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Isolation and gene analysis of interferon α-resistant cell clones of the hepatitis C virus subgenome

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Abstract

Hepatitis C virus (HCV) proteins appear to play an important role in IFN-resistance, but the molecular mechanism remains unclear. To clarify the mechanism in HCV replicon RNA harboring Huh-7 cells (Huh-9-13), we isolated cellular clones with impaired IFNα-sensitivity. Huh-9-13 was cultured for approximately 2 months in the presence of IFNα, and 4 IFNα-resistant cell clones showing significant resistances were obtained. When total RNA from clones was introduced into Huh-7 cells, the transfected cells also exhibited IFNα-resistance. Although no common mutations were present, mutations in NS3 and NS5A regions were accumulated. Transactivation of IFNα and IFNα-stimulated Stat-1 phosphorylation were reduced, and the elimination of HCV replicon RNA from the clones restored the IFNα signaling. These results suggest that the mutations in the HCV replicon RNA, at least in part, cause an inhibition of IFN signaling and are important for acquisition of IFNα resistance in Huh-9-13.

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Keywords: Hepatitis C virus; Replicon; Interferon resistance; Stat-1; Nonstructural protein NS5A

Introduction

Hepatitis C virus (HCV) is the major cause of post-transfusion non-A non-B hepatitis. Approximately 170 million individuals worldwide were estimated to be infected with HCV (Alter, 1997). It has been suggested that the development of liver cirrhosis and hepatocellular carcinoma are consequences of chronic infection with HCV (Hijikata et al., 1993b; Tong et al., 1995).

HCV, a member of the *Flaviviridae* family, has a single-stranded positive-sense linear RNA genome of about 9.5 kb (Hijikata et al., 1991; Kato et al., 1990; Takamizawa et al., 1991). The RNA encodes a single precursor polyprotein of approximately 3010 amino acids (Choo et al., 1991; Okamoto et al., 1991, 1992) that is co- and post-translationally cleaved to produce individual structural (Core, E1, E2) and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) by both host and viral proteases (Hijikata et al., 1993a,b; Houghton, 1996).

The cell line Huh-9-13, in which the HCV subgenome can self-replicate, was established by R. Bartenschlager's group (Lohmann et al., 1999). The HCV subgenomic RNA consists of the entire nonstructural coding region of the Con1 strain of the HCV genome, except for the neomycin-resistant gene. This cell line provides significant information for understanding the replication of the HCV genome and is useful as a powerful screening tool for developing anti-HCV drugs (Bartenschlager et al., 2000, 2001).

Interferon alpha (IFNα) is widely used for the treatment of patients with chronic HCV infection; however, the effectiveness of IFNα, especially in genotype 1b, is low at only about 20–30% (Lindsay, 1997), although combination therapy with Ribavirin improves treatment outcomes (up to 50–60%) (McHutchison et al., 1998). According to reports of epidemiologic analysis conducted in Japan, IFN treatment outcomes are related with mutations within a 40 amino acid sequence in NS5A (amino acid

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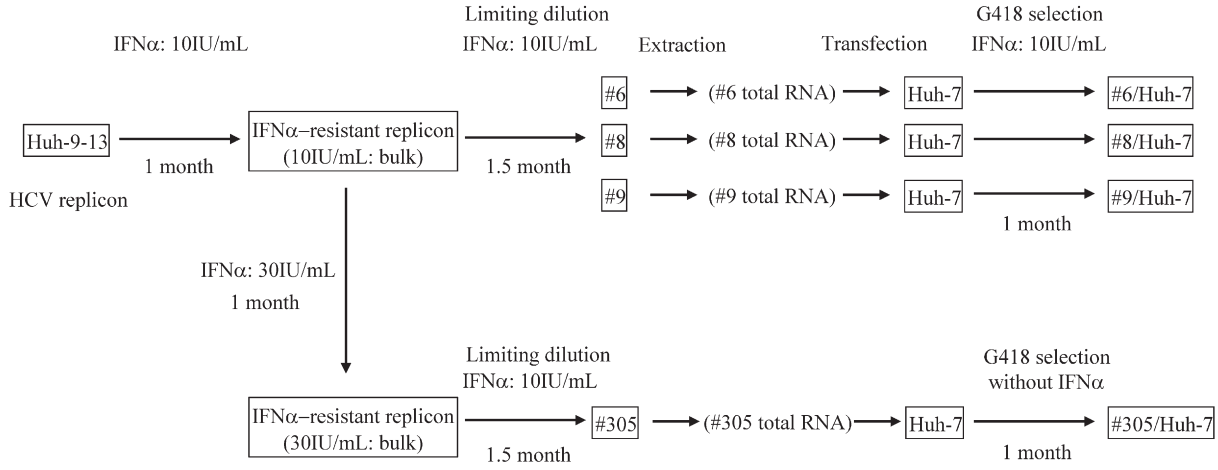

Fig. 1. An outline of the process used for isolation of replicon cells showing IFNα-resistance. Total RNA transfection derived from replicons to naive Huh-7 cells was performed using DMRIE-C reagent (Invitrogen).

