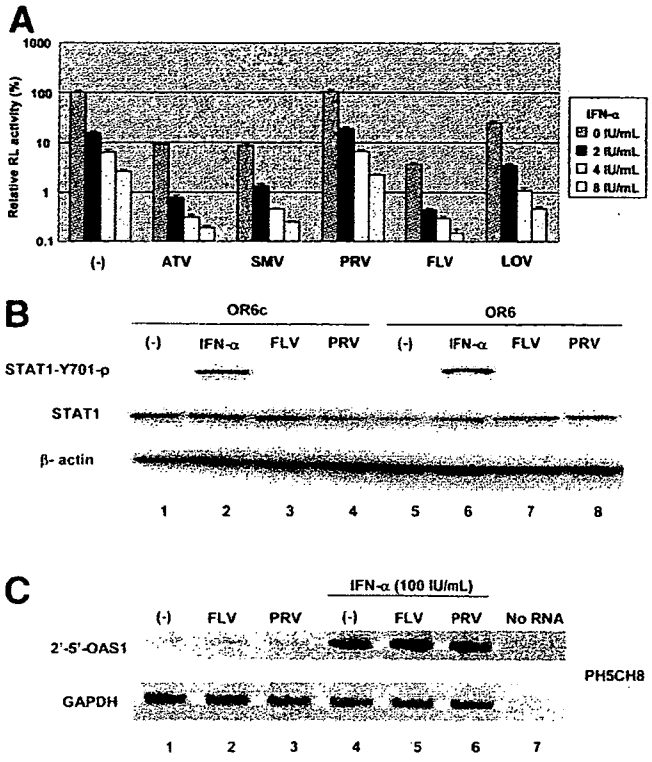

Fig. 4. Statins enhance inhibition of HCV RNA replication because of IFN-α. (A) Effects of statins on the anti-HCV activity of IFN. OR6 cells were cotreated with IFN-α (0, 2, 4, and 8 IU/mL) and ATV, SMV, PRV, FLV, or LOV (5 μmol/L each). The RL assay was performed, and the relative RL activity was calculated as shown in Fig. 1B. (B) No enhancement of type I IFN signaling by the statins. OR6c or OR6 cells were cultured for 2 hours in the absence and in the presence of IFN-α (100 IU/mL), FLV (10 μmol/L), and PRV (10 μmol/L), and the cells were subjected to Western blot analysis of STAT1 and its phosphorylation status. β-actin was used as a control for the amount of protein loaded per lane. (C) No induction of the IFN-inducible gene by the statins. PH5CH8 cells were untreated or were treated with FLV (10 μmol/L) and PRV (10 μmol/L) for 2 hours, and then total RNA extracted from the cells was subjected to RT-PCR for 2′-5′-OAS1 (25 cycles). The PH5CH8 cells were treated for 9 hours with IFN-α (100 IU/mL) alone or in combination with FLV (10 μmol/L) or PRV (10 μmol/L), and then RT-PCR for 2′-5′-OAS1 was performed. The RT-PCR products (358 bp for 2′-5′-OAS1 and 334 bp for GAPDH) were detected, as shown in Fig. 1D.

hibitory effects of the combination of IFN-α and FLV on genome-length HCV RNA replication. A dose-response curve of FLV was obtained for fixed concentrations of IFN-α of 0, 4, 8, 16, 32, and 64 IU/mL. The results revealed the curves shifted to shift markedly to the bottom as the concentration of IFN-α increased (Fig. 5A), indicating that cotreatment was drastically more effective than treatment with IFN-α alone. Furthermore, we observed that RL activity decreased to almost the background level in the OR6 reporter assay when OR6 cells were cotreated with 64 IU/mL of IFN-α and FLV at concentrations above 1.25 μmol/L (Fig. 5A). Because the data in Fig. 5A indicate the possibility of a synergistic effect of the combination of IFN-α and FLV, we examined whether the effect of this combination is synergistic or additive effect using an isobologram method.[23,24] The anti-HCV activities of IFN-α and FLV in combination were evaluated by the OR6 reporter assay. Dose-response inhibition of HCV RNA replication was evaluated for varying IFN-α concentrations (0-8 IU/mL) in the presence of various doses of FLV (0-7.5 μmol/L). The IC_{90} values of IFN-α and FLV were 4.0 IU/mL and 6.7 μmol/L, respectively. These data were used to generate isoboles, which demonstrated 90% inhibition of HCV RNA replication, and the synergistic anti-HCV action of IFN-α and FLV was revealed by the curvilinear plots of the 90% isoboles (Fig. 5B). In conclusion, we clearly demonstrated that combination treatment of IFN-α and FLV was an overwhelmingly more effective treatment,

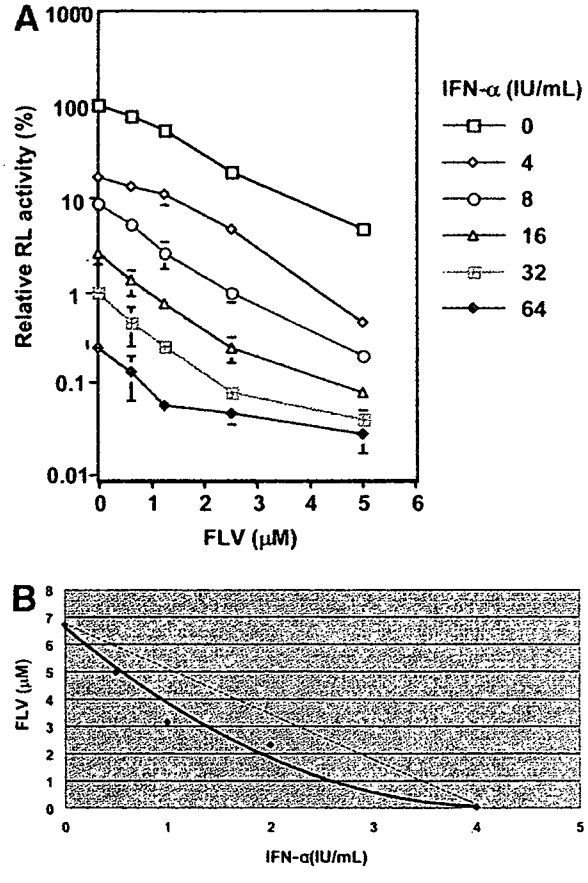

Fig. 5. Synergistic effect of FLV in combination with IFN-α on HCV RNA replication. (A) Effect of FLV in combination with IFN-α. OR6 cells were cotreated with FLV (0, 0.625, 1.25. 2.5, and 5 μmol/L) and IFN-α (0, 4, 8, 16, 32, and 64 IU/mL). The RL assay was performed after 72 hours of treatment, and the relative RL activity was calculated as shown in Fig. 1B. (B) Isobole plots of 90% inhibition of HCV RNA replication. OR6 cells were treated with IFN-α (0, 0.5, 1, 2, 4, 6, and 8 IU/mL) in combination with FLV (0, 0.625, 1.25, 2.5, 5, and 7.5 μmol/L) for 72 hours, and the RL assay was performed as shown in Fig. 1B to obtain 90% isoboles. The broken line indicates the additive effect in the isobologram method used.[23,24]

compared with the previous results for the combination treatment of IFN-α with ribavirin.[10]

Discussion

In this study, we found that different statins have different anti-HCV profiles. FLV, ATV, and SMV each exerted a stronger inhibitory effect on HCV RNA replication than did that of LOV reported previously.[11,12] However, PRV exhibited no anti-HCV activity. We also demonstrated that anti-HCV activity was drastically increased when these statins except PRV were used in combination with IFN-α. Because these statins are currently used for the clinical treatment of patients with hypercholesterolemia without inducing severe side effects, our findings suggest that these statins might be useful in combination therapy with IFN-α or IFN-α plus ribavirin.

That PRV exhibited no anti-HCV activity is interesting. From the information on LOV only[10,11] to date, the mechanism underlying statins' inhibition of HCV RNA replication has not been considered their cholesterol-lowering activity but rather their inhibition of geranylgeranylation of cellular proteins. In other words, statins' inhibition of HMG-CoA reductase leads to the reduction of intracellular mevalonate and consequently to a reduction in geranylgeranyl pyrophosphate. In fact, in OR6 cells we observed that mevalonate and geranylgeraniol restored HCV RNA replication in the FLV- or LOV-treated cells. However, we found unexpectedly that PRV did not inhibit HCV RNA replication, whereas PRV inhibited HMG-CoA reductase as effectively as other statins possessing anti-HCV activity. Although PRV is a water-soluble reagent (others are lipophilic), we confirmed PRV did induce expression of HMG-CoA reductase by a positive feedback mechanism[18] and LST-1 was expressed in our cell culture system. These findings suggest the presence of a mechanism in which PRV's inhibition of HMG-CoA reductase does not cause the depletion of geranylgeranyl pyrophosphate. Interestingly, it has been reported that PRV has a unique effect among statins on the induction of p450.[18] Therefore, further studies are needed to explain why PRV exhibits no anti-HCV activity.

We minutely examined the effect of FLV, the statin exhibiting the strongest inhibition of HCV replication of those tested in this study, in combination with IFN-α. We found that a combination treatment of IFN-α and FLV had a synergistic inhibitory effect on HCV RNA replication. Although high doses of IFN-α are more effective than low doses for eliminating HCV from a patient, the side effects increase in a dose-dependent manner. Because ribavirin enhances the effect of IFN-α slightly in a cotreatment, it is the only reagent currently used with IFN-α to treat patients with CH C. In our previous study of anti-HCV activity using the OR6 assay system, we found the IC_{50} of ribavirin to be 76 μmol/L.[10] This concentration is much higher than the clinically achievable ribavirin concentration (10-14 μmol/L) previously reported.[25] Furthermore, when administered in combination with IFN-α (2 IU/mL) and ribavirin (50 μmol/L), HCV RNA replication was reduced by only approximately 50%, compared with the effect of treatment with IFN-α alone.[10] It has been reported that the maximum blood concentration of FLV after 40 mg/day being administered orally for 4 weeks is approximately 0.6 μmol/L.[26] This concentration is rather low for the inhibition of HCV replication *in vivo*, because the IC_{90} of FLV was assigned as 6.7 μmol/L in our assay system (Fig. 5B). In addition, our study showed reatment of OR6 cells with 5 μmol/L FLV alone was almost equal to the effect of 10 IU/mL IFN-α. Although statins are known to concentrate in the liver, FLV monotherapy will not be effective for patients with CH C. However, we demonstrated that the combination of IFN-α and FLV exhibited synergistic effects on HCV RNA replication. For example, when administered in combination with IFN-α (2-8 IU/mL) and FLV (5 μmol/L), HCV RNA replication fell remarkably, to approximately 3%, compared with the effects of treatment with IFN-α alone (Fig. 4A). From these results, we propose that therapy combining FLV with IFN-α may be effective for the treatment of patients with CH C. Furthermore, additional treatment with reagents in combination (e.g., IFN-α, ribavirin, and FLV) will help to improve the SVR rate.

In conclusion, the results of the present study suggest that statins other than PRV are good reagents for combination therapy with IFN-α in patients with CH C. Although the mechanism by which PRV lacks anti-HCV activity has not been clarified in the present study, a better understanding of this mechanism may lead to the discovery of statin-related anti-HCV reagents possessing no cholesterol-lowering activity. Furthermore, our developed OR6 assay system will be useful for the time-saving screening of new anti-HCV reagents.

