did not correlate with the serum levels of HCV-RNA in the 54 patients chronically infected by HCV. The lack of correlation is difficult to explain given our current understanding of the antiviral states induced by IFN. One possible explanation is that there are mechanisms underlying IFN-induced antiviral activities independent of IRF-1¹⁴⁻¹⁶⁾, although the activation of IFN-mediated pathways is manifested by the increased expression ratio of IRF-1 to IRF-2. Consistent with this, several investigators reported that IRF-1 was necessary for the antiviral actions of IFN against some viruses, but did not affect replication of other types of viruses¹⁷⁾. In addition, current studies have shown that the variants of HCV-1b containing mutations in the NS5A region are susceptible to IFN^{5,6)}. The wild-type of the NS5A product can bind to the IFN-inducible double-stranded RNA-dependent protein kinase, a potent inhibitor of viral replication¹⁸⁾, and suppresses its function¹⁹⁾. In contrast, the mutant viral gene products cannot, resulting in sensitizing the variant viruses to IFN¹⁹⁾. This may, in part, account for the lack of correlation between the activation of the IFN-mediated pathway and the serum level of HCV-RNA, although the HCV-RNA sequences were not determined in this study.

By the analysis of factors involved in response to IFN in patients with chronic hepatitis C, the relatively lower level of viremia prior to IFN treatment was associated with complete response to IFN in this study. Similar results were described previously, where IFN treatment succeeded in viral eradication in 50-60% of patients with the baseline HCV-RNA levels of less than 1Meq/ml, but in only approximately 10% of patients with relatively higher levels of viremia¹⁻⁴⁾. With respect to relationship between the expression ratio of IRF-1 to IRF-2 mRNA in the liver tissue and response to IFN, complete response was observed more frequently in patients who had the ratios of less than the cutoff value corresponding to the mean value in the 54 patients chronically infected by HCV, compared with those who did not. Moreover, 6 (87%) of 7 patients who had both the low level of viremia and the expression ratio of less than the cutoff value resulted in complete response, in contrast to only 2 (10%) of 21 patients who did not. It is, therefore, possible that the measurement of the expression ratio of IRF-1 to IRF-2 mRNA in the liver tissue, particularly combined with the baseline serum level of HCV-RNA, serves as a predictive marker for response to IFN in patients chronically infected by HCV.

In summary, our results suggest that the activation of the IFN-mediated pathway manifested by the increased expression ratio of IRF-1 to IRF-2 mRNA in

the liver tissue occurs in a large portion of patients chronically infected by HCV, and that its preceded overactivation is related with attenuated response to IFN in these patients. The host-defense mechanisms against HCV are unlikely to result from a single-step event, but rather represent a complex process involving IFN system²⁰⁻²². Given these conditions, viral gene products or altered products caused by viral gene mutations are considered to modify the IFN-induced antiviral activities, although the IFN-mediated activation pathways play a crucial role in viral clearance.

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