(A)

	Cell	EC50(IU/mL)	Fold reduction
Original	Huh-9-13	0.7	1.0
IFNα–resistant	#6	6.9	9.5
	#8	6.7	9.2
	#9	10.2	13.9
	#305	99.2	135.6

(B)

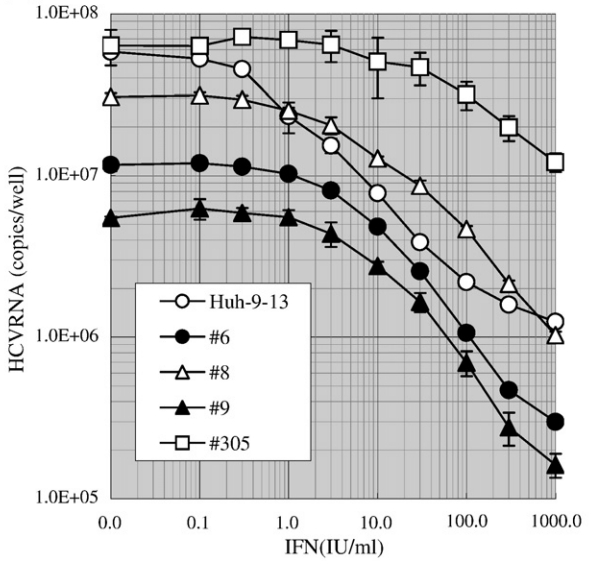

Fig. 2. Reactivity for IFNα in established IFNα-resistant replicon cells (#6, #8, #9, and #305) and original replicon cells (Huh-9-13). The cells were treated with IFNα for 48 h, and the amount of HCV RNA was measured by quantitative RT-PCR. (A) EC_{50} value (IU/mL) of IFNα in each replicon and fold reduction of the value compared to original replicon (Huh-9-13). (B) Change in copy number of HCV RNA in original and IFNα-resistant replicons by IFNα treatment. These experiments were performed in triplicate and mean values are shown.

numbers 2209–2248, based on the sequence of the prototype for HCV-J polyprotein) called the interferon sensitivity determining region (ISDR) (Enomoto et al., 1996). However, it is not clear how NS5A functionally interacts with IFN signals. Alternatively, NS5A is shown to inhibit the activity of double-stranded RNA (dsRNA)-activated protein kinase (PKR) and 2′-5′-oligoadenylate synthetase (2′-5′-OAS) induced by IFNα (Gale et al., 1997; Noguchi et al., 2001; Taguchi et al., 2004).

Recently, Meylan et al. and other groups reported that HCV-NS3-4A protease cleaved Cardif (Meylan et al., 2005) (also designated as VISA (Xu et al., 2005), MAVS (Seth et al., 2005), IPS-1 (Kawai et al., 2005)) and suppressed IFN production through RIG-I signaling. Cardif interacts with RIG-I (Yoneyama et al., 2004) mediated through CARD domains in both molecules

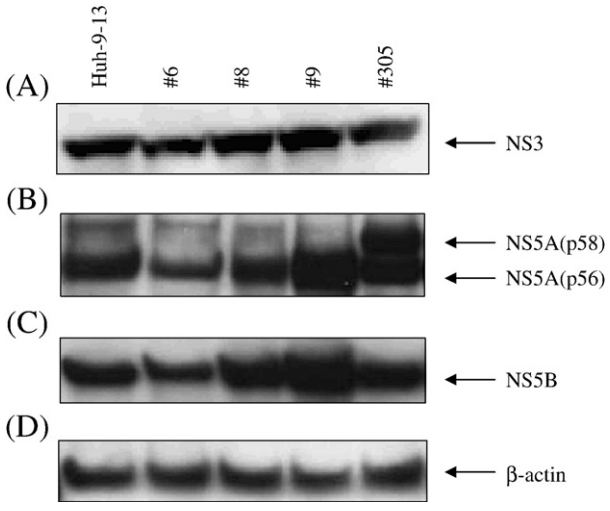

Fig. 3. Western blot analysis of the established IFNα-resistant replicon cells (#6, #8, #9, and #305) and original replicon cells (Huh-9-13). Expression of β-actin was used as an internal control of cellular protein in the replicon cells. Each cell line was inoculated on a 60-mm plate at 3×10^5 cells/well. Twenty-four hours after inoculation, the cells were lysed with SDS sample buffer. Total proteins were subjected to a 2/15% SDS gradient gel, and were subsequently immunoblotted by NS3 (A), NS5A (B), NS5B (C), and β-actin (D) antibody.

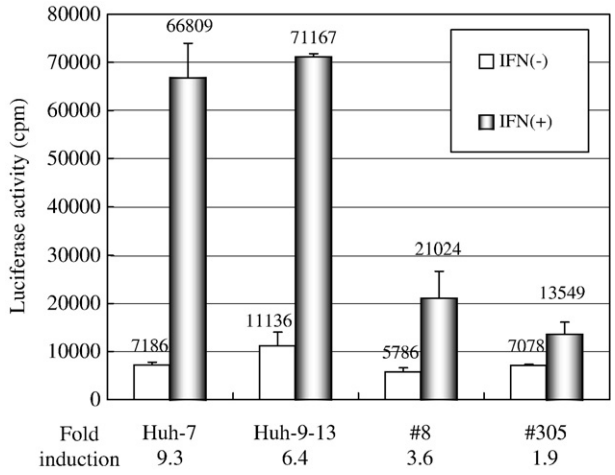

Fig. 4. Transactivation of ISRE in IFNα-resistant replicon cell lines (#8 and #305), original replicon (Huh-9-13), and parental Huh-7 cells by reporter gene (pISRE/Luc) analysis. The cells were stimulated with 1000 IU/mL of IFNα for 24 h after transfection of reporter plasmid DNA. White bars show control (no addition of IFNα) luciferase activity, and black bars show the activity under IFNα stimulation. Values of luciferase activity by IFNα stimulation relative to those of untreated cells are shown below the panel as 'fold induction'.

in a dsRNA-dependent manner, and transduce IFN production signals through the activation of nuclear factor κB (NFκB) and interferon regulatory factor 3 (IRF-3).