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References

1. Feld JJ, Hoofnagle JH. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. Nature 2005;436:967-972.
2. Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 1999;285:110-113.
3. Ikeda M, Yi M, Li K, Lemon SM. Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the

HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. J Virol 2002;76:2997-3006.
4. Pietschmann T, Lohmann V, Kaul A, Krieger N, Rinck G, Rutter G, Strand D, et al. Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. J Virol 2002;76:4008-4021.
5. Yi M, Bodola F, Lemon SM. Subgenomic hepatitis C virus replicons inducing expression of a secreted enzymatic reporter protein. Virology 2002;304:197-210.
6. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791-796.
7. Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, et al. Complete replication of hepatitis C virus in cell culture. Science 2005;309:623-626.
8. Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, et al. Robust hepatitis C virus infection in vitro. Proc Natl Acad Sci U S A 2005;102:9294-9299.
9. Ikeda M, Abe K, Dansako H, Nakamura T, Naka K, Kato N. Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system. Biochem Biophys Res Commun 2005;329:1350-1359.
10. Naka K, Ikeda M, Abe K, Dansako H, Kato N. Mizoribine inhibits hepatitis C virus RNA replication: effect of combination with interferon-alpha. Biochem Biophys Res Commun 2005;330:871-879.
11. Ye J, Wang C, Sumpter R Jr, Brown MS, Goldstein JL, Gale M Jr. Disruption of hepatitis C virus RNA replication through inhibition of host protein geranylgeranylation. Proc Natl Acad Sci U S A 2003;100:15865-15870.
12. Kapadia SB, Chisari FV. Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. Proc Natl Acad Sci U S A 2005; 102:2561-2566.
13. Wang C, Gale M Jr, Keller BC, Huang H, Brown MS, Goldstein JL, et al. Identification of FBL2 as a geranylgeranylated cellular protein required for hepatitis C virus RNA replication. Mol Cell 2005;18:425-434.
14. Naganuma A, Nozaki A, Tanaka T, Sugiyama K, Takagi H, Mori M, et al. Activation of the interferon-inducible 2′-5′-oligoadenylate synthetase gene by hepatitis C virus core protein. J Virol 2000;74:8744-8750.
15. Noguchi M, Hirohashi S. Cell lines from non-neoplastic liver and hepatocellular carcinoma tissue from a single patient. In Vitro Cell Dev Biol Anim 1996;32:135-137.
16. Dansako H, Naganuma A, Nakamura T, Ikeda F, Nozaki A, Kato N. Differential activation of interferon-inducible genes by hepatitis C virus core protein mediated by the interferon stimulated response element. Virus Res 2003;97:17-30.
17. Kato N, Sugiyama K, Namba K, Dansako H, Nakamura T, Takami M, et al. Establishment of a hepatitis C virus subgenomic replicon derived from human hepatocytes infected in vitro. Biochem Biophys Res Commun 2003;306:756-766.
18. Kocarek TA, Dahn MS, Cai H, Strom SC, Mercer-Haines NA. Regulation of CYP2B6 and CYP3A expression by hydroxymethylglutaryl coenzyme A inhibitors in primary cultured human hepatocytes. Drug Metab Dispos 2002;30:1400-1405.
19. Abe T, Kakyo M, Tokui T, Nakagomi R, Nishio T, Nakai D, et al. Identification of a novel gene family encoding human liver-specific organic anion transporter LST-1. J Biol Chem 1999;274:17159-17163.
20. Nakai D, Nakagomi R, Furuta Y, Tokui T, Abe T, Ikeda T, et al. Human liver-specific organic anion transporter, LST-1, mediates uptake of pravastatin by human hepatocytes. J Pharmacol Exp Ther 2001;297:861-867.
21. Pietschmann T, Lohmann V, Rutter G, Kurpanek K, Bartenschlager R. Characterization of cell lines carrying self-replicating hepatitis C virus RNAs. J Virol 2001;75:1252-1264.
22. Sutter AP, Maaser K, Hopfner M, Huether A, Schuppan D, Scherubl H. Cell cycle arrest and apoptosis induction in hepatocellular carcinoma cells by HMG-CoA reductase inhibitors. Synergistic antiproliferative action with ligands of the peripheral benzodiazepine receptor. J Hepatol 2005; 43:808-816.
23. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 1984;22:27-55.
24. Hwang DR, Tsai YC, Lee JC, Huang KK, Lin RK, Ho CH, et al. Inhibition of hepatitis C virus replication by arsenic trioxide. Antimicrob Agents Chemother 2004;48:2876-2882.
25. Pawlotsky JM, Dahari H, Neumann AU, Hezode C, Germanidis G, Lonjon L, et al. Antiviral action of ribavirin in chronic hepatitis C. Gastroenterology 2004;126:703-714.
26. Park JW, Siekmeier R, Lattke P, Merz M, Mix C, Schuler S, et al. Pharmacokinetics and pharmacodynamics of fluvastatin in heart transplant recipients taking cyclosporine A. J Cardiovasc Pharmacol Ther 2001;6: 351-361.

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Hepatitis C virus NS5B delays cell cycle progression by inducing interferon-β via Toll-like receptor 3 signaling pathway without replicating viral genomes

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Abstract

To clarify the pathogenesis of hepatitis C virus (HCV), we have studied the effects of HCV proteins using human hepatocytes. Here, we found that HCV NS5B, an RNA-dependent RNA polymerase, delayed cell cycle progression through the S phase in PH5CH8 immortalized human hepatocyte cells. Since treatment with anti-interferon (IFN)-β neutralizing antibody restored the cell cycle delay, IFN-β was deemed responsible for the cell cycle delay in NS5B-expressing PH5CH8 cells. The induction of IFN-β and the cell cycle delay were overridden by the down-regulation of Toll-like receptor 3 (TLR3) through RNA interference in NS5B-expressing PH5CH8 cells. Moreover, the NS5B full form was required for the cell cycle delay, the induction of IFN-β, and the activation of the IFN-β signaling pathway. Our findings revealed that NS5B induced IFN-β through the TLR3 signaling pathway in immortalized human hepatocytes even without replicating viral genomes.

Keywords: Hepatitis C virus; NS5B; Interferon-β; TLR3; Hepatocyte cells

Introduction

Since more than 170 million individuals are estimated to be infected with hepatitis C virus (HCV) worldwide, this disease is a global health problem (Thomas, 2000). HCV belongs to the family Flaviviridae, whose positive-stranded RNA genome encodes a large polyprotein precursor of approximately 3000 amino acid residues. This polyprotein is processed by a combination of the host and viral proteases into at least ten proteins in the following order: NH_2-core-envelope 1-envelope 2-p7-nonstructural protein 2 (NS2)-NS3-NS4A-NS4B-NS5A-NS5B-COOH (Kato, 2001; Kato et al., 1990). These viral proteins are not only involved in viral replication but also may affect a variety of cellular functions (Bartenschlager and Lohmann, 2000; Kato, 2001). Although persistent infection with HCV is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) (Colombo, 1996; Kato, 2001), the molecular mechanisms leading to liver cell dysplasia and HCC remain elusive.

It has been thought that unregulated cell cycle progression may be a cause of malignant transformation of normal cells. On the other hand, inhibition of cell cycle progression through the S phase may cause replication error during DNA replication, which induces genomic instability and malignant transformation. Therefore, it is important to clarify the effect of HCV proteins on cell cycle progression in order to understand the molecular mechanism underlying the pathogenesis of HCV, including the development of HCC. A number of previous reports suggested that four HCV proteins—the core, NS3, NS4B, and NS5A—are involved in modulating cell cycle progression (Arima et al., 2001; Kato, 2001; Ray and Ray, 2001; Reed and Rice, 2000). For instance, the core protein promotes cell proliferation through the Ras/Raf signaling pathway and the anti-apoptotic function (Mar-

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usawa et al., 1999; Tsuchihara et al., 1999). However, the core has been described to both enhance and repress the function of $p21^{Waf1/Cip1/Sdi1}$, a Cdk inhibitor (Dubourdeau et al., 2002; Jung et al., 2001; Lu et al., 1999; Ray et al., 1998). Recently, Scholle et al. found no significant cell cycle delay in human hepatoma HuH-7-based HCV RNA-replicating cells that were autonomously replicating genome-length HCV RNA, in comparison with cured cells of the same line from which HCV RNA had been eliminated by treatment with interferon (IFN)-α (Scholle et al., 2004). Hence, the effects of cell cycle regulation by HCV proteins are still controversial. Cancerous cell lines, such as the human hepatoma HuH-7 cell line (Hsu et al., 1993), which harbors a mutant *p53* gene, may not be suitable for addressing the effects of HCV proteins on cell cycle progression.

The PH5CH8 cell line was established by immortalization using the SV40 large-T antigen from non-neoplastic liver tissue of an HCV-related HCC patient (Ikeda et al., 1998; Noguchi and Hirohashi, 1996). PH5CH8 cells possess wild-type *p53* and *Rb* tumor suppressor genes. In nude mice, these cells reveal a non-malignant phenotype upon colony formation and tumorigenicity (Noguchi and Hirohashi, 1996), although the SV40 large-T antigen would partially repress the function of p53. Therefore, the PH5CH8 cell line is considered to be more relevant for studying the role of HCV proteins during hepatocarcinogenesis. We have previously reported that the HCV core protein activates the IFN-inducible 2′–5′-oligoadenylate synthetase gene in PH5CH8 cells (Naganuma et al., 2000). Recently, we demonstrated that the core protein's activation of this gene was mediated through the IFN-stimulated response element (ISRE) (Dansako et al., 2003). Furthermore, we found that the core protein promoted microsatellite instability in PH5CH8 cells (Naganuma et al., 2004). In fact, microsatellite instability was detected in approximately 20% of the tumor tissues from HCC patients examined, whereas no microsatellite instability was detected in normal liver tissues from the same patients (Dore et al., 2001; Kondo et al., 2000). In order to clarify the effect of HCV proteins on cell cycle progression in PH5CH8 cells, we examined cell cycle progression after the cells were released from the G1/S boundary in PH5CH8 cells expressing HCV proteins. We found that NS5B delays cell cycle progression by inducing IFN-β through the activation of the Toll-like receptor 3 (TLR3) signaling pathway without replicating viral genomes.

Results

HCV NS5B causes the delay of S phase progression

In a previous study of virus–host interactions, we examined whether or not HCV proteins affect cell cycle progression in PH5CH8 cells that stably expressed core or NS proteins. PH5CH8 cells were infected with retrovirus pCXbsr as a negative control (Ctr) or pCXbsr encoding either an HCV structural protein (HA-core) or NS protein (NS3, HA-NS4B, HA-NS5A, HA-NS5B, or NS5B), and we obtained PH5CH8 cells stably expressing each HCV protein. The expression of each HCV protein was confirmed by Western blot analysis (Fig. 1A). Then, the HCV protein-expressing cells were synchronized at the G1/S boundary, and cell cycle progression (from the S phase to the G2-M phase, then turning back to the G1 phase) was analyzed after the cells were released from synchronization. This cell cycle analysis revealed no significant differences in cell cycle progression between cells (PH/Ctr) infected with a control pCXbsr retrovirus and cells expressing core, NS3, NS4B, or NS5A (Fig. 1B). Unlike the PH/Ctr cells, the apparent delay of S phase progression was found in cells (PH/NS5B) expressing NS5B, regardless of the presence of the HA tag (Fig. 1B). To exclude the possibility that pCXbsr-derived retrovirus proteins synergistically affect the cell cycle together with NS5B, the retrovirus pCX4bsr vector (Akagi et al., 2003), which eliminates the production of any fusion proteins resulting from initiation at upstream AUG codons within the *gag* region of the vector, was used for the cell cycle analysis. As a result, the delay of S phase progression was found again in PH5CH8 cells expressing NS5B (Figs. 1A and C), suggesting that the retrovirus proteins are not involved in the delay of S phase progression. BrdUrd incorporation analysis was also carried out using PH/Ctr and PH/NS5B cells (Fig. 1D). In the PH/Ctr cells, DNA synthesis began early in the S phase (4 h after release). In the late S phase (8 h), more than 61% of the cells indicated final DNA synthesis. Thereafter, the cells either finished DNA replication in the G2-M phase (12 h) or returned to the G1 phase. In contrast, we found that DNA replication in most PH/NS5B cells predominantly remained in the early or middle S phase (8 h), and 49% of the cells were prolonged in the late S phase (12 h). To quantitatively evaluate this delay in S phase progression, the cells that had finished DNA replication were accumulated during the G2 phase by treatment with Nocodazole (Noc), which inhibits the progression of the G2 to M phases, after the cells were released from the G1/S transition. Whereas 77% of PH/Ctr cells reached the G2-M phase, only 37% of PH/NS5B cells did so (Fig. 1D). This level of decrease in cell numbers in the G2-M phase was not observed in PH5CH8 cells expressing core, NS3, NS4B, or NS5A (Fig. 1E), suggesting that NS5B specifically causes the delay of S phase progression in PH5CH8 cells. We further observed that the growth rate of PH/NS5B cells was significantly decreased relative to PH/Ctr cells (Fig. 1F), although the cell cycle distribution in asynchronous PH/NS5B cells was almost the same as that in asynchronous PH/Ctr cells (Fig. 1D). These results indicated that NS5B might delay the cell cycle progression of PH5CH8 cells in the S phase.

