Modulation of interferon signaling by hepatitis C virus non-structural 5A protein: Implication of genotypic difference in interferon treatment

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Abstract

Interferon (IFN) response rate in hepatitis C virus (HCV) patients has been varied with genotypes. In this study, we investigated the effects of HCV NS5A protein on IFN resistance and compared the genotypic differences of NS5A. We showed that IFN-α-, poly I:C-, and Sendai virus-induced ISRE transcriptional activities were inhibited by both genotype 1b and 2a NS5A protein. We demonstrated that not only genotype 1b but also genotype 2a NS5A exerted the similar extent of IFN-α-induced antiviral activity. We showed that NS5A derived from both genotype 1b and 2a showed no significant differential IFN responses as seen in HCV patients. These data imply that some other host factor may be involved in genotypic differences of IFN antagonism in HCV patients.

1. Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease, which often leads to liver cirrhosis and hepatocellular carcinoma [1]. More than 170 million people worldwide are infected with HCV. HCV is a member of the Flaviviridae family and contains a single stranded, positive-sense RNA genome of ~9600 nucleotides in length. The HCV genome encodes a single polyprotein precursor of approximately 3010 amino acids that is cleaved by both cellular signal peptidase and viral protease to generate three structural and seven non-structural proteins [2]. Core, E1, and E2 proteins are viral structural proteins, and p7, NS2, NS3, NS4A/B, and NS5A/B are non-structural proteins. The non-structural 5A (NS5A) is a multifunctional phosphoprotein consisting of 447 amino acids residues. NS5A may lead to pleiotropic responses, including regulation of cell growth and cellular signaling pathways [3,4]. NS5A is implicated in antiviral resistance to interferon (IFN). NS5A has the IFN sensitivity-determining region (ISDR). NS5A interacts with the IFN-inducible double-stranded RNA-activated protein kinase PKR through the ISDR and functions as a repressor of PKR [5]. However, the involvement of ISDR of NS5A in HCV antiviral responses is still controversial [6]. It has been reported previously that HCV E2 protein was also involved in IFN-induced antiviral response by inhibiting the PKR activity [7].

Type 1 IFNs (IFNα/β) evoke innate immune responses and remove the viruses by activating the IFN stimulated genes (ISGs) [8]. The JAK-STAT is an important signaling pathway in immune responses. Once type 1 IFNs bind to their cellular receptors, IFN-alpha receptor 1 (IFNAR1) becomes activated by phosphorylation. The activated receptor subsequently stimulates Janus kinases (JAKs). JAKs have a tyrosine kinase activity that leads to phosphorylation of signal transducer and activator of transcription (STATs) 1 and 2 [9]. The phosphorylated STAT1 and STAT2 form either homo- or hetero-dimer that binds further with either IRF9 or P48. The resulting IFN-stimulated gene factor 3 (ISGF3) complex interacts with the IFN-stimulated response elements (ISREs) sequences in the promoter region and thus stimulates ISGs [10].

To elucidate how genotypic difference of HCV occurs in IFN treatment, we investigated the effects of NS5A protein on antiviral activities using IFN, poly I:C, and Sendai virus treatments. Our data demonstrate that NS5A inhibits IFN signaling via JAK-STAT pathway and induction of interleukin-8/CXCL-8 (IL-8) (also referred to as CXCL-8). Nevertheless, NS5A derived from genotype 1b and 2a exerted no significant genotypic differences in the responses to IFN, poly I:C, and Sendai virus treatments.

2. Materials and methods

2.1. Plasmid constructions

cDNA encoding HCV NS5A (genotype 2a) was amplified by PCR using the JFH-1 clone of HCV [11] as a template and subcloned into...
Luciferase reporter gene assay

Luciferase activities were then determined. Fig. 1A showed that vector, 1b NS5A, 2a NS5A expression plasmid, individually with or without poly I:C treatment, and then luciferase activities were determined. As expected, ISRE transcriptional activity was markedly increased by poly I:C in control vector cells (Fig. 1C). Similar to IFN-α, Poly I:C-induced ISRE-luc reporter activity was inhibited by NS5A protein. We also found that the inhibitory effect of NS5A protein on Poly I:C-induced ISRE gene expression between two genotypes was also insignificant.