Despite bearing an HCV-1b genotype-derived replicon with mutations in ISDR, the replicon cells do not show resistances to IFN (Frese et al., 2002; Guo et al., 2001, 2004). Concerning this point, some reports regarding IFN-resistance acquisition and analysis of this property in the replicon cells (Namba et al., 2004; Sumpter et al., 2004; Zhu et al., 2005) showed involvement of various factors such as viral and/or host gene alterations participating in IFNα-resistance in replicon cells.

Here, we isolated IFNα-resistant clones of the HCV subgenome with accumulated mutations, especially in NS3 and NS5A regions. We observed impairment of phosphorylation of Stat-1 in cells bearing the IFNα-resistant HCV replicon. Our findings suggest that NS5A contributes to the acquisition of IFNα-resistant phenotype in HCV replicon cells.

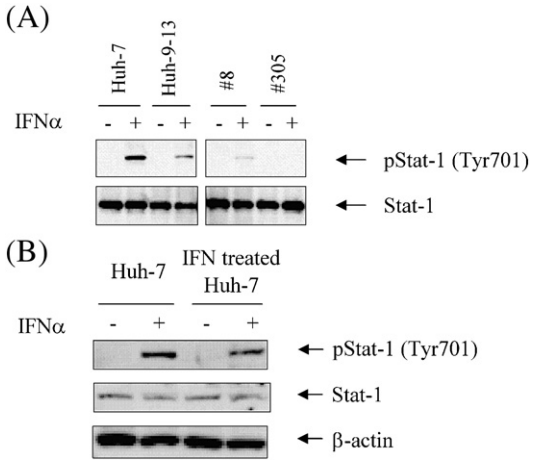

Fig. 5. (A) Change in phosphorylation of Stat-1 in IFNα-resistant replicon cell lines (#8 and #305), original replicon (Huh-9-13) and parental Huh-7 cell. Phosphorylation of Stat-1 was analyzed by western blot analysis using anti-phospho-Stat-1 (Tyr701) antibody. The cells were cultured in medium with or without 500 IU/mL of IFNα for 30 min. Upper panel represents a phospho-Stat-1 (Tyr701) and lower panel shows a Stat-1. Western blot analysis was performed as described in Materials and methods. (B) Change in phosphorylation of Stat-1 in Huh-7 cells maintained in the presence or absence of IFNα (10 IU/mL) for 4 weeks. Upper panel represents a phospho-Stat-1 (Tyr701), middle panel shows a Stat-1 and lower panel shows a β-actin. Phosphorylation of Stat-1 in these cells was examined as described above.

Results

Establishment of IFNα-resistant replicon cell lines

HCV replicon cells were cultured for approximately 1 month in the presence of 10 IU/mL IFNα. HCV RNA titer decreased during the culture; however, the appearance of cells less sensitive to IFNα during prolonged culture was observed by quantitative RT-PCR. The resistant cells were then cloned by limiting dilution. Three clones (Fig. 1: #6, #8, and #9) were obtained, and mixed pools of these resistant cells were further selected in the presence of 30 IU/mL IFNα for another 4 weeks. After confirming decreased sensitivity to IFNα at this dose, the clone

(A)

	Cell	IFN selection	EC50 (IU/mL)	Fold Reduction
Total RNA transfection	Huh-9-13/Huh-7	(-)	**0.7**	**1.0**
	#305/Huh-7	(-)	**4.1**	**5.9**
original	Huh-9-13	(-)	**0.6**	**1.2**

(B)

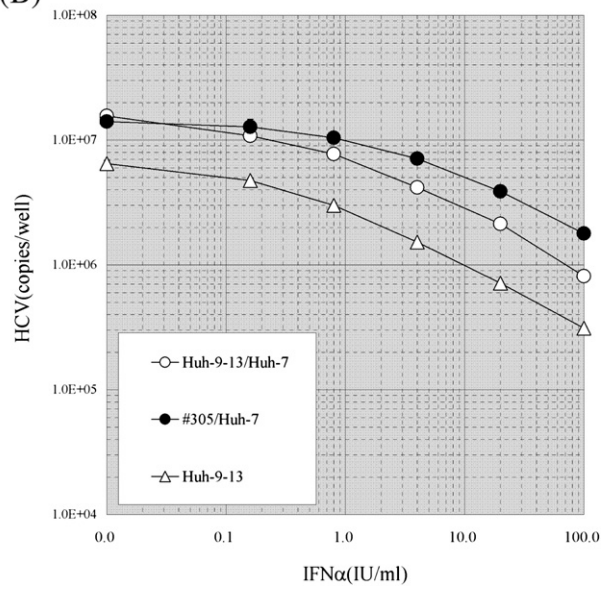

Fig. 6. Reactivity for IFNα in the Huh-7 cells, #305/Huh-7, transfected with total RNA of #305 replicon cells and in the Huh-7 cells, Huh-9-13/Huh-7, transfected with total RNA of original replicon cells (Huh-9-13). These transfected cells were selected with G418 in the absence of IFNα. The amount of HCV RNA was analyzed by quantitative RT-PCR, as described in Fig. 2. (A) EC_{50} value (IU/mL) of IFNα in Huh-9-13/Huh-7 and #305/Huh-7. (B) Change in copy number of HCV RNA in Huh-9-13/Huh-7 and #305/Huh-7 by IFNα treatment. These experiments were performed in triplicate and mean values are shown.