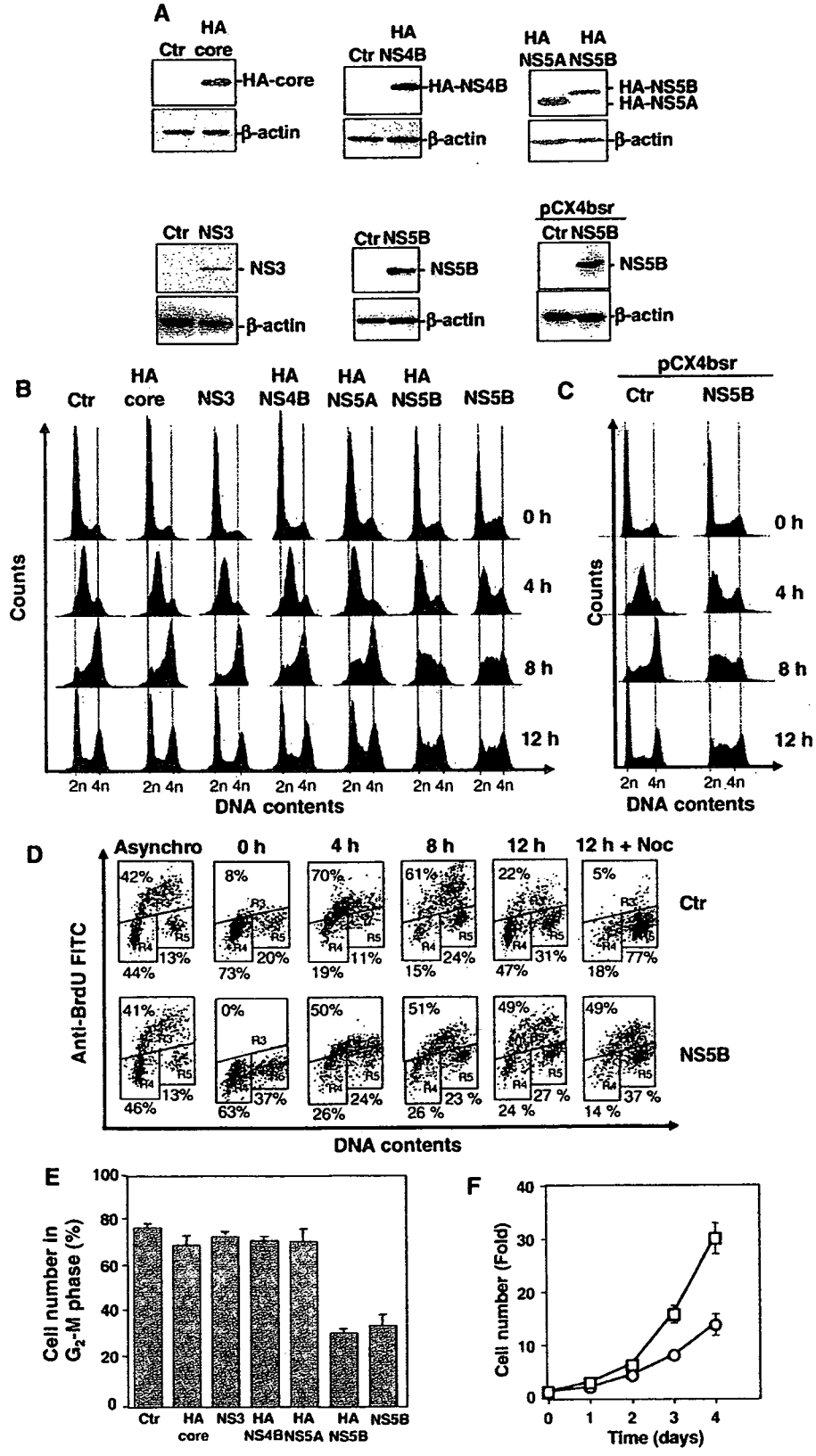
Cell cycle delay by NS5B is also found in other immortalized human hepatocytes

To clarify whether or not the delay of S phase progression by NS5B occurs in other human cell lines, we prepared three cell lines (Fig. 2A) that stably express NS5B–non-neoplastic human hepatocyte NKNT-3 (Kobayashi et al., 2000), hepatoma

HuH-7, and cervical carcinoma HeLa–and subjected them to the cell cycle analysis described above. The results revealed that S phase progression was delayed in NKNT-3, but not in HuH-7 or HeLa cells (Fig. 2B). This indicates that the delay of the cell cycle by NS5B expression is not limited to PH5CH8 cells.

IFN-β mediates the delay of S phase progression by NS5B

Since it has been reported previously that IFN-β induced the delay of S phase progression in human cultured cells (Vannucchi et al., 2000), we speculated that IFN-β was



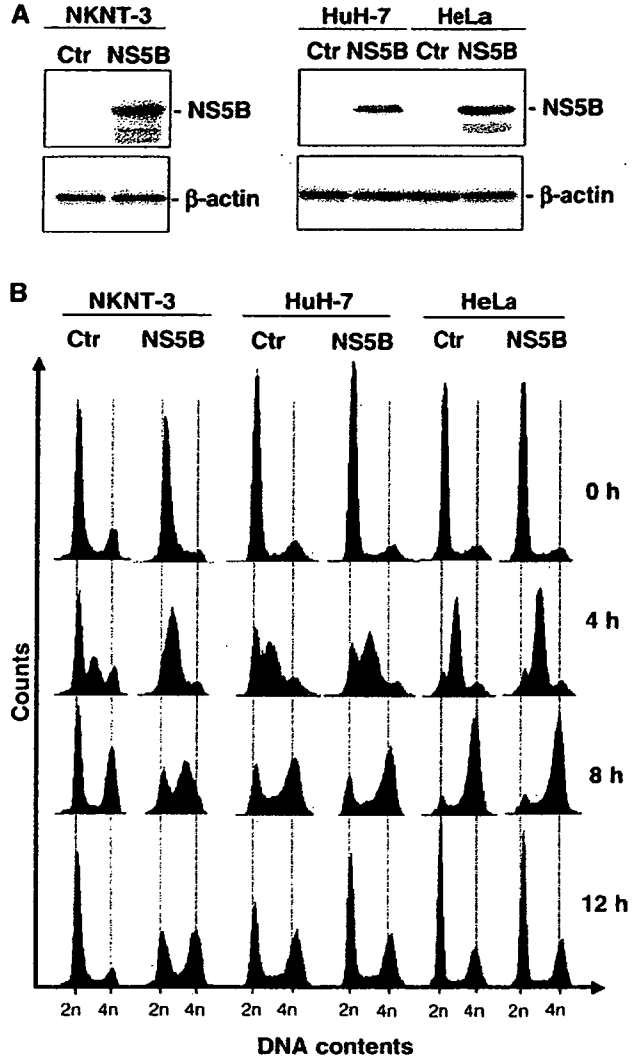


Fig. 2. NS5B delays S phase progression in another immortalized human hepatocytes. (A) Western blot analysis of NKNT-3, HuH-7, and HeLa cells infected with pCXbsr retrovirus encoding NS5B. The pCXbsr retrovirus was used as a control infection (Ctr). Anti-NS5B and anti-β-actin antibodies were used for the immunoblotting analysis. (B) Cell cycle analysis of NKNT-3, HuH-7, and HeLa cells expressing NS5B. NKNT-3, HuH-7, and HeLa cells that expressed NS5B (NS5B series) were synchronized, and cell cycle progression was analyzed as indicated in Fig. 1B. NKNT-3, HuH-7, and HeLa cells that were infected with the pCXbsr retrovirus were also analyzed as a control (Ctr series).

induced by NS5B. To evaluate this hypothesis, we examined whether or not PH/NS5B and NKNT-3 cells expressing NS5B (NK/NS5B) induce the expression of IFN-β. RT-PCR analysis clearly indicated that they did, and, at the same time, we found that HuH-7 and HeLa cells did not, despite their expression of NS5B (Fig. 3A). We next examined whether or not S phase progression is delayed in PH5CH8 and NKNT-3 cells treated with IFN-β prior to release from the G1/S boundary. As we expected, the S phase progression was stalled in PH5CH8 and NKNT-3 cells treated with IFN-β (Fig. 3B). We also observed that IFN-γ did not possess this activity of IFN-β (data not shown). These results suggest that the induction of IFN-β is implicated in the cell cycle delay in two immortalized human hepatocyte cell lines, PH5CH8 and NKNT-3.

To confirm the effect of IFN-β on cell cycle delay, we further examined whether or not treatment with anti-IFN-β neutralizing antibody can restore the cell cycle delay in PH/NS5B cells. The results showed that this treatment canceled the delay of S phase progression in PH/NS5B cells (Fig. 4A). BrdUrd incorporation analysis also showed that the proportion of cells reaching the G2-M phase was increased by the treatment with anti-IFN-β antibody in PH/NS5B cells (Fig 4B). These observations indicated that the expression of IFN-β mediated cell cycle delay during the S phase in PH/NS5B cells and suggested that the expression of NS5B induced IFN-β in PH5CH8 and NKNT-3 cells even without replication of the viral genome.

Activation of TLR3 signaling pathway by NS5B

Since IFN-β is known as a major cytokine induced by the activation of the TLR3 and TLR4 signaling pathways (Takeda et al., 2003), we next focused on which TLR pathway was activated for the production of IFN-β in PH/NS5B cells. To answer this question, TLR3- and TLR4-specific siRNAs were used to knock down TLR3 and TLR4 expression in PH/NS5B cells. TLR3 and TLR4 mRNAs were drastically decreased in PH/NS5B cells transfected with TLR3 and TLR4 siRNAs, respectively, but not in PH/NS5B cells transfected with GL2 siRNA (Elbashir et al., 2001) used as a control (Fig. 5A). This result indicates that the siRNAs used specifically contribute well to the degradation of TLR3 and TLR4 mRNAs. In this condition, IFN-β mRNA was significantly decreased in only PH/NS5B cells transfected with TLR3 siRNA (Fig. 5A), indicating that IFN-β expression in PH/NS5B cells is mediated through the TLR3 signaling pathway. The growth rate of PH/NS5B cells transfected with TLR3 siRNA was also accelerated, although TLR4 siRNA showed a rather lethal effect (Fig. 5B).

Fig. 1. HCV NS5B causes the delay of S phase progression. (A) Expression of HCV proteins in human cells introduced by retrovirus-mediated gene transfer. Western blot analysis of PH5CH8 cells infected with pCXbsr retroviruses encoding HCV proteins (HA-core, NS3, HA-NS4B, HA-NS5A, HA-NS5B, and NS5B) or pCX4bsr retrovirus encoding NS5B. pCXbsr or pCX4bsr retrovirus was used as a control infection (Ctr). Anti-HA (3F10, Roche), anti-NS3 (Novacastra), anti-NS5B, and anti-β-actin (Sigma) antibodies were used for the immunoblotting analysis. (B) Cell cycle analysis of PH5CH8 cells expressing HCV proteins. PH5CH8 cells that expressed HA-core, NS3, HA-NS4B, HA-NS5A, HA-NS5B, or NS5B were synchronized at G1/S boundary, and then cell cycle progression was monitored by flow cytometry after the release of the cells into the S phase at the indicated times. PH/Ctr cells that were infected with pCXbsr retrovirus were also analyzed as a control. (C) Cell cycle analysis of PH5CH8 cells infected with pCX4bsr retrovirus encoding NS5B. The cell cycle analysis was performed as described in panel B. (D) BrdUrd incorporation analysis of PH/Ctr and PH/NS5B cells. Cell cycle distribution of dot-plots of BrdUrd fluorescence versus DNA contents was analyzed in asynchronous or synchronized PH/Ctr and PH/NS5B cells. To measure the cells reaching the G2-M phase at 12 h after release, the cells were accumulated by Noc treatment. (E) Analysis of the cells reaching the G2-M phase. The percentage of cells at that phase was assessed by Noc treatment as indicated in panel D. The data are means ± SD of values from three independent experiments. (F) Growth curve of PH/Ctr and PH/NS5B cells. PH/Ctr (squares) or PH/NS5B (circles) were plated onto 6-well plates (3×10^4 cells per well), and the kinetics of cell proliferation during 4 days in culture were determined by trypan blue treatment. The data indicate average values ± SD from three independent experiments.

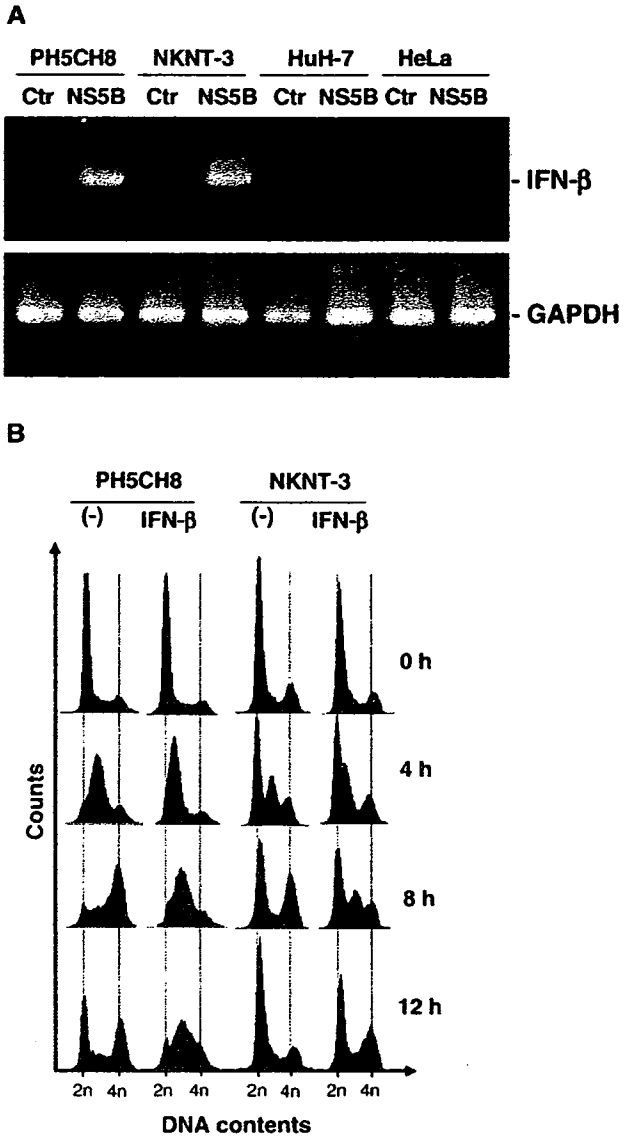


Fig. 3. IFN-β mediates the delay of S phase progression by NS5B. (A) RT-PCR analysis of IFN-β mRNA. The total RNAs were extracted from PH5CH8, NKNT-3, HuH-7, and HeLa cells (Ctr and NS5B series) and were subjected to RT-PCR analysis using primer sets for IFN-β (341 bp) and GAPDH (587 bp). (B) Cell cycle analysis of PH5CH8 and NKNT-3 cells treated with or without IFN-β. PH5CH8 and NKNT-3 cells were treated with or without IFN-β (500 IU/ml) at 12 h prior to release, and cell cycle progression was analyzed as indicated in Fig. 1B.