Since Sendai virus infection induces ISRE gene expression [14], we investigated whether Sendai virus–induced ISRE-luc reporter activity was also regulated by NS5A protein. Huh7 cells cotransfected with ISRE-luc reporter plasmid and vector, 1b NS5A, 2a NS5A expression plasmid, individually were infected with Sendai virus for 6 h and then luciferase activities were determined. Fig. 1D showed that Sendai virus–induced ISRE transcriptional activity (lane 2) was also inhibited by NS5A protein of both genotype 1b (lane 3) and 2a (lane 4). The inhibitory effect of NS5A on Sendai virus–induced ISRE reporter activity showed no significant difference between two genotypes.

3.2. NS5A protein derived from both genotype 1b and 2a inhibits IFN-α-induced STAT1 phosphorylation and its nuclear translocation

Stimulation of IFN-α signaling leads to the phosphorylation of STAT1 protein. Phosphorylated STAT1 then forms ISGF3 complex together with STAT2 and IRF9, and this complex is translocated to the nucleus and induces ISRE gene expression [15]. To elucidate the underlying mechanism of inhibitory role of NS5A in IFN signaling, we investigated IFN-α–induced STAT1 phosphorylation in cells expressing NS5A protein. Stable cells expressing 1b NS5A, 2a NS5A, and vector, respectively were treated with increasing amounts of IFN-α for 12 h and then STAT1 phosphorylation level was determined. NS5A is a key regulator of the type I IFN-induced immune responses, and thereby NS5A strongly inhibits STAT1 activation in Huh7 cells. As shown in Fig. 2A, phosphorylation level of STAT1 was increased in a dose-dependent manner of IFN in vector stable cells (top panel). However, IFN-α–induced STAT1 phosphorylation was significantly inhibited by both 1b and 2a NS5A proteins. Next, we investigated whether inhibition of STAT1 phosphorylation by NS5A protein could interrupt nuclear translocation of phosphorylated STAT1. Both cytosolic and nuclear fractions prepared from stable cells either untreated or treated with IFN-α were immunoblotted with anti-phospho-STAT1 antibody. In the absence of IFN-α treatment, STAT1 was not phosphorylated (Fig. 2B, left panel) and endogenous STAT1 expression level was not affected by NS5A protein in all cells. However, IFN-α–induced STAT1 phosphorylation and nuclear translocation of STAT1 protein in vector stable cells (Fig. 2B, lanes 1 and 4 in right panel). On the other hand, IFN-α–induced phosphorylation and nuclear translocation of STAT1 were dramatically inhibited in cells expressing NS5A protein derived from both genotypes 1b and 2a (Fig. 2B, lanes 5 and 6 in right panel). This result showed that not only STAT1 phosphorylation but also nuclear translocation of the phosphorylated STAT1 was down-regulated by NS5A protein. To further demonstrate whether inhibition of nuclear translocation of phosphorylated STAT1 might occur in the context of HCV RNA replication, both cytosolic and nuclear fractions of IFN-cured and HCV replicon cells prepared from either untreated or treated with IFN-α were immunoblotted with anti-phospho-STAT1 antibody. In the absence of IFN-α stimulation, phosphory-
lated STAT1 was not detected in both IFN-cured and HCV replicon cells (Fig. 2C, lanes 1–4). Upon IFN-α stimulation, both cytosolic and nuclear STAT1 protein levels were increased in IFN-cured cells (Fig. 2C, lanes 5 and 7). We found that phosphorylated STAT1 level in cytosolic fraction of HCV replicon was lower than that of IFN-cured cells (Fig. 2C, lane 5 vs. lane 6). Furthermore, IFN-α-stimulated nuclear translocation of STAT1 was remarkably decreased in HCV replicon cells as compared with IFN-cured cells (Fig. 2C, lanes 6 and 8). Together, these data suggest that the inhibitory effect of NS5A on IFN-α-induced immune response may be due to the inhibition of phosphorylation and subsequent nuclear translocation of STAT1 protein.