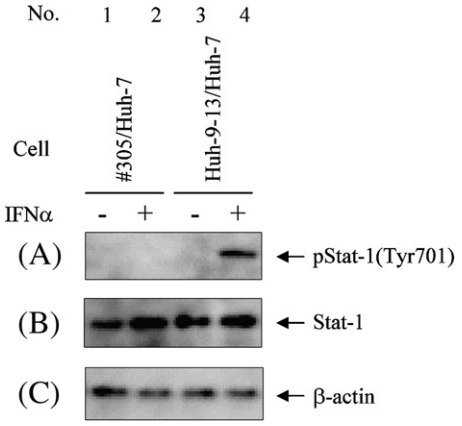

Fig. 7. Phosphorylation of Stat-1 in #305/Huh-7 and Huh-9-13/Huh-7 described in Fig. 6. The experiment was performed as described in Fig. 5. Each panel shows (A) phospho-Stat-1 (Tyr701), (B) Stat-1, and (C) β-actin. (Lanes 1 and 2) Huh-7 cells transfected with IFNα-resistant replicon (#305) total RNA (#305/Huh-7). (Lanes 3 and 4) Huh-7 cells transfected with original replicon (Huh-9-13) total RNA (Huh-9-13/Huh-7).

(Fig. 1; #305) showing highest resistance to IFNα was obtained. Sensitivities of these clones to IFNα are shown in Fig. 2. The basal HCV RNA levels in these cells (#6, #8, #9, and #305) were almost equal to that in the original replicon cells (Huh-9-13). The EC_{50} value of IFNα for the original replicon (Huh-9-13) was 0.7 IU/mL, compared to 6.9 IU/mL, 6.7 IU/mL, 10.2 IU/mL, and 99.2 IU/mL for resistant clones #6, #8, #9, and #305, respectively. These results demonstrate that sensitivity to IFNα based on EC_{50} value decreased 9 to 135-fold in the IFNα-resistant clones.

Characterization of IFNα-resistant replicon cell lines

First, expression of HCV NS proteins (NS3, NS5A, and NS5B) in IFNα-resistant replicon cell lines (#6, #8, #9, and #305) was analyzed by western blot. We detected expression of all the NS proteins in these cell lines as well as in original replicon cell (Huh-9-13) at almost at the same levels, although the levels of NS5A and NS5B in clone #6 were slightly low (Fig. 3). Interestingly, only clone #305 exhibited a different migration of NS5A, corresponding to the size of hyper-phosphorylated form (p58) in addition to the size of basal phosphorylated form (p56).

To analyze the change in IFNα signal transduction in two representative IFNα-resistant replicon cell lines (#8 and #305), we carried out a reporter gene assay using a firefly luciferase gene fused with three repeats of an ISG15-type IFN-stimulated responsive element (ISRE) as a reporter construct (pISRE/Luc). After transfection of pISRE/Luc to these replicon cells, the cells were stimulated with 1000 IU/mL of IFNα for 24 h. As shown in Fig. 4, the transactivation by IFNα in original replicon cells (Huh-9-13) was slightly reduced compared with that of parental cell line Huh-7 (Huh-7, 9.3-fold; Huh-9-13, 6.4-fold). Luciferase activity of #8 and #305 was more diminished than that of Huh-9-13 (#8, 3.6-fold; #305, 1.9-fold). The extent of decline of transactivation by IFNα treatment in these resistant replicon cell lines was dependent on the extent of IFNα-resistance, as quantified by RT-PCR (Fig. 2). It is suggested that the genetic alteration in HCV replicon RNA confers on IFNα-resistance in these cell lines.

In relation to the reporter gene analysis, JAK-STAT pathway activated by type I IFN was analyzed in IFNα-resistant replicons containing cells (#8 and #305). Phosphorylation of Stat-1, one of the important molecules in the JAK-STAT signal transduction pathway, was lowered in original replicon cells (Huh-9-13) compared with that in parental Huh-7 (Fig. 5A). However, severely impaired phosphorylation of Stat-1 was observed in the IFNα-resistant replicons containing cells (#8 and #305) compared with original replicon cells (Huh-9-13) (Fig. 5A). Furthermore, phosphorylation of Stat-1 was also decreased in #305 containing cells maintained in the absence of IFNα for 4 weeks, and the degree of decrease of Stat-1 phosphorylation was almost equal to that maintained in the presence of IFNα (data not shown). In contrast to these observations, Huh-7 cells, the parental cell of Huh-9-13 that was maintained in the presence of IFNα for 4 weeks did not show the significant alteration of Stat-1 phosphorylation compared with that maintained in the absence of IFNα (Fig. 5B). These results suggest that reduction of phosphorylation of Stat-1 in these IFNα-resistant replicon cell lines is caused by alteration of HCV replicon RNA and it may correlate with suppression of transcription from the reporter gene (Fig. 4).