Furthermore, BrdUrd incorporation analysis using the Noc treatment revealed that 56% of PH/NS5B cells transfected with TLR3 siRNA reached the G2-M phase at 12 h after release, while only 33% of PH/NS5B cells transfected with GL2 siRNA reached that phase (Fig. 5C). The percentage of G2-M phase cells at 12 h after release was also 34% in PH/NS5B cells transfected with TLR4 siRNA, although the growth rate of these cells was lower than that of cells transfected with GL2 siRNA. These results indicated that the induction of IFN-β by NS5B expression was mediated through the activation of the TLR3 signaling pathway. This, in turn, demonstrated that TLR3 siRNA could override the delay of S phase progression in PH/NS5B cells.

To obtain further evidence that the induction of IFN-β by NS5B is mediated through TLR3, we prepared human embryonic kidney (HEK) 293 cells stably expressing TLR3 derived from PH5CH8 cells since it has been reported that ectopic expression of TLR3 can reconstruct the TLR3 signaling pathway in HEK293 cells (Alexopoulou et al., 2001). First, HEK293 cells were infected with retrovirus pCXbsr encoding NS5B or pCXbsr as a negative control (Ctr), yielding HEK293 cells (HEK/NS5B) stably expressing NS5B and control HEK293 cells (HEK/Ctr). Next, HEK/NS5B and HEK/Ctr cells were infected with

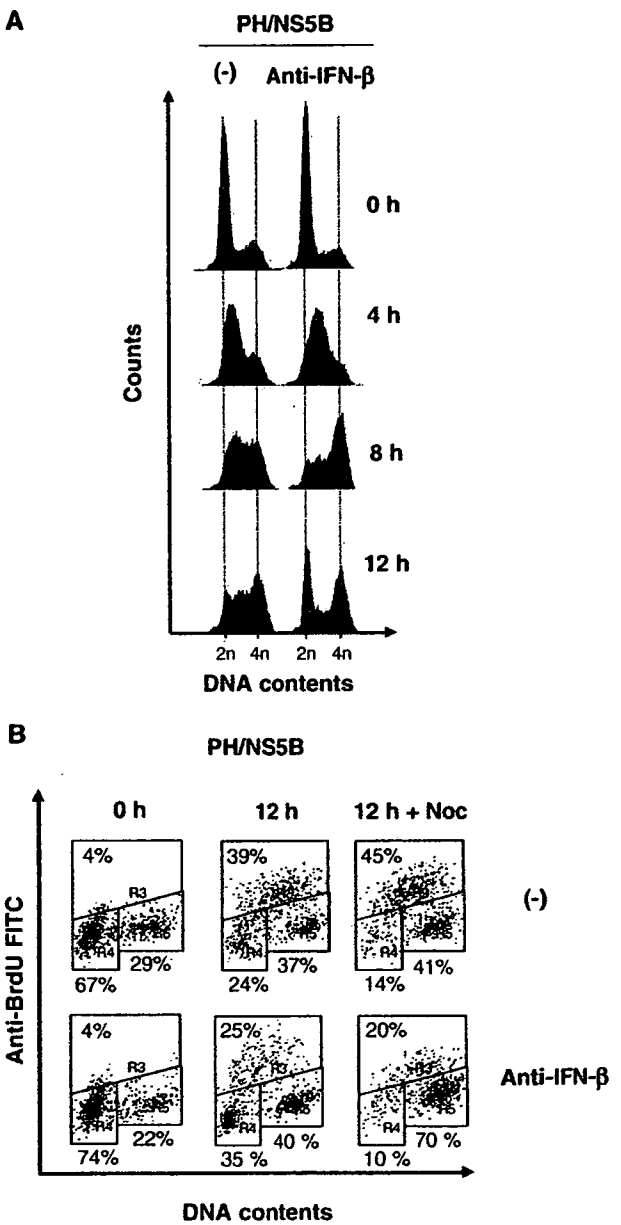


Fig. 4. Treatment with anti-IFN-β antibody canceled the delay of S phase progression. (A) Cell cycle analysis of PH/NS5B cells treated with or without anti-IFN-β antibody. PH/NS5B cells were treated with anti-IFN-β antibody (70 U/ml, Oxford Biotechnology) during cell cycle synchronization and after release from the G1/S boundary. (B) BrdUrd incorporation analysis of PH/NS5B cells treated with or without anti-IFN-β antibody. The antibody was used as indicated in panel A. Noc was used as indicated in Fig. 1D.

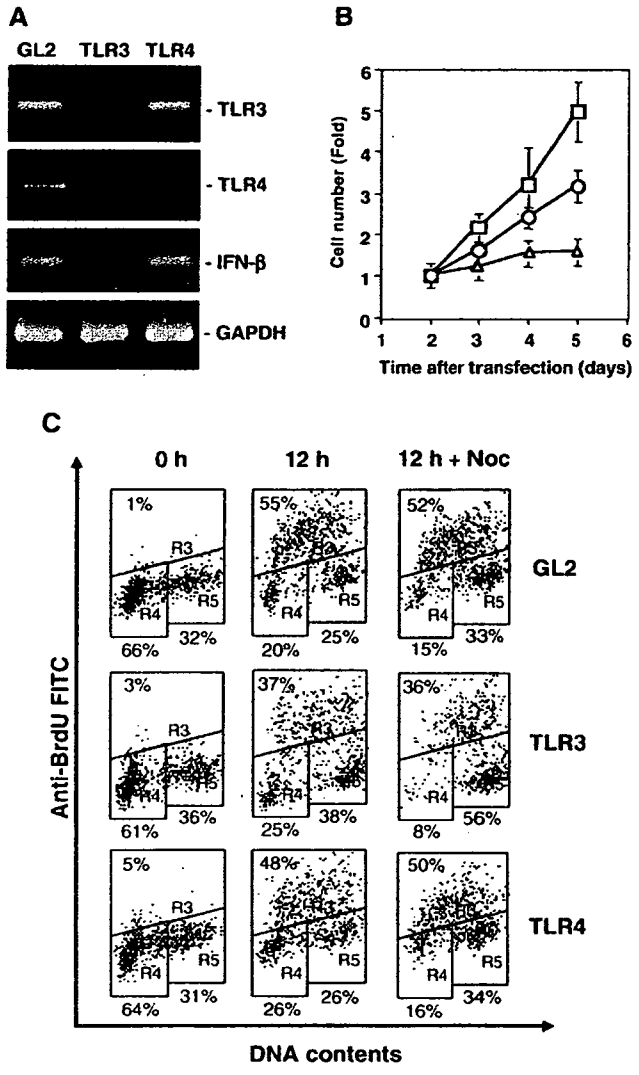


Fig. 5. Activation of IFN-β gene by NS5B is mediated through the TLR3 signaling pathway. (A) Down-regulation of IFN-β mRNA by transfection of TLR3 siRNA. PH/NS5B cells were transfected with dsRNA duplexes targeting TLR3, TLR4, or luciferase GL2. After 3 days, the expression levels of TLR3, TLR4, IFN-β, and GAPDH mRNAs were examined by RT-PCR. (B) Growth curve of PH/NS5B cells transfected with siRNAs. After 2 days of transfection, the proliferation kinetics of PH/NS5B cells transfected with GL2 (circles), TLR3 (squares), and TLR4 (triangles) siRNAs were analyzed as indicated in Fig. 1F. (C) BrdUrd incorporation analysis of PH/NS5B cells transfected with GL2, TLR3, and TLR4 siRNAs. After 2 days of transfection, the cells were synchronized, and cell cycle progression was analyzed as indicated in Fig. 1D.

retrovirus pCXpur encoding TLR3 or pCXpur as a negative control, yielding cells stably expressing TLR3 and control HEK293 cells. The expression of NS5B or TLR3 was confirmed by Western blot analysis (Fig. 6A). We then performed a dual-luciferase reporter assay using an IFN-β gene promoter. The results revealed that the luciferase activity was enhanced in only the HEK293 cells stably expressing both NS5B and TLR3 (Fig. 6B). This suggests that TLR3 mediates NS5B's induction of IFN-β. However, since the enhancement of luciferase activity was approximately two-fold, we failed to detect the enhancement of the mRNA expression levels for IFN-β and one of its target genes, ISG56 (data not shown). To accurately assess the enhancement, high expression levels of NS5B and TLR3 in HEK293 cells will be needed.

The RIG-I-mediated signaling pathway is not implicated in the induction of IFN-β in PH/NS5B cells

Recently, RIG-I, a cellular DExD/H box helicase, was found to be a double-stranded RNA (dsRNA) binding protein that functions independently of TLR3 to induce IFN-β in response to viral infection (Yoneyama et al., 2004). Since another recent study showed that both the TLR3- and RIG-I-mediated signaling pathways are functional in PH5CH8 cells (Li et al., 2005a, 2005b), we examined whether or not the RIG-I-mediated signaling pathway is involved in NS5B's induction of IFN-β. First, PH/NS5B and PH/Ctr cells were infected with retrovirus pCXpur encoding myc-tagged RIG-IC, a dominant negative inhibitor of RIG-I harboring only the helicase domain but not the two N-terminal CARD domains (Yoneyama et al., 2004), or pCXpur as a negative control. Cells that stably expressed myc-tagged RIG-IC were thus obtained. The expression of myc-

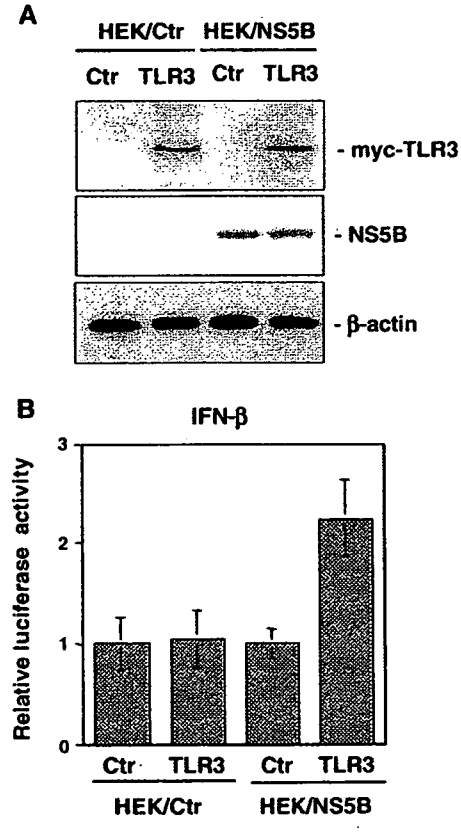


Fig. 6. Ectopic expression of TLR3 enhances the IFN-β gene promoter in only the HEK293 cells stably expressing NS5B. (A) Expression of TLR3 and NS5B in HEK293 cells introduced by retrovirus-mediated gene transfer. Western blot analysis of HEK/Ctr or HEK/NS5B cells infected with pCXpur retrovirus encoding myc-tagged TLR3 was performed. The pCXpur retrovirus was used as a control infection. Anti-myc, anti-NS5B, and anti-β-actin antibodies were used for the immunoblotting analysis. (B) Dual luciferase reporter assay of the IFN-β gene promoter. The cells shown in panel A were transfected with pIFN-β(-125)-Luc, and the dual luciferase assay was performed as described previously (Dansako et al., 2003). Data are means ± SD from three independent triplicate experiments.

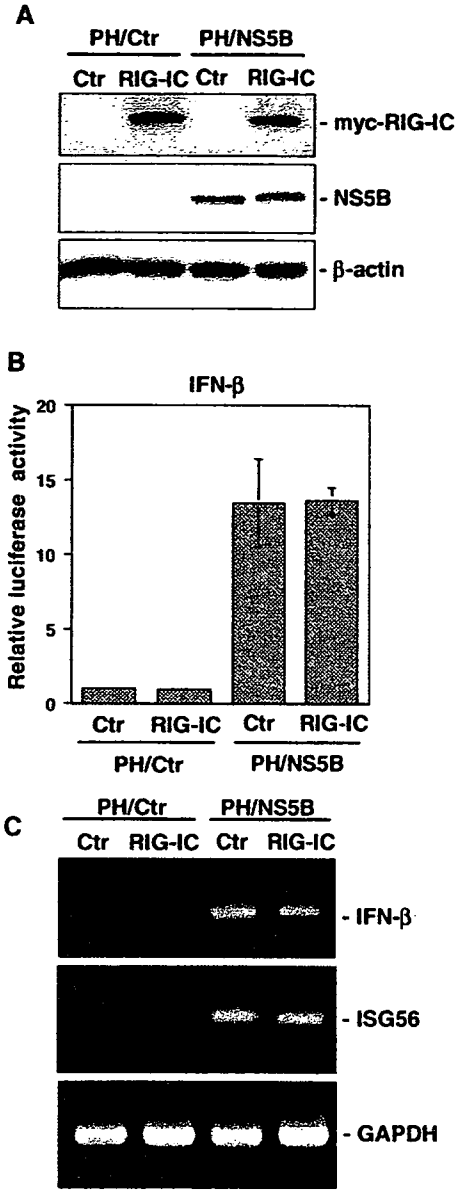


Fig. 7. Induction of IFN-β by NS5B is not mediated through the RIG-I signaling pathway. (A) Expression of RIG-IC and NS5B in PH5CH8 cells introduced by retrovirus-mediated gene transfer. Western blot analysis of PH/Ctr or PH/NS5B cells infected with pCXpur retrovirus encoding myc-tagged RIG-IC was performed. The pCXpur retrovirus was used as a control infection. Anti-myc, anti-NS5B, and anti-β-actin antibodies were used for the immunoblotting analysis. (B) Dual luciferase reporter assay of the IFN-β gene promoter. The cells shown in panel A were transfected with pIFN-β(-125)-Luc, and the dual luciferase assay was performed as indicated in Fig. 6B. (C) RT-PCR analysis of IFN-β and ISG56 mRNAs. The total RNAs were extracted from the cells shown in panel A and subjected to RT-PCR analysis using primer sets for IFN-β (341 bp), ISG56 (320 bp), and GAPDH (587 bp).

tagged RIG-IC was confirmed by Western blot analysis (Fig. 7A). Using PH/Ctr cells stably expressing myc-tagged RIG-IC, we confirmed that IFN-β production was markedly suppressed after infection with Sendai virus (data not shown), as initially observed in Newcastle disease virus infection (Yoneyama et al., 2004). This indicates that RIG-IC functions as a dominant negative inhibitor of RIG-I in PH5CH8 cells. We then performed a dual-luciferase reporter assay using an *IFN-β* gene promoter. The results revealed that the enhancement of luciferase activity in PH/NS5B cells was not suppressed regardless of RIG-IC expression (Fig. 7B). Furthermore, the mRNA expression levels for IFN-β and one of its target genes, ISG56, were also unchanged by the expression of RIG-IC (Fig. 7C). These results suggest that NS5B's induction of IFN-β is not mediated through the RIG-I signaling pathway.

