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DOI: 10.1515/ii-2017-0037

Original Article

Inhibition on IFN-β Expression by HCV NS3 and NS5A in HepG2 Cells

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Objective To observe the effects of HCV protein, NS3 and NS5A on IFN-β in HepG2 cells and its regulation mechanism.

Methods Human liver hepatocellular carcinoma cells HepG2 were transfected with recombinant eukaryotic plasmid pcDNA3.1/myc-His-core, NS3 or NS5A to overexpress these proteins, and the expression of IFN-β were detected by qRT-PCR, Western blotting and ELISA. Luc2P reporter plasmids pGL4.10-IFNβ-P were constructed and transfected into HepG2 cells, and the activity of IFN-β promoter were determined through luciferase assay for regulation mechanism study.

Results Both mRNA level and protein expression of IFN-β were significantly decreased \( (P < 0.05) \) in the presence of NS3 or NS5A protein. Luciferase assay revealed that NS3 or NS5A protein downregulated IFN-β promoter activity \( (P < 0.05) \). Meanwhile, HCV core protein had little effect on IFN-β expression.

Conclusions HCV protein NS3 and NS5A could inhibit innate IFN-β expression and thus escape immune selection and hinder the host immune responses.

Key words: Hepatitis C; Viral nonstructural proteins; Interferon-beta; Gene expression regulation

Hepatitis C virus (HCV) is an important human pathogen that may cause acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma worldwide. At least 200 million people are currently infected with HCV, accounting for 3.3% of the world population.1 According to statistics, there are about 4 million people newly infected with HCV every year.2 Hepatocytes, as hepatitis viruses-harboring cells, are the main site of HCV replication, which contain the maximum viral load in infected peoples. Human liver hepatocellular carcinoma cells HepG2 are reported to possess the potential to inductively express interferon-beta (IFN-β).1 The large HCV polyprotein is cleaved by proteases into at least 10 proteins, including four structural proteins (core, E1, E2 and p7) and six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). This positively stranded RNA virus is extremely efficient in establishing persistent infection by escaping immune detectionor and hindering the host immune responses. There are two important signaling pathways that activate the host innate immunity against viral infection. One utilizes Toll-like receptor (TLR) family and the other take the RNA helicase retinoic acid inducible gene I (RIG-I) as the receptors for intracellular viral double stranded RNA (dsRNA).4,5 Data from PubMed and Google Scholar suggested that HCV proteins core, NS3/4A and NS5A have direct interactions with these two pathways. Therefore in the present study, we focus on the effects of core, NS3 and NS5A on IFN related genes. We identified IFNB1 (the coding gene for IFN-β) was down-regulated significantly by NS3 and NS5A protein through PC-PCR, Western blotting and ELISA. Luc2P reporter plasmids pGL4.10-IFNβ-P were constructed and transfected into HepG2 cells, and the activity of IFN-β promoter were determined through luciferase assay for regulation mechanism study.

Materials and Methods

Cell culture and transient transfection

HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum in a humidified chamber at 37 °C and 5% CO2. The pcDNA3.1/myc-His-core, pcDNA3.1/myc-His-NS3 and pcDNA3.1/myc-His-NS5A which contain the complete coding region of HCV core, NS3 and NS5A proteins (1b genotype) and pcDNA3.1/myc-His (vector set as negative control) were conserved by Institute of Infectious Diseases, Beijing Ditan Hospital,
Capital Medical University. Cells were seeded into 6-well plates at 70% confluence 24 hours before transfection. And then cells were transfected with 2µg of the above four plasmids using jetPRIME transfection reagent (Polyplus – transfection, France) according to the manufacturer’s protocols.