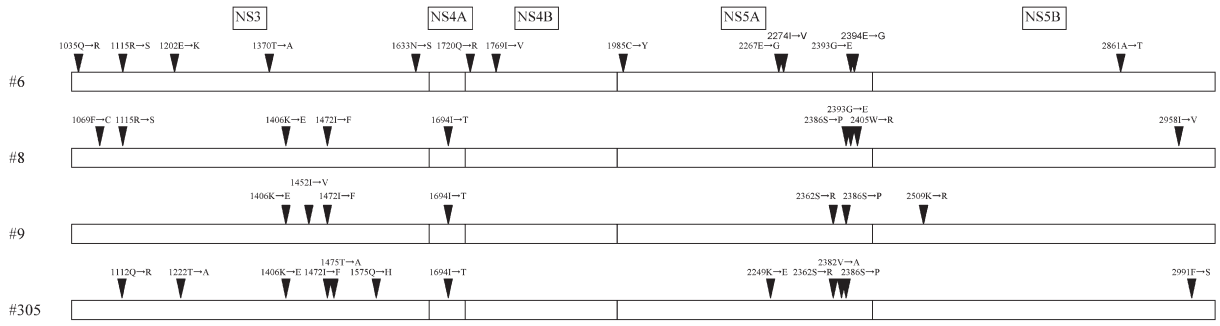

Fig. 8. The amino acid sequence deduced from nucleotide sequence in IFNα-resistant replicon cells. The nucleotide sequence was determined by an RT-PCR direct sequencing method. Arrows indicate the amino acid substitutions that were detected only in IFNα-resistant replicons compared with original replicon (Huh-9-13). The numbering of amino acids was referred to that of complete polyprotein of the isolate.

HCV replicon RNA confers IFNα-resistance

To confirm the role of HCV subgenomic RNA from clone #305 for acquisition of IFNα-resistance, total RNA was extracted from the cells and transfected to naive Huh-7 cells. The transfected cells were selected with G418 in the absence of IFNα. HCV negative-stranded replicon RNA, replication intermediate, and HCV NS proteins (NS3, NS5A and NS5B) were detected in the cells (data not shown).

Concerning the cells transfected with total RNA from IFNα-resistant #305 cell (#305/Huh-7) or the cells transfected with total RNA from original Huh-9-13 replicon cell (Huh-9-13/Huh-7), IFNα-sensitivity (EC_{50}) was analyzed (Fig. 6). IFNα-sensitivity (EC_{50}) of the Huh-9-13/Huh-7 showed 0.7 IU/mL, whereas the #305/Huh-7 showed 4.1 IU/mL. EC_{50} values of the Huh-7 cells bearing IFNα-resistant replicon derived from clone #305 were approximately 6-fold higher than that of Huh-7 cells bearing the original replicon. Although IFNα-resistance (EC_{50}) of the cells bearing #305 RNA was not as high as that of original #305, this finding suggests that acquisition of IFNα-resistance of these cells was due to genetic alteration of the replicon RNA.

We investigated the phosphorylation status of Stat-1 by stimulation of IFNα in these cells. As shown in Fig. 7, phosphorylation of Stat-1 in #305/Huh-7 (lane 2) was suppressed compared with that in Huh-9-13/Huh-7 (lane 4), suggesting that the IFNα-resistant HCV replicon derived from #305 is responsible for acquisition of the decreasing response to Stat-1 phosphorylation stimulated by IFNα.

Direct sequencing analysis of IFNα-resistant replicons

Nucleotide sequences in the NS region of each resistant clone were determined by RT-PCR direct sequencing. Sites of mutation that were detected only in IFNα-resistant replicons are shown by arrowheads and numbers (N-terminus of NS3 was denoted as 1027 based on the numbering of the complete polyprotein of the isolate), together with conversion of amino acids by arrows (Fig. 8). Although synonymous mutations are clustered in NS3 and the C-terminal region of NS5A, there were no common mutations among these resistant clones. Moreover, no mutations located at the positions as in IFNα-resistant replicons established by Namba et al. (2004) and Sumpter et al. (2004) were found in the present study. Mutations in the ISDR of NS5A were reported

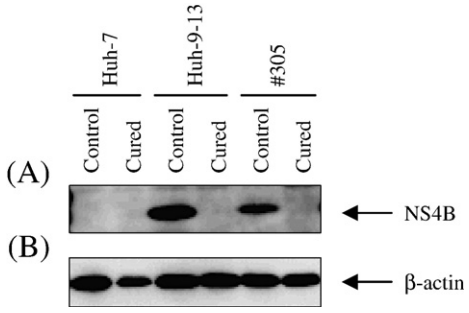

Fig. 9. Expression of NS protein (NS4B) (A) and β-actin (B) was confirmed in 'cured cells' by western blot analysis. Huh-7 cells with JTP-71892 as well as replicon cells (Huh-9-13 and #305) were analyzed likewise.

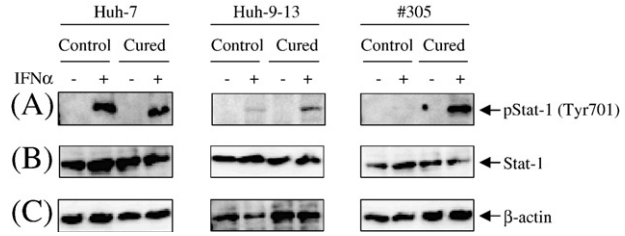

Fig. 10. Phosphorylation of Stat-1 (Tyr701) in 'cured cells'. Phosphorylation of Stat-1 (Tyr701) (A) by IFNα stimulation was investigated by western blot analysis. Stat-1 (B) and β-actin (C) were also analyzed. IFNα stimulation and western blot analysis were performed as described in Fig. 5.

to play an important role in outcome of IFN treatment to patients with genotype 1b of HCV in Japan (Enomoto et al., 1996); however, the amino acid sequence of ISDR was preserved among these replicon cell lines in our experiments.

Characterization of 'cured cells' obtained by IFNα-resistant HCV replicon cells

To clarify the role of HCV replicon RNA in resistance to IFNα, the replicon cells (Huh-9-13 and #305) were treated with JTP-71892 (1 μM) for more than 1 month to establish 'cured cells', as described in Materials and methods. JTP-71892 is a JTK-109-derivative synthesized in our laboratory, which has a potent inhibitory effect on HCV replication (Hirashima et al., 2006). The amounts of HCV replicon RNA in both replicon-bearing cell types were decreased less than what could be detected by quantitative RT-PCR, while the amounts of GAPDH mRNA used as a control did not show any difference (data not shown). The representative HCV NS protein, NS4B, was not detected in the 'cured cells' (Fig. 9).