NS5B does not interact with TLR3 adaptor protein

Since we showed that NS5B's induction of IFN-β was mediated through the TLR3 but not the RIG-I signaling pathway, we further examined the mechanism underlying IFN-β induction by testing the possibility of interaction between NS5B and the TLR3 adaptor protein TRIF (Yamamoto et al., 2002). We prepared HEK/NS5B cells stably expressing myc-tagged NS5A or myc-tagged TRIF and examined whether or not NS5B interacts with TRIF by an immunoprecipitation method following Western blot analysis. The results clearly showed that NS5B and myc-tagged NS5A were co-immunoprecipitated by anti-myc antibody as reported previously (Shirota et al., 2002). However, co-immunoprecipitation of NS5B and myc-tagged TRIF was clearly not observed (Fig. 8). This result suggests that the activation of the TLR3 signaling pathway by NS5B occurs through one or more factors other than TRIF.

Induction of IFN-β depends on RNA-dependent RNA polymerase (RdRp) activity of NS5B

Since dsRNA, an intermediate of viral replication, is known as a natural ligand for the activation of TLR3 (Alexopoulou et

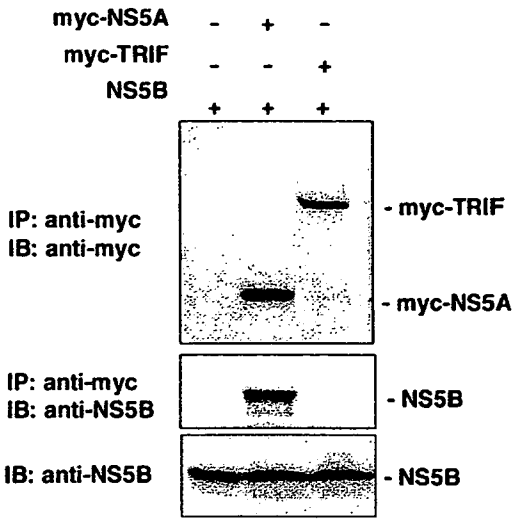


Fig. 8. NS5B does not interact with TRIF. HEK/NS5B cells were infected with pCXpur retrovirus encoding myc-tagged NS5A (middle lane) or myc-tagged TRIF (right lane). pCXpur retrovirus was used as a control infection (left lane). Cell lysate was immunoprecipitated (IP) with anti-myc antibody-conjugated agarose beads. The immunoprecipitates were resolved by SDS-PAGE, and anti-myc (upper panel) and anti-NS5B (middle panel) antibodies were used for the immunoblotting (IB) analysis. To confirm the expression level of NS5B, cell lysates were subjected to immunoblotting analysis using anti-NS5B antibody (lower panel).

al., 2001; Takeda et al., 2003), we next examined whether or not the induction of IFN-β in human hepatocytes expressing NS5B (591 amino acids; amino acids 2420 to 3010 in the HCV-1b genotype) (Kato et al., 1990) depends on NS5B's RdRp activity. Since this activity is already well characterized (Hagedorn et al., 2000), we constructed several NS5B mutants to evaluate this subject (Fig. 9A). One is the substitution mutant G2736V of the GDD motif (amino acids 2736–8) located in the catalytic site, and the other is the deletion mutant Δ2575–7 (R2753T, K2754S, and Δ2575–7) at the priming and interrogation sites, all of which are essential for NS5B's RdRp activity (Behrens et al., 1996; Bressanelli et al., 2002). We also constructed three carboxyl-truncated forms (ΔC21, ΔC56, and ΔC97, lacking 21, 56, and 97 amino acids, respectively) of NS5B. These truncated mutants of NS5B lack the last 21 hydrophobic amino acids, which are necessary and sufficient to target NS5B to the cytosolic side of the endoplasmic reticulum (ER) membrane (Schmidt-Mende et al., 2001; Yamashita et al., 1998). Although ΔC21 and ΔC56, but not ΔC97, possess RdRp activity in vitro, ΔC56 shows higher RdRp activity than ΔC21 because only the latter possesses a regulatory motif inhibiting RNA binding and polymerase activity (Leveque et al., 2003). We prepared PH5CH8 cells stably expressing these NS5B mutants and then performed cell cycle analysis using these

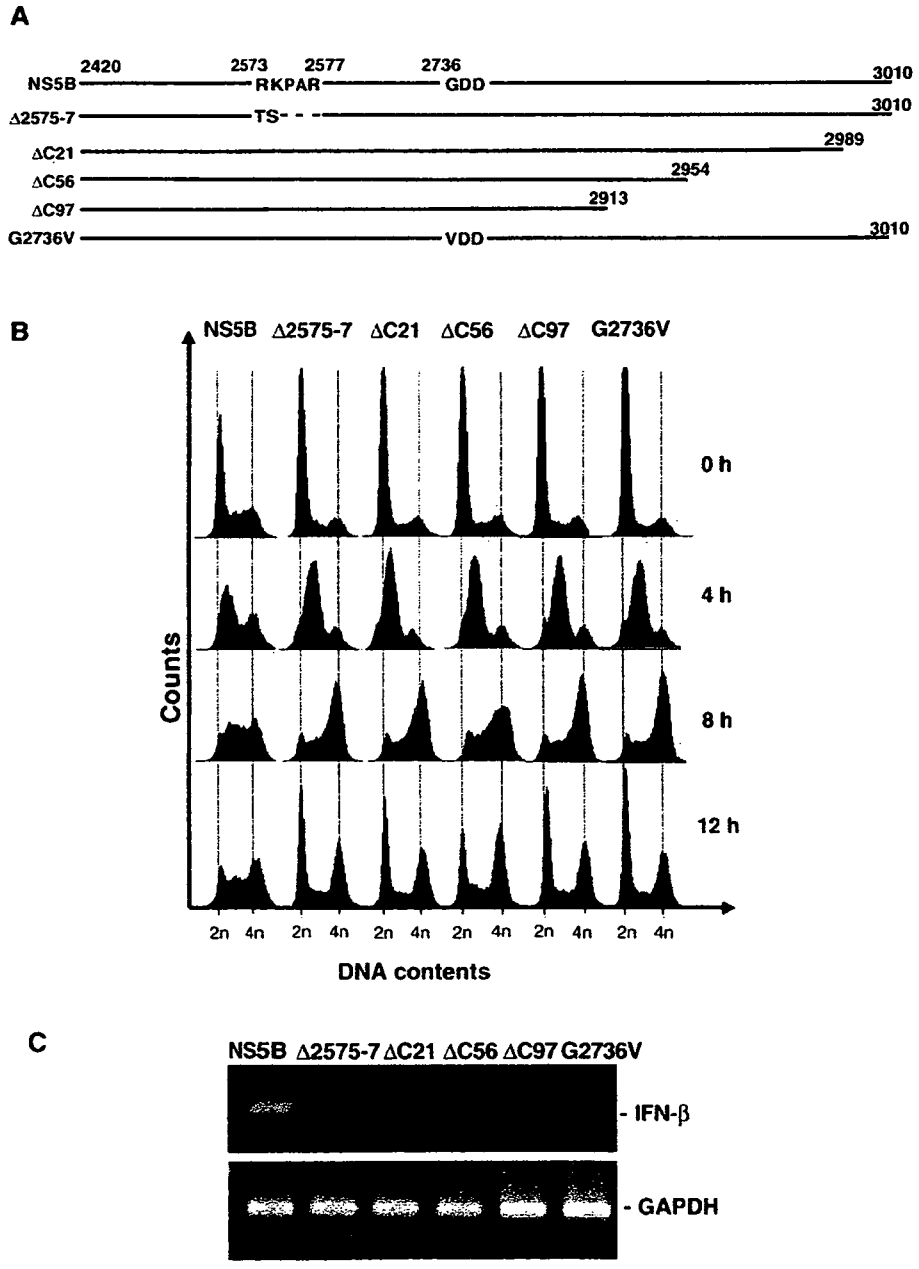


Fig. 9. The RdRp activity of NS5B anchoring on ER membrane is required for induction of IFN-β and following the delay of S phase progression. (A) Schematic presentation of the NS5B mutants used. Only amino acid sequences in the mutated regions of NS5B are indicated. (B) Cell cycle analysis of PH/NS5B and PH5CH8 cells expressing NS5B mutants. Cell cycle distribution was analyzed as described in Fig. 1B. (C) RT-PCR analysis of IFN-β mRNA in PH/NS5B and PH5CH8 cells expressing NS5B mutants. RT-PCR analysis was performed as described in Fig. 3A.

prepared cells. The results revealed no effect on S phase progression in the PH5CH8 cells expressing NS5B mutants (Fig. 9B), although PH5CH8 cells expressing ΔC56 showed a slight delay of S phase progression. Induction of IFN-β mRNA was also not observed in the PH5CH8 cells expressing NS5B mutants (Fig. 9C). These results revealed that the delay of S phase progression and the induction of IFN-β depend on the RdRp activity of NS5B, and these effects are coupled with ER membrane anchorage of NS5B in cells.

To examine the activation of IRF3, a factor specifically induced by stimulated TLR3 or TLR4, by the expression of NS5B and its mutants, we performed a dual-luciferase reporter assay using a synthetic promoter having five repeats of the consensus ISRE, which was the same as the IRF3 target sequence in the *IFN-β* gene promoter (Fig. 10A) and an intrinsic *IFN-β* gene promoter (Fig. 10B). The results showed that the luciferase activity was enhanced approximately five-fold (Fig. 10A) and eight-fold (Fig. 10B) only in PH/NS5B cells,