**RNA isolation and qRT-PCR analysis**

Total RNA was extracted from cultured cells 24 hours after transfection through Maxwell 16 Total RNA Purification Kit (Promega, America) according to the manufacturer’s instructions. A total of 2 µg RNA from each sample was applied to generate cDNA by reverse transcription with the One-step RT-PCR kit (TaKaRa, Japan). A SYBR real-time quantitative PCR assay was performed on an ABI Prism 7500 following the manufacturer’s protocol. β-actin was taken as reference for normalizing data. Primer sequences were shown in Table 1. The cycle parameters were as follows: 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 1 min; 40 cycles. The PCR products were electrophoresed in a 1.5% agarose gel containing ethidium bromide and visualized by UV illumination. Each sample was tested in triplicate.

**Western blotting analysis for IFN-β protein**

The HepG2 cells were collected, washed with PBS and lysed in ice-cold lysis buffer (20 mmol/L Tris HCl, pH 7.5, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Nonidet P-40, 1 mg/L Aprotinin, 100 mg/L PMSF). The protein concentration of whole cell extract was measured through Bio-Rad DC protein assay kit. Equal amounts of cell lysates (80 µg protein) were separated by 10% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked for 2 hours in TBS containing 5% nonfat milk and 0.1% Tween-20 and then incubated with each specific primary antibody, mouse anti-human IFN-β (Santa Cruz Biotechnology) and mouse anti-human β-actin (Santa Cruz Biotechnology) overnight at 4 °C. After washing thrice with TBS-T for 10 minutes, the membrane was incubated with the appropriate HRP-conjugated secondary antibody (goat anti-mouse from Santa Cruz Biotechnology), diluted in TBS-T for 1 hour at room temperature and washed 3 more times. Proteins were visualized with Pierce ECL Western Blotting Substrate. Cells treated with poly (I:C) were set as positive control.

**Detection of soluble IFN-β by ELISA**

The cell culture supernatant from HepG2 cells transfected with pcDNA3.1/myc-His-core, pcDNA3.1/myc-His-NS3 and pcDNA3.1/myc-His-NS5A for 48 hours were collected and the levels of IFN-β was detected by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s protocols (CSB-E09889h, cusabio). Cells treated with poly (I:C) were set as positive control.

**Genomic amplification and construction of expression plasmids**

Genomic DNA was isolated from HepG2 cells using a Genomic DNA Purification kit (Promega, USA). A series of 5'-flanking DNA fragments upstream of the transcription initiation site of IFN-β (−1433 bp

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
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<tbody>
<tr>
<td>IRF3</td>
<td>sense: 5'-ACCAGCCGTGGGACCAAGAG-3'</td>
</tr>
<tr>
<td></td>
<td>antisense: 5'-TACCAAGGCCCTGGAC-3'</td>
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<tr>
<td>IRF7</td>
<td>sense: 5'-TGTCCTCTGCTGAAGCTGAA-3'</td>
</tr>
<tr>
<td></td>
<td>antisense: 5'-GTGGTCTGATAGAGGCTG-3'</td>
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<tr>
<td>IRF9</td>
<td>sense: 5'-CCCGAAAATCTCCGAAACTG-3'</td>
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<tr>
<td></td>
<td>antisense: 5'-CAGCACACTCGCGAAACT-3'</td>
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<tr>
<td>STAT1</td>
<td>sense: 5'-GTGGAAAGACACAGCTGCA-3'</td>
</tr>
<tr>
<td></td>
<td>antisense: 5'-ACTGGACCCCCTGTCTCTCAA-3'</td>
</tr>
<tr>
<td>STAT2</td>
<td>sense: 5'-CGACCAGGCACTGGGAGGCG-3'</td>
</tr>
<tr>
<td></td>
<td>antisense: 5'-TCACTCTCAGGCAACTGGATAGG-3'</td>
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<td>OAS1</td>
<td>sense: 5'-CTTGCCAGACACGTGGTCTCCC-3'</td>
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<td>antisense: 5'-TGGAGCGGACACTGAGCAAAGG-3'</td>
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</tr>
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<tr>
<td></td>
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<td>PKR</td>
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<td>sense: 5'-CTTCTGTTTTTTCAGAAGATTCA-3'</td>
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<tr>
<td></td>
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<td>antisense: 5'-ATGGCCCAAGGCTCTCCTG-3'</td>
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<tr>
<td></td>
<td>antisense: 5'-AGTACCTTGGGCCTGAGGAG-3'</td>
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- + 83 bp) were inserted into Eco R V and Hind III restriction sites of a pGL-4.10-basic vector (Promega, USA). The PCR primers were as follows, sense: 5’- GATATCACAAGCTCCCTGCTGATGG -3’; antisense: 5’- AAGCTTTCGCCTACTACCTGTTGTG -3’.