The phosphorylation status of Stat-1 was then analyzed in these cells. The Stat-1 phosphorylation (Tyr701) by IFNα stimulation has restored remarkably in 'cured cells' (derived from both Huh-9-13 and #305) (Fig. 10). There was no obvious difference in the extent of Stat-1 phosphorylation by JTP-71892 treatment in Huh-7, indicating that restoration of Stat-1 phosphorylation was not due to JTP-71892. There was no clear difference in the amount of non-phosphorylated Stat-1 and β-actin expression by the IFNα stimulation or JTP-71892 treatment among these cell clones. These results suggest that HCV replicon RNA contributes to IFNα-resistance through impairment of phosphorylation of Stat-1, at least in part.

Discussion

We cultured HCV replicon cells in the presence of 10 and 30 IU/mL IFNα to isolate IFNα-resistant clones. Four different resistant clones with differing sensitivities to IFNα were isolated. The sensitivity for IFNα attenuated more than 100-fold in the #305 replicon, which was isolated in the presence of 30 IU/mL of IFNα and showed the most remarkable resistance in our study.

We analyzed the appearance of G418-resistant cells, #305/Huh-7, obtained by transfection of total RNA from the IFNα-

resistant replicon-bearing cells to Huh-7 by culturing them in the absence of IFNα, as shown in Fig. 1. IFNα sensitivities of the Huh-7 cells transduced with HCV replicon RNA of #305 cells were about 6-fold lower than those transfected with total RNA of original replicon cells (Huh-9-13), in coincident with a reduction of Stat-1 phosphorylation. However, #305/Huh-7 conferred a lesser extent of IFNα-resistance compared with that of parental #305 (Figs. 2 and 6). Although some factors other than HCV replicon RNA itself may participate in acquisition of IFNα-resistance in #305 cells, these results suggest that replicon RNA derived from #305 was significantly involved in regulation of IFNα signaling. The 'cured cells', from which HCV genomic RNA was removed from IFNα-resistant replicon cell line (#305) after treatment with 1 μM of JTP-71892, a potent HCV replication inhibitor, resulted in restoration of IFNα signaling to parental Huh-7. This finding suggests that HCV replicon RNA plays important roles in suppression of Stat-1 function. Moreover, this effect is dependent on mutation of HCV replicon RNA.

Mutations of amino acids were clustered throughout the whole region of NS3 and the C-terminus of NS5A in the IFNα-resistant replicon RNAs; however, there were no common amino acid mutations among the clones. This result may suggest the possibility that a change of plural functions participates in the acquisition of resistance. Whereas we did not identify common mutations, four amino acid mutations, K1406E, I1472F, I1694T, and S2386P, in NS3/4A and NS5A were shown to be common in #8, #9, and #305. In particular, the mutation at S2386P in NS5A located near region V3, one of the important prediction factors of the outcome in clinical IFN therapy (Nousbaum et al., 2000; Puig-Basagoiti et al., 2005), is found in #9 and #305. The nucleotide sequence of ISDR region was preserved between original replicon and IFNα-resistant replicons.

Concerning the mutations in NS5A region of #305, we established 3 chimeric replicon cell clones harboring Huh-9-13 replicon that was substituted with NS5A coding region derived from #305, which was selected by G418 in the absence of IFNα. These cell clones showed reduction of IFNα sensitivity (EC_{50}) as 20 to 30 times as those of normal replicon cell (Huh-9-13). Although chimeric replicons harboring #305 NS5A showed lesser extent of IFNα-resistance than that of #305 replicon cell, NS5A of #305 plays an important role in acquisition of IFNα-resistance in the replicon cell (data not shown).

Naka et al. (2005) reported that nonsense mutations and deletions of type I IFN receptor genes (IFNAR1, IFNAR2c) were found in certain clones of replicon cells that gained IFNα-resistance. However, we did not detect any such mutation or deletion in either of these genes in this work. Furthermore, we were not able to obtain resistant phenotype by IFN treatment at high concentrations of more than 1000 IU/mL.

In #305, among other IFNα-resistant clones, substantial amount of slow migrating form of NS5A was observed. From previous reports (Asabe et al., 1997, Ide et al., 1997; Kaneko et al., 1994; Kim et al., 1999; Reed et al., 1997, 1998; Tanji et al., 1995), it is supposed that this form is hyper-phosphorylated NS5A with 58 KD. Hyper-phosphorylated form of NS5A (p58) negatively participates in replication of HCV RNA in replicon cells (Appel et al., 2005; Evans et al., 2004; Huang et al., 2006; Neddermann et al., 2004). However, the quantity of basal HCV replication in #305 was almost the same as in other replicon cells, including Huh-9-13. Thus, it is likely that the hyper-phosphorylation of NS5A does not contribute to suppression of replication of HCV replicon. Rather, it may be related to a potent IFNα-resistance in #305 via un-identified mechanisms. Further studies are needed to clarify the role of hyper-phosphorylated NS5A in IFNα-resistance.