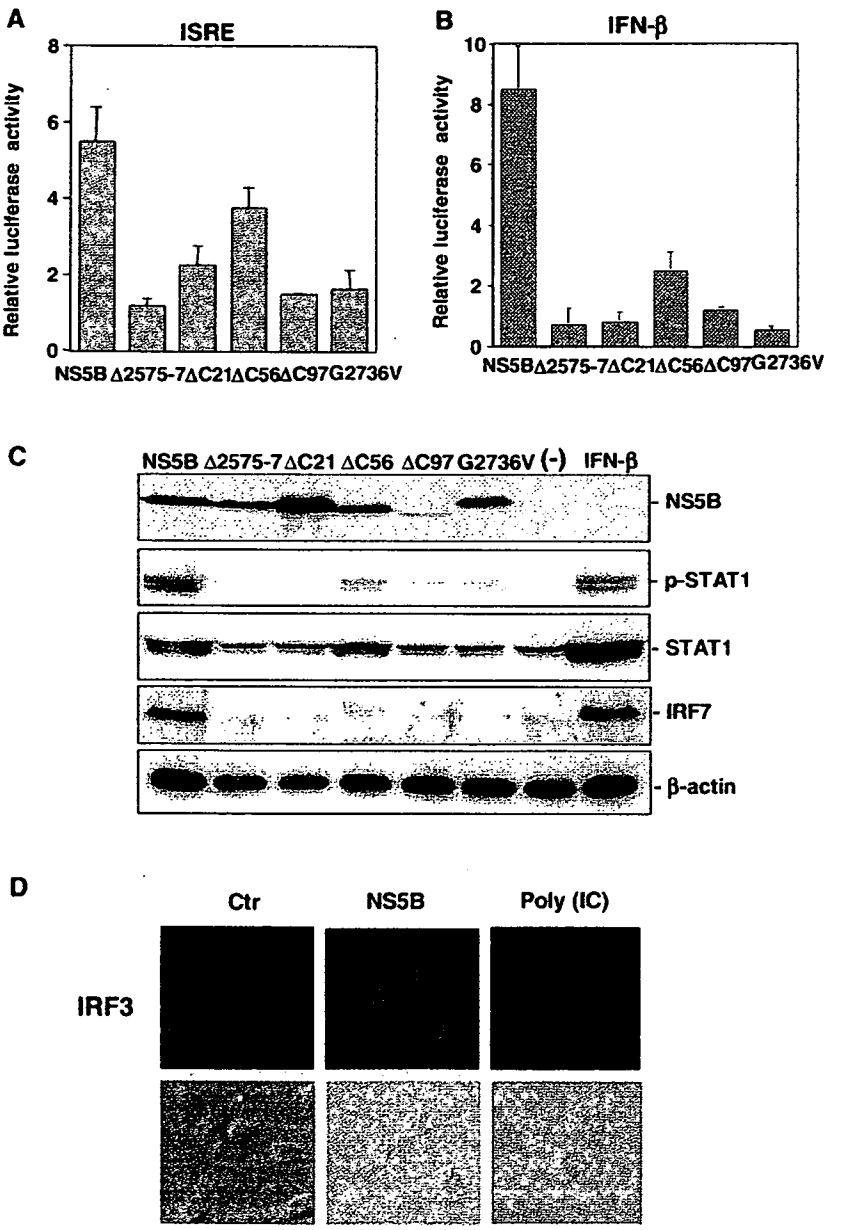


Fig. 10. NS5B full form is required for activation of IRF3 target sequences and IFN-β signaling pathway. (A) Dual luciferase reporter assay toward IRF3 target sequences. PH5CH8 cells were transfected with the pISRE-Luc (Stratagene) and pCXbsr encoding NS5B or its mutant, and the dual luciferase assay was performed as indicated in Fig. 6B. The lysates of cells transfected with pCXbsr were used as a control. (B) Dual luciferase reporter assay of the IFN-β gene promoter. Dual luciferase assay was performed as described in panel A except using pIFN-β(-125)-Luc instead of pISRE-Luc. (C) Western blot analysis of the components involved in the IFN-β signaling pathway. The lysates of PH/NS5B and PH5CH8 cells expressing NS5B mutants were subjected to immunoblotting using anti-NS5B, anti-p-STAT1(Y701), anti-STAT1, anti-IRF7, and anti-β-actin antibodies. PH5CH8 cells treated with or without IFN-β (500 IU/ml for 24 h) were also analyzed as a control. (D) Subcellular distribution of endogenous IRF3. PH/Ctr and PH/NS5B cells were processed and stained with anti-IRF3 antibody and an FITC-conjugated secondary antibody. PH5CH8 cells treated with poly (IC) were also used as a positive control.

suggesting that IRF3 is activated by the NS5B full form. Interestingly, however, luciferase activity was enhanced approximately four-fold (Fig. 10A) and three-fold (Fig. 10B) in PH5CH8 cells expressing ΔC56, although the enhancement was not as great as the five-fold (Fig. 10A) and eight-fold (Fig. 10B) in PH/NS5B cells, respectively. We then examined the phosphorylation status of STAT1 on Y701 and the level of IRF7, one of the downstream targets of the IFN-β signaling pathway (Katze et al., 2002). Western blot analysis revealed marked phosphorylation of STAT1 and IRF7 expression in PH/NS5B cells as well as in PH5CH8 cells treated with IFN-β (Fig. 10C). Although slight phosphorylation of STAT1 was observed in the PH5CH8 cells expressing ΔC56, IRF7 expression was not observed (Fig. 10C). Unlike PH/NS5B cells and PH5CH8 cells expressing ΔC56, neither the phosphorylation of STAT1 nor the expression of IRF7 was detected in PH5CH8 cells expressing other NS5B mutants. These results indicated that ΔC56 had an extremely low ability to induce IFN-β after activation of TLR3, although ΔC56 was still able to enhance the IRF3 target promoter. To obtain further evidence of the activation of IRF3, we examined the subcellular distribution of endogenous IRF3 in PH/Ctr and PH/NS5B

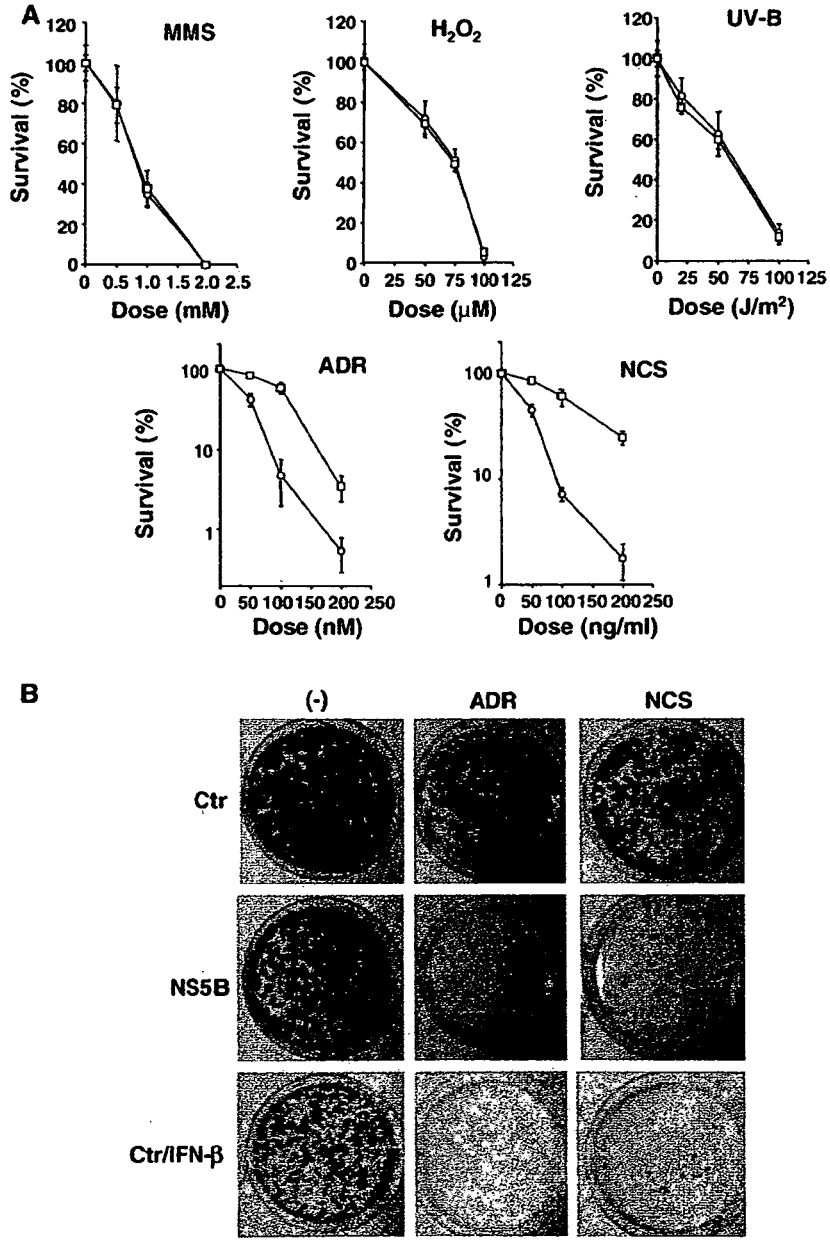


Fig. 11. Sensitivity of PH/NS5B cells against DNA-damaging reagents. (A) Clonogenic assays for PH/Ctr (square) and PH/NS5B (circle) cells after treatment with increasing doses of DNA-damaging reagents. Cells were treated with MMS, H_2O_2, UV-B, ADR, and NCS. Ten days after the treatment, cells were fixed and stained with Coomassie brilliant blue. Only colonies containing >50 cells were scored as being derived from viable clonogenic cells. Data are means ± SD from two independent triplicate experiments. (B) PH/Ctr, PH/NS5B, and IFN-β-treated (20 IU/ml) PH/Ctr cells were treated with ADR (100 nM) or NCS (100 ng/ml). The panels show survived colonies that are stained with Coomassie brilliant blue at 10 days after the treatment.

cells. In PH/Ctr cells, IRF3 was distributed in a perinuclear and/or cytoplasmic context. However, in PH/NS5B cells as well as PH5CH8 cells treated with poly (IC), IRF3 was distributed to the nucleus, a finding consistent with its activated state (Fig. 10D). Taken together, our findings indicate that the RdRp activity of HCV NS5B anchoring on ER membrane is necessary and sufficient to activate the TLR3 signaling pathway.

PH/NS5B cells are more susceptible than PH/Ctr cells to DNA-damaging reagents

To better understand the effect of IFN-β induction in PH/NS5B cells, we next examined the susceptibilities of PH/NS5B and PH/Ctr cells against various types of DNA-damaging reagents. A clonogenic assay using PH/NS5B and PH/Ctr cells was performed by treatment with MMS (a DNA alkylating reagent) and H_2O_2 (a DNA oxidative reagent) and by UV-B irradiation, which induces DNA single-strand breaks and/or thymidine dimer formation in DNA. ADR and NCS, which induce DNA double-strand breaks, were also used for the clonogenic assay. As shown in Fig. 11A, PH/NS5B and PH/Ctr cells were susceptible to the MMS treatment, the H_2O_2 treatment, and the UV-B irradiation, and no differences were observed between their susceptibilities. Interestingly, however, PH/NS5B cells were more susceptible than PH/Ctr cells against ADR or NCS treatment (Fig. 11A). These results suggest that PH/NS5B cells are more sensitive than PH/Ctr cells to damage in the form of DNA double-strand breaks. To clarify whether or not IFN-β induction increases the susceptibility against ADR or NCS treatment, we examined the effect of ADR or NCS in PH/Ctr cells treated with IFN-β. In this treatment, the cells changed to susceptible phenotype against ADR or NCS treatment, as observed in PH/NS5B cells (Fig. 11B). These results suggest that IFN-β induced by NS5B in PH5CH8 cells changes the cells into the hypersensitive phenotype, making them susceptible to DNA damage in the form of double-strand breaks.

Discussion

In the present study, we found that HCV NS5B induced IFN-β in two kinds of immortalized human hepatocyte cell lines, PH5CH8 and NKNT-3. We showed that NS5B's induction of IFN-β was mediated through the TLR3 but not the RIG-I signaling pathway. The induction of IFN-β caused the delay of cell cycle progression through the S phase in these cells. Since it has been generally known that the activation of the TLR3 signaling pathway is caused by dsRNA, a molecular pattern associated with replicating viral genomes, we first obtained data suggesting that dsRNA is generated by NS5B even without replication of the viral genome.

TLRs belong to a family of evolutionarily conserved innate immune recognition molecules, and ten members of the TLR family have been identified in human (Medzhitov, 2001; Takeda et al., 2003). TLR3 recognizes dsRNA and induces the antiviral immune responses (Alexopoulou et al., 2001; Matsumoto et al., 2002). TLR3 activates transcription factor IRF3 through TRIF, leading to IFN-β production (Oshiumi et al., 2003; Yamamoto et al., 2002, 2003). We speculated on two possible mechanisms underlying the activation of the TLR3 signaling pathway by NS5B. The first possibility is that protein–protein interaction between NS5B and TRIF is involved in the activation of the TLR3 signaling pathway. However, we failed to obtain evidence of direct interaction between NS5B and TRIF. The second possibility is that the RdRp activity of NS5B contributes to the activation of TLR3. To evaluate this hypothesis, we examined whether or not several NS5B mutants, including carboxyl-truncated mutants or an RdRp activity-defective mutant (G2736V), could induce IFN-β. The experimental data clearly showed that neither the G2736V mutant nor the carboxyl-truncated mutants could induce IFN-β. Therefore, we suggested that NS5B RdRp activity anchoring the ER membrane is critical for the activation of the TLR3 signaling pathway.

The finding that NS5B RdRp activity on the ER membrane was a critical factor for the induction of IFN-β surprised us because we expected that dsRNA, a natural ligand for TLR3, was produced in NS5B-expressing hepatocyte cells without replication of the viral RNA genome. Therefore, we now presume a daring hypothesis: that NS5B can produce dsRNA using cellular RNA as a template on the ER membrane. Since no direct evidence has been found to support this hypothesis at this stage, further experiments are necessary to evaluate this hypothesis. For instance, if possible, the detection of newly synthesized dsRNA in NS5B-expressing cells or the detection of newly synthesized dsRNA by recombinant NS5B using cellular RNA in vitro may become positive evidence. Furthermore, since the formation of a membrane-associated replication complex is a characteristic of positive-stranded RNA viruses, including HCV (Shi et al., 2003), it will also be interesting to examine whether or not the RdRps of the other RNA viruses possess novel activity similar to that observed in this study. At least we recently detected that NS5B derived from an HCV-2a genome designated JFH-1, which produces virus particles infectious for HuH-7 cells (Wakita et al., 2005), also strongly induced IFN-β in PH5CH8 cells (Ikeda et al., unpublished data). In addition, we are not able to completely exclude the possibility that NS5B-encoding RNA, but not NS5B, specifically activates the TLR3 signaling pathway. However, this possibility is unlikely because the G2736V mutant with only one nucleotide substitution could not activate the TLR3 signaling pathway.