3.3. NS5A derived from both genotype 1b and 2a inhibits IFN-α-induced ISGs expressions

In response to viral infection, type I IFN induces activation of STAT1 and stimulates the transcription of ISGs, which contain the ISRE in the promoter regions [10,16]. To investigate the effects of NS5A on the IFN-induced ISGs expression levels, stable cells expressing vector, 1bNS5A, 2aNS5A, respectively, were treated with IFN-α for the indicated time. Total cell lysates were immunoblotted with anti-ISGs protein antibodies. As shown in Fig. 3A, both MxA and ISG15 proteins were highly expressed at 8 h after IFN-α stimulation in vector stable cells and the expression levels of both...
Fig. 2. HCV NS5A protein inhibits IFN-α-induced STAT1 phosphorylation and nuclear translocation. (A) IFN-α induced STAT1 phosphorylation was suppressed by HCV NS5A protein in stable cells. Stable cells expressing either genotype 1b or 2a NS5A protein, or vector stable cells were treated with selected amounts (0, 50, 100, 200, 500, 1000 U/ml) of IFN-α for 12 h. Cells were harvested and the level of STAT1 phosphorylation was determined by immunoblotting with anti-phospho-STAT1 antibody. Protein expression of GAPDH was used as a loading control for the same amount of cell lysates. (B) HCV NS5A protein inhibited IFN-α induced STAT1 phosphorylation and nuclear translocation. Stable cells expressing either genotype 1b or 2a NS5A protein, or vector stable cells were either left untreated or treated with 500 U/ml of IFN-α for 12 h. Both cytosolic and nuclear fractions were prepared and the level of STAT1 phosphorylation was determined by immunoblotting with anti-phospho-STAT1 antibody (1st panel). The same cell lysates were analyzed for total STAT1 level by immunoblotting with anti-nonphospho-STAT1 antibody (2nd panel). Protein expression of NS5A was confirmed by immunoblotting with anti-Myc antibody (3rd panel). Both cytosolic and nuclear fractions were verified by immunoblot analysis using either anti-GAPDH (4th panel) or B23 antibodies (5th panel). (C) IFN-α-induced STAT1 phosphorylation and nuclear translocation were inhibited by HCV subgenomic replicon. HuH7 cells harboring HCV replicon and IFN-cured cells were either left untreated or treated with 500 U/ml of IFN-α for 12 h. Both cytosolic and nuclear fractions were prepared and the levels of both total and phosphorylated STAT1 were determined as described in (B). C, IFN-cured cells; R, HCV subgenomic replicon cells.
MxA and ISG15 proteins were continuously increased up to 24 h. On the other hand, PKR was expressed as early as 1 h after IFN-α stimulation in vector stable cells. It was striking that MxA, ISG15, and PKR expressions were remarkably inhibited by both genotype 1b and 2a NS5A protein (Fig. 3A, middle and right panels). We found that the expression level of STAT1 was not affected by NS5A protein. These data indicate that NS5A inhibits ISGs expressions through the inhibition of STAT1 phosphorylation. To further investigate whether IFN-α-induced ISGs expressions were inhibited in the context of HCV RNA replication, ISGs protein expression levels in both IFN-cured and replicon cells were compared in the absence or presence of IFN-α treatment. In the absence of IFN-α stimulation, none of ISGs proteins were expressed (Fig. 3B, lanes 1 and 2). Upon IFN-α treatment, protein levels of ISGs, including MxA, ISG15, and 2′-5′-oligoadenylate synthetase (OAS) were increased in IFN-cured cells (Fig. 3B, lane 3). As expected, all ISGs protein levels were drastically decreased in cells harboring HCV replicon (Fig. 3B, lane 4). These data confirmed that HCV counteracted IFN-induced signaling pathways by inhibiting ISGs protein expressions.

3.4. Both intracellular and extracellular IL-8 levels are increased in cells expressing NS5A protein derived from both genotype 1b and 2a

Since NS5A inhibits nuclear translocation of STAT1 and ISGs expressions, mRNA microarray analysis was performed using NS5A stable cells to investigate possible involvement of other regulatory genes in IFN responses. Following IFN-α treatment for 12 h, total mRNAs isolated from vector stable, 1b NS5A stable, and 2a NS5A stable cells, respectively were subjected to microarray analysis. Supplementary Fig. 2 is the summary of selected genes from microarray data. Interestingly, IL-8 level was highly increased in both genotype 1b and 2a NS5A stable cells as compared with vector stable cells (3.94- and 4.17-fold increase, respectively). IL-8 is a key component of innate immune response because IL-8 inhibits ISGF3 complexes of STAT1, STAT2, and IRF9[17]. When ISGF3 fails to function properly, the cellular level of IL-8 is increased. It has been previously reported that CXCL-8/IL-8 induction was regulated by HCV infection[18]. To examine whether IL-8 level was regulated by NS5A protein, Huh7 cells transfected with the control vector, 1b NS5A, and 2a NS5A expression plasmid, individually were immunoblotted with anti-IL-8 antibody. Indeed, IL-8 protein levels were highly increased in cells expressing both genotypes 1b and 2a NS5A protein (Supplementary Fig. 3, lanes 3 and 4) as compared with control vector cells.