Concerning effects of NS5A on IFN signaling, it was reported that transiently- or stably-transfected NS5A inhibits IFN-stimulated Stat-1 phosphorylation and transactivation of ISRE in hepatocyte-derived cell lines, including Huh-7 cell (Gong et al., 2007; Lan et al., 2007). These authors also suggested the interaction of NS5A with Stat-1. Although these evaluation methods were different from that of our replicon system, they lend additional credibility to the suggestion that NS5A plays an important role in regulation of IFN signaling via inhibition of Stat-1 phosphorylation.

Stat-1 phosphorylation by IFNα stimulation was suppressed in IFNα-resistant replicon cells. The degree of suppression of Stat-1 phosphorylation was related to the sensitivity of IFNα in IFNα-resistant replicons (Fig. 5A). Moreover, the decrease of Stat-1 phosphorylation in #305 cells maintained in the absence of IFNα for 4 weeks was almost same level as that maintained in the presence of IFNα, suggesting that IFNα pressure did not induce a negative feedback (i.e. leading to the degradation of IFN receptor) loop in our experimental system. In contrast, Stat-1 phosphorylation was not changed significantly in parental Huh-7 cells that were maintained in the presence of IFNα compared with that maintained in the absence of IFNα (Fig. 5B), suggesting that Stat-1 phosphorylation in the parental Huh-7 cells was not affected with IFNα pressure and that the alteration of HCV replicon confers the IFNα-resistance. Stat-1 phosphorylation was also suppressed in the Huh-7 cells transfected with total RNA from IFNα-resistant replicon (Fig. 7). Moreover, the 'cured cells' showed a restoration of Stat-1 phosphorylation (Fig. 10). These observations suggest that IFNα-resistance in IFNα-resistant replicon cells depends on a change in Stat-1 phosphorylation, at least in part. For unknown reasons, we could not detect phosphorylation of Stat-2 (Tyr689), Stat-3 (Tyr705) (Sarcar et al., 2004; Zhu et al., 2005), JAK-1 (Tyr1022), or Tyk-2 (Tyr1054) in these cells. Concerning these proteins in the replicon cells, further investigation is needed to understand their roles in acquisition of IFNα-resistance.

Although the underlying mechanism of acquisition of IFNα-resistance gained by HCV replicon RNA remains unclear, clarification of detailed analysis of the role of Stat-1 in regard to IFN signaling in HCV replicon cells may contribute to the development therapeutic agents.

Materials and methods

Cell culture

Huh-9-13 cells harboring HCV subgenomic (NS3-3′X) replicon and parental Huh-7 cells were purchased from ReBLikon GmbH. Cells were cultured in Dulbecco's modified Eagle's

medium (DMEM) supplemented with 10% fetal bovine serum. To Huh-9-13 cells, 1 mg/mL of G418 (Geneticin; Invitrogen), a selective marker for replicated HCV subgenome was added.

IFN treatment

Huh-9-13 cells were seeded in a 75-cm^2 flask at a density of 3×10^5 cells/flask. Twenty-four hours after cell seeding, human IFNα (Sumiferon®300; Dainippon Sumitomo Pharma) was added so that the final concentration in medium was 10 IU/mL. Control cells were cultured in medium with no other additional substances. Cell passages were performed approximately every 7 days and the cells were cultured for approximately 1 month in the presence of IFNα (10 IU/mL). After decreases in sensitivity to IFNα were confirmed in the IFNα-treated groups by quantitative RT-PCR, IFNα-resistant cell phenotypes were further cultured for about 1 month in the presence of 30 IU/mL IFNα, and sensitivity to IFNα was then also measured in these cells. The cells cultured in the presence of 10 or 30 IU/mL of IFNα were cloned by a limiting dilution method using 96-well plates: cells were seeded at 1 cell/well and cultured in medium containing 10 IU/mL IFNα. After culture for about two to three weeks, survival and growth of cloned cells were confirmed, and then colonies were isolated and added to 48-well plates containing the test substance in 500 μL of culture medium per well. The proliferated cells in the 48-well plates were transferred to 6-well plates, and these were further put into 75-cm^2 cell culture flasks for subculture. Thereafter, subculture passage was performed approximately every 7 days. Cloning and subculture were performed in the presence of IFNα.

Measurement of IFN-sensitivity (quantitative analysis of HCV replicon and GAPDH mRNA)

IFN-sensitivity of IFNα-treated replicon cells was measured by quantitative RT-PCR. Cells (1×10^4 cells/well) were seeded in 96-well plates in the presence of 0, 0.1, 0.3, 1, 3, 10, 30, 100, 300, or 1000 IU/mL of IFNα. Forty-eight hours after cultivation with IFNα, the cells were harvested to extract total RNA using a total RNA extraction kit (RNeasy® 96; Qiagen) in accordance with the instruction manual. Quantification of HCV replicon RNA in the prepared RNA was performed using TaqMan® EZ RT-PCR Core Reagent (ABI) using a sequence detector under the following conditions: sense-primer: 5′-CGGGAGAGCCATAGTGG-3′ (130-S17; Greiner), antisense-primer: 5′-AGTACCACAAGGCCTTTCG-3′ (290-R19; Greiner), probe: 5′(FAM)-CTGCGGAACCGGTGAGTACAC (TAMRA)-3′ (148-S21FT; TaKaRa) (Takeuchi et al., 1999), RT-PCR reaction conditions: 50 °C, 2 min→60 °C, 30 min→95 °C, 5 min→45 cycles×(95 °C, 20 s→62 °C, 1 min). The number of copies in the samples was determined using a standard curve calibrated with 10^4 to 10^8 copies of synthesized HCV RNA standards encoding from 5′ terminus to E2 region, and recorded as amount of HCV RNA.