Since the activation of the TLR3 signaling pathway in NS5B-expressing PH5CH8 or NKNT-3 cells is considered to be due to a novel function of NS5B, it is important to clarify whether or not this function occurs in the HCV life cycle. Although HCV replicon systems carrying autonomously replicating HCV RNA genomes developed using HuH-7 (Blight et al., 2000; Ikeda et al., 2002; Lohmann et al., 1999) and HeLa (Zhu et al., 2003) cells have become powerful tools for basic studies of HCV, these systems would not be suitable to prove our hypothesis because the induction of IFN-β by NS5B was not observed in HuH-7 or HeLa cells, in which the TLR3 signaling pathway was suggested to be defective. In fact, it has been recently reported that HuH-7 cells lack a TLR3

response to external dsRNA (Lanford et al., 2003). Therefore, a new HCV replicon system needs to be developed using other human cell lines possessing intact TLR3 signaling pathways. We are currently making a trial to establish an HCV replicon system using PH5CH8 or NKNT-3 cells.

On the other hand, it has been recently found that an HCV serine protease, NS3-4A, can block the phosphorylation and effector action of IRF3 (Foy et al., 2003). This finding using HuH-7 cells suggests that NS3-4A mediates the proteolysis of cellular proteins within an antiviral signaling pathway upstream of IRF3, leading to persistent viral infection. The recently identified TRIF (Li et al., 2005a, 2005b) and RIG-I (Foy et al., 2005) are possible candidates for these cellular proteins. Therefore, it was thought that IFN-β induction by NS5B through the activation of TLR3 might be suppressed by NS3-4A in PH5CH8 cells. In fact, our recent study showed that NS3-4A, in a serine protease activity-dependent manner, suppressed NS5B's activation of the IFN system (Dansako et al., 2005). However, the synergistic induction of IFN-β in PH5CH8 cells co-expressing Core and NS5B was only partially suppressed by NS3-4A, whereas the induction of IFN-β by NS5B only was drastically suppressed by NS3-4A (Dansako et al., 2005). We speculate that a biological implication of this phenomenon is that HCV proteins contribute to the maintenance of a low steady state of the virus by controlling the expression level of IFN-β in the infected cells.

In addition to the delay of cell cycle progression through the S phase, enhanced susceptibility to DNA-damaging reagents was found in NS5B-expressing PH5CH8 cells. This phenomenon was attributed to IFN-β induced by NS5B. Further characterization of this phenomenon may contribute to the understanding of IFN-β's biological effects on hepatocytes and effects on the pathogenesis of hepatocellular carcinoma caused by HCV. Furthermore, the findings of the present study may contribute to an understanding of the mechanisms underlying the TLR3 activation involved in innate immunity against viral infection. In addition, our findings suggest that an antiviral state in uninfected cells may be induced by the expression of a viral protein, NS5B.

Materials and methods

Cell culture and cell cycle analysis

The non-neoplastic immortalized human hepatocyte cell lines, PH5CH8 and NKNT-3 cells, were maintained as described previously (Ikeda et al., 1998; Kobayashi et al., 2000). Human hepatoma cell line HuH-7 cells, human cervical carcinoma HeLa cells, and HEK 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

To synchronize the cells at the G1/S transition, growing cells were treated with thymidine (Sigma, St. Louis, MO) (2.5 mM) for 19 h, washed in PBS, and released into fresh medium for 11 h. The cells were then treated with aphidicolin (Sigma) (5 μM) for 13 h, washed in PBS, and released into fresh medium. The cells were pulse-labeled with 10 μM bromodeoxyuridine (BrdUrd; Sigma) for 1 h, fixed with 70% ethanol at indicated time points, stained with fluorescein-isothiocyanate (FITC)-conjugated mouse monoclonal antibody to BrdUrd (BD Pharmingen, San Diego, CA), and counterstained with propidium iodide (PI) (Sigma). The cellular content of DNA was determined by flow cytometry with FACScalibur instrument, and data were analyzed with CELL Quest software (BD Biosciences, San Jose, CA) (Naka et al., 2004). To determine the population of G2-M phase reached cells, the cells were treated with Nocodazole (Noc; Sigma) (200 ng/ml) at 5 h after release into the S phase. Then, after 7 h (post release from 12 h), the cell population that had accumulated in the G2-M phase was analyzed by flow cytometry. To examine the effects of IFN-β, PH5CH8 and NKNT-3 cells were treated with or without IFN-β (500 IU/ml) at 12 h prior to release, and cell cycle progression was analyzed. To assess the effect of anti-IFN-β neutralizing antibody, NS5B-expressing cells were treated with anti-IFN-β antibody (70 U/ml, OBT0377, Oxford Biotechnology, Oxfordshire, UK) during cell cycle synchronization and after release from the G1/S boundary.

Vector construction and retrovirus infection

Retroviral vectors pCXbsr (Akagi et al., 2000) and pCX4bsr (Akagi et al., 2003), which contain the resistance gene for blasticidin, were used in this study. The DNA fragments encoding the influenza hemagglutinin tagged (HA)-core, NS3, HA-NS4B, HA-NS5A, HA-NS5B, and NS5B were amplified from pMILE (HCV 1B-1 strain belonging to genotype 1b; accession no. AB080299) by PCR using KOD-plus DNA polymerase (Toyobo, Osaka, Japan). The obtained DNA fragments were subcloned into the *Eco*RI (*Bam*HI for NS5B) and *Not*I sites of pCXbsr or pCX4bsr. The DNA fragment encoding myc-tagged NS5A was also amplified from pMILE by PCR. The obtained DNA fragment was subcloned into the *Eco*RI and *Not*I sites of pCXpur (Akagi et al., 2000), which contains the resistance gene for puromycin. The DNA fragments encoding TLR3 (accession no. NM_003265), Toll-IL-1 receptor (TIR) domain-containing adaptor-inducing IFN-β (TRIF or TICAM-1, accession no. NM_182919), and RIG-IC, a dominant negative inhibitor of retinoic acid-inducible gene-I (RIG-I) (Yoneyama et al., 2004), were amplified from cDNAs obtained from PH5CH8 cells. The primer sequences containing the *Sph*I (for forward) or *Not*I (for reverse) recognition sites for TLR3, TRIF, and RIG-IC were designed to enable expression of the TLR3, TRIF, and RIG-IC open-reading frames, respectively. The obtained DNA fragments were subcloned into the *Sph*I and *Not*I sites of pCXpur/myc, which can express myc-tagged protein. The *IFN-β* gene promoter region (−125 to +19) described previously (Fujita et al., 1988) was amplified using genomic DNA derived from PH5CH8 cells and a primer set of 5′-ACGGGGTACC-GAGTTTTAGAAACTACTAAAATG-3′ containing the *Kpn*I recognition site (underlined) and 5′-AGGAAGATCTTC-GAAAGGTTGCAGTTAGAATG-3′ containing the *Bgl*II recognition site (underlined). The obtained DNA fragment was

subcloned into the *Kpn*I and *Bgl*II sites of pGL3-Basic (Promega) and was termed pIFN-β(-125)-Luc. Retrovirus infections were performed as described previously (Naganuma et al., 2004).

RT-PCR and RNA interference

RT-PCR was carried out as described previously (Dansako et al., 2003). The sequences of sense and antisense primers for IFN-β (accession no. V00547) were 5′-CCCTGAGGAGATTAAGCAGCTGC-3′ and 5′-AGTTCCTTAGGATTTCCACTCTGAC-3′. The sequences of primer set for ISG56 (accession no. X03557) were 5′-AGAAGCAGGCAATCACAGAAAAGCTG-3′ and 5′-CCAGGGCTTCATTCATATTTCCTTCC-3′. Small-interference RNA (siRNA) duplexes targeting the coding regions of human TLR3, TLR4, and luciferase GL2 (Elbashir et al., 2001) as a control were chemically synthesized (Greiner, Tokyo, Japan). The sequences of the human TLR3 oligonucleotides were: 5′-CCUCCAGCACAAUGAGCUATT-3′ and 5′-UAGCUCAUUGUGCUGGAGGTT-3′. The sequences of the human TLR4 oligonucleotides were: 5′-CCUCCCCUUCUCAACCAAGTT-3′ and 5′-CUUGGUUGAGAAGGGGAGGTT-3′. The cells were transfected with the indicated siRNA duplex using OligofectAMINE (Invitrogen, Carlsbad, CA). Total RNAs were extracted after 3 days, and RT-PCR was performed using primer sets for TLR3 (Kadowaki et al., 2001), TLR4 (Kadowaki et al., 2001), and GAPDH (Dansako et al., 2003).

Western blot analysis

The preparation of cell lysates, SDS-PAGE, and immunoblotting analysis were performed according to standard procedures using primary antibodies, rat monoclonal anti-HA (3F10; Roche Molecular Biochemicals, Mannheim, Germany), mouse monoclonal anti-NS3 (clone MMM33; Novacastra Laboratories, Newcastle upon Tyne, UK), anti-NS5B (a gift from Dr. M. Kohara), anti-myc (PL14; Medical and biological laboratories, Nagoya, Japan), anti-β-actin (AC-15, Sigma), anti-STAT1 (clone 42; BD Transduction Laboratories, San Diego, CA), rabbit polyclonal anti-phospho-STAT1(Y701) (Cell Signaling Technology, Beverly, MA), and anti-IRF7 (H-246, Santa Cruz Biotechnology, Santa Cruz, CA), and horseradish-peroxidase-conjugated secondary antibodies. The immune complex was visualized using the ECL Western blot detection system (Amersham Bioscience, Piscataway, NJ).

Reporter assay

The luciferase activity was measured by dual-luciferase assay system (Promega, Madison, WI) as previously described (Dansako et al., 2003). Briefly, cells were transfected with pISRE-Luc (Stratagene, LaJolla, CA) or pIFN-β(-125)-Luc reporter plasmid together with phRL-CMV (Promega) as an internal control reporter plasmid by FuGENE6 (Roche). After 48 h of transfection, cell lysates were then prepared and assayed for luciferase activities; transfection efficiency was normalized by renilla luciferase activity (internal control) derived from phRL-CMV. Three independent triplicate transfection experiments were conducted in order to verify the reproducibly of the results.

Immunoprecipitation

Cells were lysed in a buffer containing 50 mM Tris (pH 7.4), 125 mM NaCl, 0.1%(v/v) Nonidet P-40 (NP-40; Sigma), 5 mM EDTA, 0.1 M NaF, and a mixture of protease inhibitors (Complete; Roche). Pre-cleared cell lysates were subjected to immunoprecipitation using agarose-conjugated anti-myc antibody (PL14, MBL). Bound proteins were eluted from beads by boiling in SDS sample buffer, and immunoblotting analysis was performed using anti-myc or anti-NS5B antibody, and HRP-conjugated anti-mouse IgG TrueBlot (eBioscience, San Diego, CA).

Immunofluorescence analysis

To examine the intracellular protein localization, 2×10^4 cells were cultured and treated on chamber slides then fixed and probed with polyclonal rabbit anti-IRF3 antibody (FL-425, Santa Cruz Biotechnology) and FITC-conjugated donkey anti-rabbit secondary antibody according to a method described previously (Foy et al., 2003). PH5CH8 cells treated with poly (IC) (2.5 μg/ml for 6 h; Amersham Biosciences) were used as a positive control for the activation of IRF3.

Evaluation of sensitivity to DNA damage

Cells in an exponential growth phase were plated onto 10-cm plates (5×10^3 cells/plate) and cultured for 4 days. The cells were treated with hydrogen peroxide (H_2O_2; Wako Pure Chemical, Osaka, Japan), methylmethane sulfonate (MMS; Sigma), Adriamycin (ADR; doxorubicin; Sigma), and neocarzinostatin chromophore (NCS; generously provided by Kayaku, Tokyo, Japan) for 2 h at 37 °C. For UV-B treatment (UV-B radiation at 302 nm), the medium was aspirated prior to exposure, the cells were washed twice with PBS, and then a fresh culture medium was added. Ten days later, the cells were fixed and stained with Coomassie brilliant blue as described previously (Naganuma et al., 2004). Only colonies containing >50 cells were scored as being derived from viable clonogenic cells.

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References

Akagi, T., Shishido, T., Murata, K., Hanafusa, H., 2000. v-Crk activates the phosphoinositide 3-kinase/AKT pathway in transformation. Proc. Natl. Acad. Sci. U.S.A. 97, 7290–7295.

Akagi, T., Sasai, K., Hanafusa, H., 2003. Refractory nature of normal human diploid fibroblasts with respect to oncogene-mediated transformation. Proc. Natl. Acad. Sci. U.S.A. 100, 13567–13572.

Alexopoulou, L., Holt, A.C., Medzhitov, R., Flavell, R.A., 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 413, 732–738.

Arima, N., Kao, C.Y., Licht, T., Padmanabhan, R., Sasaguri, Y., 2001. Modulation of cell growth by the hepatitis C virus nonstructural protein NS5A. J. Biol. Chem. 276, 12675–12684.

Bartenschlager, R., Lohmann, V., 2000. Replication of hepatitis C virus. J. Gen. Virol. 81, 1631–1648.

Behrens, S.E., Tomei, L., De Francesco, R., 1996. Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. EMBO J. 15, 12–22.

Blight, K.J., Kolykhalov, A.A., Rice, C.M., 2000. Efficient initiation of HCV RNA replication in cell culture. Science 290, 1972–1974.

Bressanelli, S., Tomei, L., Rey, F.A., De Francesco, R., 2002. Structural analysis of the hepatitis C virus RNA polymerase in complex with ribonucleotides. J. Virol. 76, 3482–3492.