We then determined the extracellular IL-8 level in NS5A stable cells. Culture supernatants collected from either vector stable or NS5A stable cells were concentrated with Centricon and then IL-8 protein levels were determined by enzyme-linked immunosorbent assay (ELISA) kit. As shown in Fig. 4, IL-8 production was increased 2- to 3-fold at 12 h and 4- to 5-fold at 24 h in both genotypes 1b and 2a NS5A stable cells as compared with vector stable cells (left panel). MTT data indicated that cell viability was not affected by both genotypes 1b and 2a NS5A (Fig. 4, right panel).
We confirmed that IL-8 production was also increased 2- to 4-fold in cells transiently expressing either genotype 1b or 2a NS5A protein (Supplementary Fig. 4). Since cell viability was not affected by NS5A protein, the increase of IL-8 production was not due to cell damage (Supplementary Fig. 4, right panel). To determine the region in NS5A that is responsible for IL-8 induction, various deletion mutants of NS5A (Supplementary Fig. 5A) were transiently expressed in Huh7 cells and then IL-8 production was measured by using an IL-8 specific ELISA kit. As shown in Supplementary Fig. 5B, IL-8 production was increased ~14-fold in wild-type NS5A as compared with vector control. IL-8 productions in both middle- and C-terminal NS5A mutants maintained basal levels as in the vector control. However, IL-8 production was increased ~8-fold in cells expressing N-terminal region of NS5A, indicating that N-terminal region of NS5A mediated IL-8 production. To further demonstrate whether the N-terminal NS5A protein could block ISRE transcription, we performed ISRE transcription assay using various deletion mutants of NS5A (Supplementary Fig. 5A). As shown in Supplementary Fig. 5C, N-terminal NS5A protein, but not middle and C-terminal NS5A, blocked ISRE activity. This further confirms that the N-terminal NS5A conferred IFN-α-induced antiviral immune responses.

3.5. IL-8 augments the inhibitory effect of NS5A protein on IFN-α-induced ISRE reporter activity

To investigate whether NS5A-induced IL-8 production was involved in JAK-STAT pathway, Huh7 cells expressing NS5A protein were treated with IFN-α, further incubated with IL-8, and then ISRE-luc reporter activity was determined. As shown in Fig. 5A, IFN-α-induced ISRE reporter activity was decreased by both genotype 1b and 2a NS5A protein. The inhibitory function was enhanced by IL-8 in a dose-dependent manner in both genotypes. Next, we determined the effect of IL-8 on IFN-α-induced antiviral activity of NS5A protein. Stable cells expressing either vector or genotype 1b NS5A protein were treated with IFN-α in the absence or presence of IL-8, and total cell lysates were immunoblotted with anti-ISG15 antibody. Fig. 5B showed that ISG15 protein expression level in vector stable cells was increased by IFN-α in a dose-dependent manner (Fig. 5B, left panel); whereas, IFN-α-induced ISG15 expression was inhibited by NS5A protein (Fig. 5B, middle panel). Moreover, inhibition of IFN-α-induced ISG expression was significantly enhanced by addition of IL-8 in cells expressing NS5A protein (Fig. 5B, right panel). We found the similar results using stable cells expressing NS5A protein derived from genotype 2a (data not shown). These in vitro data further imply that some host factor may be involved in genotypic differences of IFN antagonism in HCV patients.

3.6. Silencing of IL-8 inhibits anti-IFN activity of NS5A IL-8

To verify the role of IL-8 in the anti-IFN response of NS5A, Huh7 cells transfected with siRNA targeting two different sites of IL-8 were treated with IFN-α and then luciferase activities were determined. As shown in Supplementary Fig. 6, anti-IFN activity of NS5A was abrogated by knockdown of IL8 with siRNA. It is noteworthy that each siRNA alone targeting different site of IL8 nullified the inhibitory effect of NS5A, demonstrating that IL-8 produced by NS5A played an important role in anti-IFN activity.

4. Discussion

Patients infected with HCV genotype 1 had lower SVR rate to IFN than those infected with genotype 2 or 3. Current combination therapy using pegylated IFN-α plus ribavirin for the majority of genotypes 2 or 3 results in a SVR, whereas only 50% of patients infected with genotype 1 attain SVR. However, the underlying mechanisms of HCV to IFN resistance are not clearly understood.