Direct sequencing analysis of HCV replicon RNA

Nucleotide sequences of HCV replicon RNA were analyzed by direct sequencing method. The NS region of total RNA extracted from IFNα-resistant replicon clones was divided into four fragments and amplified using an RT-PCR kit (ReverTra Dash®; TOYOBO). Four primers (HCV-NS-1RV: 5′-ATAGCACTCGCACAGAACCGA-3′; Greiner, HCV-NS-2RV: 5′-GGAACCGTTTTTCACATGTCC-3′; Greiner, HCV-NS-3RV: 5′-ATGTGGTTAACGGCCTTGCT-3′; Greiner, HCV-NS-4RV: 5′-TCATCGGTTGGGGAGTAGATAGA-3′; Greiner) were used for reverse transcription (RT). For polymerase chain reaction (PCR), another four primers (HCV-NS-1FW: 5′-ATGGCGCCTATTACGGCCTA-3′; Greiner, HCV-NS-2FW: 5′-TGTTCGATTCCTCGGTTCTGT-3′; Greiner, HCV-NS-3FW: 5′-CCCCTTCTTCTCATGTCAACG-3′; Greiner, HCV-NS-4 FW: 5′-GGAACCTATCCAGCAAGCCC-3′; Greiner) were used in addition to the primers for RT.

RT and PCR reactions were conducted in accordance with the instruction manual provided with the kit. RT reaction was conducted at 42 °C, 20 min, and the reaction mixtures were then heated to 99 °C, 5 min. The PCR reaction was performed for 30 cycles under the following conditions: 98 °C, 10 s; 60 °C, 2 s; then 74 °C, 90 s.

Sequencing was performed using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (ABI). One μL of amplified RT-PCR product for each clone was purified using QIAquick Gel Extraction kit (Qiagen) and the sequence primers were used to prepare each of the reaction solutions in accordance with the manufacturer's procedure. Twenty μL of each solution was allowed to react for 25 cycles under the conditions: 96 °C, 10 s; 50 °C, 5 s; 60 °C, 4 min; then 72 °C, 7 min. The solutions were then purified by Dye EX 2.0 (Qiagen) in accordance with the instruction manual. After that, the samples were applied for sequencing analysis using an ABI PRISM 3100 genetic analyzer (ABI).

The NS region (5952 bp, 1984 amino acids) in sequenced samples underwent gene analysis using Vector NTI analysis software (Invitrogen). In a comparison of deduced amino acid sequences based on nucleotide sequences among the four IFNα-resistant replicon clones and original replicons, the NS regions were compared to that of the original replicon clone to identify mutations. The amino acid sequence of the original replicon cells was included among the materials provided with the Huh-9-13 cell line product from ReBLikon GmbH.

Reporter gene analysis

We attempted to clarify IFNα transactivation in IFNα-resistant replicons. Firefly luciferase fused gene with three repeats of an ISG15-type IFN-stimulated responsive element (ISRE) was used as a reporter construct (pISRE/Luc). HCV replicon cells or Huh-7 cells (3×10^5 cells/well) were seeded on a 60-mm plate in the absence of IFNα. Eight hours after cell seeding, the reporter construct (3 μg) was transfected using FuGENE6 (Roche) as a transfection reagent, following the instruction manual. The transfected cells were cultured further 12 to 14 h, and then the cells (1×10^4 cells) were inoculated on a 96-well plate and cultured for 24 h with or without 1000 IU/mL of IFNα. The luciferase activity was measured by adding Steady Glo® to the cells using TopCount (Packard).

Western blot analysis

The cell lysates were prepared in Laemmli buffer (BIO-RAD) and subjected to SDS-2/15% gradient PAGE and transferred onto nitrocellulose membranes. To detect expression of HCV NS proteins, antibodies against NS3, NS4B, NS5A, and NS5B were used. Anti-β-actin antibody (Sigma) was also used for detection of β-actin as an internal control.

To investigate the phosphorylation of Stat-1 at Tyr701 in HCV replicon cells and its parental Huh-7 cells, the cells were cultured in the medium containing 500 IU/mL of IFNα for 30 min. After cell lysates were prepared as previously described, western blot analysis was performed using an anti-phospho-Stat-1 (Tyr701) antibody (Cell Signaling Technology) or an anti-Stat-1 antibody (BD Transduction Laboratories). Immunocomplexes were detected by visualization using enhanced chemiluminescence (Amersham Biosciences).

Transfection of total RNA derived from replicon cells to naive Huh-7

Total RNA (5 μg) extracted from HCV replicon cells was transfected to Huh-7 cells using DMRIE-C transfection reagents, in accordance with the instruction manuals provided with the reagents. The transfected cells were cultured in the absence of IFNα and selected with 1000 μg/mL of G418 for 4 weeks. Drug-resistant cells were collected and reactivity to IFNα was measured as described in previous section.

Elimination of HCV replicon RNA from replicon cells (Isolation of 'cured' replicon)

To remove HCV replicon RNA from replicon cells, HCV replicon cells were treated ('cured') with HCV RNA-dependent RNA polymerase NS5B inhibitor, JTP-71892, JTK-109-derivatives synthesized in our laboratory (Hirashima et al., 2006; Ishida et al., 2006). The replicon cells (5×10^4 cells) were inoculated on a 60-mm plate and further cultured in the presence of the compound (1 μM) for about 4 weeks. The cell culture was performed in the absence of G418, to prevent survival of the compound-resistant clones. Medium was exchanged with fresh medium containing the compound twice per week. The finding that 1 μM of JTP-71892 does not exhibit any toxicity or growth inhibition in long-term culture had been previously confirmed.

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