**Luciferase reporter assay**

HepG2 cells were seeded into 48-well plate at 70% confluence 24 hours before transfection. Cells were co-transfected with 0.083 μg pGL4.10-IFNB1, 0.0083 μg internal control phRL-TK plasmids, and 0.166 μg pcDNA3.1/myc-His-core, pcDNA3.1/myc-His-NS3 or pcDNA3.1/myc-His-NS5A through jetPRIME transfection reagent (Polyplus-transfection, France) according to the manufacturer’s protocols. At 24 hours post-transfection, cells were washed with PBS and lysed in 50 μl passive lysis buffer. Firefly luciferase activity and Renilla luciferase activity were measured by the Dual-Luciferase reporter assay system (Promega, USA). Cells treated with poly(I:C) were set as positive control. All transfections were performed in triplicate, and the promoter activities were expressed as mean ± SD in three independent experiments.

**Statistical analysis**

All data are shown as mean ± SD. The differences between test group and control group were analyzed using the Student’s t test. *P < 0.05 was considered statistically significant.

**RESULTS**

**mRNA expression of IFN signaling pathway-related genes**

To understand the effect of HCV proteins core, NS3 and NS5A on mRNA expression of IFN signaling pathway related genes, we analyzed the relative mRNA level in HepG2 cells through RT-PCR. Among the 11 genes: IRF3, IRF7, IRF9, STAT1, STAT2, OAS1, OAS2, OAS3, PKR, IFNB1 and MxA, we found that only IFN-β expression was down-regulated by 63% and 54% in NS3 and NS5A group, respectively (n = 3, *P < 0.05) (Figure 1), and mRNA levels of the rest genes between the negative control group and NS3/NS5A.
Figure 3. Influences of HCV NS5A protein on IFN signal pathway-related genes.
Notes: Total RNA from HepG2 cells transfected with pcDNA3.1/myc-His-NS5A for 24 h was analyzed for the expression of IFN signal pathway-related genes by qRT-PCR. The values from control samples were set to 1. Statistical significance was determined using Student’s t test. *P < 0.05 (n = 3).

Figure 4. Influences of HCV Core protein on IFN signal pathway-related genes.
Notes: Total RNA from HepG2 cells transfected with pcDNA3.1/myc-His-core for 24 h was analyzed for the expression of IFN signal pathway-related genes by qRT-PCR. The values from control samples were set to 1. Statistical significance was determined using Student’s t test. *P < 0.05 (n = 3).

Figure 5. Downregulation of IFN-β expression by HCV proteins.
Notes: Total protein lysates were obtained from HepG2 cells transfected with pcDNA3.1/myc-His-core, pcDNA3.1/myc-His-NS3 and pcDNA3.1/myc-His-NS5A for 48 hours and the levels of IFN-β protein by Western blotting were analyzed. Band intensities of IFN-β were quantified by the Image J program, and the values from control samples were set to 1.
A: Western blotting was used to verify the expression of HCV core, NS3 and NS5A in HepG2 cells after transfection. B: The expression of IFN-β after overexpress HCV core, NS3 and NS5A.
NS5A group had no significant difference (n = 3, P > 0.05) (Figure 2 and 3). None of those genes were significantly regulated by core (Figure 4).