Modulations of IFN signaling by HCV proteins have been reported previously. HCV proteins inhibit IFN-α-induced JAK-STAT pathway in the liver of transgenic mice [19]. Among the HCV viral proteins, NS5A and E2 proteins disrupted the normal IFN-α-induced antiviral activities by interaction with PKR [5,7]. Both HCV core and NS5A proteins inhibited IFN-α-induced JAK-STAT signal pathway by interaction with STAT1 protein [20]. Since IFN resistance has been controversial depends on HCV genotypes of NS5A, we extensively compared IFN-α-induced ISRE activities between genotype 1b and 2a NS5A using Poly I:C and Sendai virus as well as IFN treatment. We demonstrated that IFN-α-induced ISRE reporter activity was inhibited by both genotype 1b and 2a NS5A. Furthermore, NS5A proteins derived from both genotypes inhibited ISRE-luc reporter activity in a dose-dependent manner in Huh7 cells. It was noteworthy that genotype 1b NS5A inhibited ISRE-luc reporter activity slightly, but not significantly, more than 2a NS5A. Both Poly I:C and Sendai virus are known to stimulate IFN signaling pathways. In fact, we showed that both Poly I:C- and Sendai virus-induced ISRE transcriptional activities were also inhibited by NS5A protein. However, the differences of inhibitory function between two genotypes of NS5A protein on Poly I:C- and Sendai virus-induced ISRE gene expressions were indistinguishable from each other. Collectively, these results suggest that HCV NS5A, irrespective of its genotype, specifically inhibits IFN-α-, Sendai virus-, and poly I:C-induced ISRE-luc reporter activities.

To see if there is any difference in IFN-α signaling between two genotypes, we determined IFN-α-induced phosphorylated STAT1, an active form of STAT1, protein level in cells expressing either 1b or 2a NS5A protein. Indeed, IFN-α-induced STAT1 phosphoryla-

![Fig. 4. HCV NS5A protein induces IL-8 production. IL-8 production was increased in cells stably expressing NS5A protein. Culture supernatants were collected from stable cells expressing either genotype 1b or 2a NS5A protein, or vector stable cells at 12 and 24 h after plating. Supernatants were further concentrated with Centricon Filter-70 (Millipore) and IL-8 levels were determined by using an IL-8 specific ELISA kit (left panel). An MTT assay was performed by treating cells with tetrazolium substrate and reduction values were expressed as percentage of the control stable cells (right panel).](image-url)
teins induce IL-8 [23], and NS5A protein is also able to inhibit IFN responses partially by inducing IL-8 [24]. Indeed, both NS5A protein expressions remarkably decreased in stable cells expressing both genotype 1b and 2a NS5A protein. We further demonstrated that ISG protein expression levels were drastically inhibited in HCV replicon cells. These data indicate that both genotypes of NS5A counteract IFN signaling by inhibiting STAT1 phosphorylation and subsequent ISG family protein expressions. Recently, Garaigorta and Chisari [21] reported that HCV-induced PKR activation suppressed the ISG proteins in HCV-infected cells. This study demonstrated that the activation of PKR by HCV actually gave the positive effect on HCV in antiviral activity. However, genotypic differences in IFN antagonism between genotype 1b and 2a NS5A were not significant as opposed to previous report [25]. Our in vitro data imply that some other host factors, other HCV proteins, and host genetics such as IL28B may be implicated in genotype differences in IFN responses of HCV patients. Nevertheless, NS5A appears to play the crucial role in the viral evasion from host innate immune response and thus contribute to persistence and pathogenesis of HCV-induced liver disease.

5. Conflict of interest

None.

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Fig. 5. IL-8 promotes the inhibitory effect of NS5A protein on IFN-α-induced immune response. (A) IL-8 potentiates inhibitory effect of NS5A protein on IFN-α induced ISRE reporter activity. Huh7 cells were cotransfected with ISRE-luc reporter plasmid together with 0.5 μg of either 1b NS5A or 2a NS5A expression plasmid. At 24 h after transfection, cells were either left untreated or treated with 500 U/ml of IFN-α for 18 h, and then incubated with the selected amounts (0, 15, 30, and 45 ng/ml) of human IL-8 for 6 h. The data shown represent triplicate experiments. (B) IL-8 enhances the antiviral activity of NS5A protein. Stable cells expressing either vector or genotype 1b NS5A protein were treated with increasing amounts of IFN-α (0, 50, 100, 200, 500, 1000 U/ml) for 12 h in the absence or presence of IL-8 for 12 h. Total cell lysates were immunoblotted with anti-ISG15 antibody. GAPDH was used as a loading control.

![Graph showing IFN-α and IL-8 effects on ISRE-luc reporter activity]
ology, Korea. The authors thank Dr. Takaji Wakita (National Institute of Infectious Disease, Tokyo) for providing us with an HCV JFH-1 cDNA clone.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.08.032.

References