**HCV NS3 and NS5A decrease IFN-β expression in HepG2 cells**

Based on the results of RT-PCR, we know that IFN-β expression was inhibited by HCV protein NS3 and NS5A. To further verify these results, the expression levels of IFN-β protein in HepG2 cells were measured by Western blotting and ELISA analysis. Compared with the control group, IFN-β expression was almost 4.59 folds of poly (I:C) group, and 0.71, 0.61 folds of NS3, NS5A group by Western blotting, respectively (n = 3, P < 0.05) (Figure 5). While IFN-β expression was almost 5.12 folds of poly (I:C) group, and 0.80, 0.76 folds of NS3, NS5A group detected by ELISA, respectively (n = 3, P < 0.05) (Figure 6). But the impact of core protein was not significantly different, which were coincident with previous results.

**Transcriptional activity of IFN-β promoter in HCV core/NS3/NS5A positive or negative cells**

To clarify the mechanism underlying suppression by HCV proteins on IFN-β, we detected the activity of IFN-β promoter. The IFN-β promoter sequence was amplified by PCR using human genomic DNA as template. As shown in Figure 7, a 1516 bp visible fragment consistent with the predicted size was obtained by electrophoresis. Sequencing analysis confirmed that the cloned gene was identical with the original sequence in GenBank (accession number NM002176). The IFN-β promoter was put upstream of the firefly luciferase gene in the pGL4.10-basic

![Figure 6. Downregulation of IFN-β expression by HCV protein NS3 and NS5A.](image)

Notes: The cell culture supernatant from HepG2 cells transfected with pcDNA3.1/myc-His-core, pcDNA3.1/myc-His-NS3 and pcDNA3.1/myc-His-NS5A for 48 hours were collected and the levels of IFN-β were detected by enzyme-linked immunosorbent assay (ELISA). The values from control samples were set to 1.

![Figure 7. PCR product of IFN-β promoter.](image)

Notes: A 1516 bp visible fragment consistent with the predicted size was observed by electrophoresis. DL2000 marker; 1: PCR products.

![Figure 8. Analysis of dual enzymatic digestion of pGL4.10-IFNB1 by agarose gel electrophoresis.](image)

Notes: A 1512 bp visible fragment consistent with the predicted size was observed by electrophoresis. Lane 1: the plasmid of pGL4.10-IFNB1. Lane 2, 3: the plasmid of pGL4.10-IFNB1 was digested by EcoRV and Hind III, and fragment of 1516 bp was obtained.
vector which lacks eukaryotic promoter and enhancer sequences. The resultant pGL4.10-IFNB1-Luci plasmid was confirmed by double enzyme digestion with EcoRI and HindIII (Figure 8). To examine the transcriptional activity of IFN-β gene promoter, HepG2 cells were co-transfected with 0.083 μg pGL4.10-IFNB1, 0.0083 μg TK and 0.166 μg pcDNA3.1/myc-His-(-), pcDNA3.1/myc-His-core, pcDNA3.1/myc-His-NS3 or pcDNA3.1/myc-His-NS5A. As a positive control, 25 ng/ml poly (I:C) were added to the culture medium. HepG2 cells co-transfected with pGL4.10, pcDNA3.1/myc-His-(-) and TK were set as blank control. Twenty-four hours after transfection, cells were harvested and luciferase activity was measured. The normalized luciferase activities were calculated by assigning a value of 1 to the levels of activity of cells that were co-transfected with pcDNA3.1/myc-His-(-) and pGL4.10-IFNB1. *P < 0.05.

**Figure 9. Effects of HCV protein core, NS3, NS5A on IFN-β promoter activity.**

Notes: HepG2 cells were co-transfected with 0.083 μg pGL4.10-IFNB1, 0.0083 μg TK and 0.166 μg pcDNA3.1/myc-His-(-), pcDNA3.1/myc-His-core, pcDNA3.1/myc-His-NS3 or pcDNA3.1/myc-His-NS5A. As a positive control, 25 ng/ml poly (I:C) were added to the culture medium. HepG2 cells co-transfected with pGL4.10, pcDNA3.1/myc-His-(-) and TK were set as blank control. Twenty-four hours after transfection, cells were harvested and luciferase activity was measured. The normalized luciferase activities were calculated by assigning a value of 1 to the levels of activity of cells that were co-transfected with pcDNA3.1/myc-His-(-) and pGL4.10-IFNB1. *P < 0.05.

Discussion

As the defense mechanism of the organism to viral infection, interferon is an extremely important part, especially IFN-β, the first key factor in the innate immune response against viral infection. When virus infects the host, the virus RNA could be recognized by the pathogen-associated molecular patterns (PAMPs) such as Toll-like receptors (TLRs), retinoic acid-inducible gene 1 (RIG-1) and the melanoma differentiation-associated gene 5 protein (MDA5). Among these receptors, TLRs recruited the adaptor molecule myeloid differentiation primary-response gene 88 (MyD88) and Toll/IL-1 receptor domain-containing adaptor inducing IFN (TRIF), while RIG-I and MDA-5 recruited virus-induced signaling adapter (VISA), then through TANK-binding kinase 1 (TBK1) or IκB kinase-ε (IKK-ε) phosphorylate IRF-3 and finally induce IFN-β transcription. Then, IFN-β induced the IFN-regulated genes responsible for the antiviral response. But as for virus, the inhibition of IFN production is an indispensable function against the defense mechanism. During the co-evolution with the host cells, many viruses encoded proteins to inhibit the interferon production, making it difficult for host cells to clear viruses.

The mechanisms for persistent viral infection maybe the destruction of host innate immune response against viruses. Many researches suggested that HCV protein core, NS3/4A and NS5A had effects on host innate immune signaling pathway, which provide conditions for viruses to escape immune response. Among more than 10 HCV proteins, only NS5B, a viral RNA-dependent RNA polymerase (RdRp), activated the IFN-β promoter, NS4A, NS4B and NS5A efficiently inhibited this activation of NS5B on IFN-β promoter. The LCMV-infected NS5A-Tg mice had a decreased induction of IFN-β, 2', 5'-OAS and PKR, which reflected the inhibitory interference of NS5A with the antiviral interferon response. Recent studies revealed that based on the application of a peptidomimetic active site NS3 protease inhibitor, we could identify signaling intermediates of the TLR3 as well as the RIG-I signal transduction pathway as proteolytical targets of the NS3/4A protease. Thus, NS3/4A blocks the TLR3 signal transduction pathway by specific proteolytical cleavage of the TRIF adaptor rendering TRIF incapable...
to recruit TBK1 which resulted in an impaired TLR3 mediated activation of the IFN-β promoter. Besides, it is reported that hepatitis C virus core protein can abrogate the DDX3 function that enhances IPS-1-mediated IFN-β induction. Also, HCV core could attenuate IL-6 stimulated acute-phase response, damage the innate immune response and enhance inflammatory response to form persistent infection.

However, the influence of HCV on innate immunity has not been elucidated. Therefore, in this study, we analyzed the effect of HCV proteins on the innate immune system, especially the induction of IFN-β. In our research, we observed the impact of HCV protein-core, NS3 and NS5A on the expression of IFN-β in human liver hepatocellular carcinoma cell line HepG2 cells. Through the analysis by RT-PCR, Western blotting and ELISA, the results suggested that NS3 and NS5A can efficiently suppress IFN-β in HepG2 cells. To further understand the mechanisms, we carried out luciferase activity assay of IFN-β promoter, which showed that NS3 and NS5A can inhibit IFN-β promoter activity. This suggests that NS3 and NS5A suppression of IFN-β promoter activity may be one of the mechanisms for IFN-β inhibition by HCV. Meanwhile, our results show HCV core had almost no effect on IFN-β expression and promoter activity, this may explained by distinguish effects of different forms of HCV core. HCV core proteins in monomeric form is sensed by TLR2, on the contrary, neither recombinant nor serum derived infectious HCV results in efficient activation of TLR2 signaling and IFN production. For HCV, impairment of core-TLR interaction in infectious particles may have roles to escape from innate antiviral immune responses and facilitate the persistence of HCV infection. Inhibition of NS3 and NS5A on IFN-β expression reveals that HCV as an ever developing virus against host immunity is able to use its proteins accumulating mechanisms for near unbeatable survival.

REFERENCES