Colombo, M., 1996. The natural history of hepatitis C. Bailliere's Clin. Gastroenterol. 10, 275–288.

Dansako, H., Naganuma, A., Nakamura, T., Ikeda, F., Nozaki, A., Kato, N., 2003. Differential activation of interferon-inducible genes by hepatitis C virus core protein mediated by the interferon stimulated response element. Virus Res. 97, 17–30.

Dansako, H., Naka, K., Ikeda, M., Kato, N., 2005. Hepatitis C virus proteins exhibit conflicting effects on the interferon system in human hepatocyte cells. Biochem. Biophys. Res. Commun. 336, 458–469.

Dore, M.P., Realdi, G., Mura, D., Onida, A., Massarelli, G., Dettori, G., Graham, D.Y., Sepulveda, A.R., 2001. Genomic instability in chronic viral hepatitis and hepatocellular carcinoma. Hum. Pathol. 32, 698–703.

Dubourdeau, M., Miyamura, T., Matsuura, Y., Alric, L., Pipy, B., Rousseau, D., 2002. Infection of HepG2 cells with recombinant adenovirus encoding the HCV core protein induces p21(WAF1) down-regulation—Effect of transforming growth factor beta. J. Hepatol. 37, 486–492.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T., 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411, 494–498.

Foy, E., Li, K., Wang, C., Sumpter Jr., R., Ikeda, M., Lemon, S.M., Gale Jr., M., 2003. Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. Science 300, 1145–1148.

Foy, E., Li, K., Sumpter Jr., R., Loo, Y.M., Johnson, C.L., Wang, C., Fish, P.M., Yoneyama, M., Fujita, T., Lemon, S.M., Gale Jr., R., 2005. Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signaling. Proc. Natl. Acad. Sci. U.S.A. 102, 2986–2991.

Fujita, T., Sakakibara, J., Sudo, Y., Miyamoto, M., Kimura, Y., Taniguchi, T., 1988. Evidence for a nuclear factor(s), IRF-1, mediating induction and silencing properties to human IFN-beta gene regulatory elements. EMBO J. 7, 3397–3405.

Hagedorn, C.H., van Beers, E.H., De Staercke, C., 2000. Hepatitis C virus RNA-dependent RNA polymerase (NS5B polymerase). Curr. Top. Microbiol. Immunol. 242, 225–260.

Hsu, I.C., Tokiwa, T., Bennett, W., Metcalf, R.A., Welsh, J.A., Sun, T., Harris, C.C., 1993. p53 gene mutation and integrated hepatitis B viral DNA sequences in human liver cancer cell lines. Carcinogenesis 14, 987–992.

Ikeda, M., Sugiyama, K., Mizutani, T., Tanaka, T., Tanaka, K., Sekihara, H., Shimotohno, K., Kato, N., 1998. Human hepatocyte clonal cell lines that support persistent replication of hepatitis C virus. Virus Res. 56, 157–167.

Ikeda, M., Yi, M., Li, K., Lemon, S.M., 2002. Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. J. Virol. 76, 2997–3006.

Jung, E.Y., Lee, M.N., Yang, H.Y., Yu, D., Jang, K.L., 2001. The repressive activity of hepatitis C virus core protein on the transcription of p21(waf1) is regulated by protein kinase A-mediated phosphorylation. Virus Res. 79, 109–115.

Kadowaki, N., Ho, S., Antonenko, S., Malefyt, R.W., Kastelein, R.A., Bazan, F., Liu, Y.J., 2001. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J. Exp. Med. 194, 863–869.

Kato, N., 2001. Molecular virology of hepatitis C virus. Acta Med. Okayama 55, 133–159.

Kato, N., Hijikata, M., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., Sugimura, T., Shimotohno, K., 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc. Natl. Acad. Sci. U.S.A. 87, 9524–9528.

Katze, M.G., He, Y., Gale Jr., M., 2002. Viruses and interferon: a fight for supremacy. Nat. Rev., Immunol. 2, 675–687.

Kobayashi, N., Fujiwara, T., Westerman, K.A., Inoue, Y., Sakaguchi, M., Noguchi, H., Miyazaki, M., Cai, J., Tanaka, N., Fox, I.J., Leboulch, P., 2000. Prevention of acute liver failure in rats with reversibly immortalized human hepatocytes. Science 287, 1258–1262.

Kondo, Y., Kanai, Y., Sakamoto, M., Mizokami, M., Ueda, R., Hirohashi, S., 2000. Genetic instability and aberrant DNA methylation in chronic hepatitis and cirrhosis-A comprehensive study of loss of heterozygosity and microsatellite instability at 39 loci and DNA hypermethylation on 8 CpG islands in microdissected specimens from patients with hepatocellular carcinoma. Hepatology 32, 970–979.

Lanford, R.E., Guerra, B., Lee, H., Averett, D.R., Pfeiffer, B., Chavez, D., Notvall, L., Bigger, C., 2003. Antiviral effect and virus–host interactions in response to alpha interferon, gamma interferon, poly(i)–poly(c), tumor necrosis factor alpha, and ribavirin in hepatitis C virus subgenomic replicons. J. Virol. 77, 1092–1104.

Leveque, V.J., Johnson, R.B., Parsons, S., Ren, J., Xie, C., Zhang, F., Wang, Q.M., 2003. Identification of a C-terminal regulatory motif in hepatitis C virus RNA-dependent RNA polymerase: structural and biochemical analysis. J. Virol. 77, 9020–9028.

Li, K., Chen, Z., Kato, N., Gale Jr., M., Lemon, S.M., 2005a. Distinct poly(I–C) and virus-activated signaling pathways leading to interferon-β production in hepatocytes. J. Biol. Chem. 280, 16739–16747.

Li, K., Foy, E., Ferreon, J.C., Nakamura, M., Ferreon, A.C.M., Ikeda, M., Ray, S.C., Gale Jr., M., Lemon, S.M., 2005b. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor-3 adaptor protein, TRIF. Proc. Natl. Acad. Sci. U.S.A. 102, 2992–2997.

Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L., Bartenschlager, R., 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285, 110–113.

Lu, W., Lo, S.Y., Chen, M., Wu, K., Fung, Y.K., Ou, J.H., 1999. Activation of p53 tumor suppressor by hepatitis C virus core protein. Virology 264, 134–141.

Marusawa, H., Hijikata, M., Chiba, T., Shimotohno, K., 1999. Hepatitis C virus core protein inhibits Fas- and tumor necrosis factor alpha-mediated apoptosis via NF-kappaB activation. J. Virol. 73, 4713–4720.

Matsumoto, M., Kikkawa, S., Kohase, M., Miyake, K., Seya, T., 2002. Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. Biochem. Biophys. Res. Commun. 293, 1364–1369.

Medzhitov, R., 2001. Toll-like receptors and innate immunity. Nat. Rev., Immunol. 1, 135–145.

Naganuma, A., Nozaki, A., Tanaka, T., Sugiyama, K., Takagi, H., Mori, M., Shimotohno, K., Kato, N., 2000. Activation of the interferon-inducible 2′-5′-oligoadenylate synthetase gene by hepatitis C virus core protein. J. Virol. 74, 8744–8750.

Naganuma, A., Dansako, H., Nakamura, T., Nozaki, A., Kato, N., 2004. Disturbance of the DNA repair system by HCV core protein. Cancer Res. 64, 1307–1314.

Naka, K., Tachibana, A., Ikeda, K., Motoyama, N., 2004. Stress-induced premature senescence in hTERT-expressing ataxia telangiectasia fibroblasts. J. Biol. Chem. 279, 2030–2037.

Noguchi, M., Hirohashi, S., 1996. Cell lines from non-neoplastic liver and hepatocellular carcinoma tissue from a single patient. In Vitro Cell. Dev. Biol. 32, 135–137.

Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T., Seya, T., 2003. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. Nat. Immunol. 4, 161–167.

Ray, R.B., Ray, R., 2001. Hepatitis C virus core protein: intriguing properties and functional relevance. FEMS Microbiol. Lett. 202, 149–156.

Ray, R.B., Steele, R., Meyer, K., Ray, R., 1998. Hepatitis C virus core protein represses p21WAF1/Cip1/Sid1 promoter activity. Gene 208, 331–336.

Reed, K.E., Rice, C.M., 2000. Overview of hepatitis C virus genome structure, polyprotein processing, and protein properties. Curr. Top. Microbiol. Immunol. 242, 55–84.

Schmidt-Mende, J., Bieck, E., Hugle, T., Penin, F., Rice, C.M., Blum, H.E., Moradpour, D., 2001. Determinants for membrane association of the hepatitis C virus RNA-dependent RNA polymerase. J. Biol. Chem. 276, 44052–44063.

Scholle, F., Li, K., Bodola, F., Ikeda, M., Luxon, B.A., Lemon, S.M., 2004. Virus–host cell interactions during hepatitis C virus RNA replication: impact of polyprotein expression on the cellular transcriptome and cell cycle association with viral RNA synthesis. J. Virol. 78, 1513–1524.

Shi, S.T., Lee, K.J., Aizaki, H., Hwang, S.B., Lai, M.M., 2003. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. J. Virol. 77, 4160–4168.

Shirota, Y., Luo, H., Qin, W., Kaneko, S., Yamashita, T., Kobayashi, K., Murakami, S., 2002. Hepatitis C virus (HCV) NS5A binds RNA-dependent RNA polymerase (RdRP) NS5B and modulates RNA-dependent RNA polymerase activity. J. Biol. Chem. 277, 11149–11155.

Takeda, K., Kaisho, T., Akira, S., 2003. Toll-like receptors. Annu. Rev. Immunol. 21, 335–376.

Thomas, D.L., 2000. Hepatitis C epidemiology. Curr. Top. Microbiol. Immunol. 242, 25–41.

Tsuchihara, K., Hijikata, M., Fukuda, K., Kuroki, T., Yamamoto, N., Shimotohno, K., 1999. Hepatitis C virus core protein regulates cell growth and signal transduction pathway transmitting growth stimuli. Virology 258, 100–107.

Vannucchi, S., Percario, Z.A., Chiantore, M.V., Matarrese, P., Chelbi-Alix, M.K., Fagioli, M., Pelicci, P.G., Malorni, W., Fiorucci, G., Romeo, G., Affabris, E., 2000. Interferon-beta induces S phase slowing via up-regulated expression of PML in squamous carcinoma cells. Oncogene 19, 5041–5053.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., habermann, A., Krausslich, H.G., Mizokami, M., Bartenschlager, R., Liang, T.J., 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11, 791–796.

Yamamoto, M., Sato, S., Mori, K., Hoshino, K., Takeuchi, O., Takeda, K., Akira, S., 2002. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. J. Immunol. 169, 6668–6672.

Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., Akira, S., 2003. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 301, 640–643.

Yamashita, T., Kaneko, S., Shirota, Y., Qin, W., Nomura, T., Kobayashi, K., Murakami, S., 1998. RNA-dependent RNA polymerase activity of the soluble recombinant hepatitis C virus NS5B protein truncated at the C-terminal region. J. Biol. Chem. 273, 15479–15486.

Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S., Fujita, T., 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat. Immunol. 5, 730–737.

Zhu, Q., Guo, J.T., Seeger, C., 2003. Replication of hepatitis C virus subgenomes in nonhepatic epithelial and mouse hepatoma cells. J. Virol. 77, 9204–9210.

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Genetic variation and dynamics of hepatitis C virus replicons in long-term cell culture

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Hepatitis C virus (HCV) genomic sequences are known to vary widely among HCV strains, but to date there have been few reports on the genetic variations and dynamics of HCV in an experimental system of HCV replication. In this study, a genetic analysis of HCV replicons obtained in long-term culture of two HCV replicon cells (50-1 and 1B-2R1), which were established from two HCV strains, 1B-1 and 1B-2, respectively, was performed. One person cultured 50-1 cells for 18 months, and two people independently cultured 50-1 cells for 12 months. 1B-2R1 cells were also cultured for 12 months. The whole nucleotide sequences of the three independent replicon RNA clones obtained at several time points were determined. It was observed that genetic mutations in both replicons accumulated in a time-dependent manner, and that the mutation rates of both replicons were approximately $3{\cdot}0 \times 10^{-3}$ base substitutions/site/year. The genetic diversity of both replicons was also enlarged in a time-dependent manner. The colony formation assay by transfection of total RNAs isolated from both replicon cells at different time points into naïve HuH-7 cells revealed that the genetic mutations accumulating with time in both replicons apparently improved colony formation efficiency. Taken together, these results suggest that the HCV replicon system is useful for the analysis of evolutionary dynamics and variations of HCV. Using this replicon cell culture system, it was demonstrated further that neither ribavirin nor its derivative mizoribine accelerated the mutation rate or the increase in the genetic diversity of HCV replicon.

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INTRODUCTION

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis (Choo *et al.*, 1989; Kuo *et al.*, 1989), which progresses to liver cirrhosis and hepatocellular carcinoma (Ohkoshi *et al.*, 1990; Saito *et al.*, 1990). HCV belongs to the family *Flaviviridae*, whose genome consists of a positive-stranded RNA molecule of 9·6 kb and encodes a large polyprotein precursor of about 3000 aa residues (Kato *et al.*, 1990a; Tanaka *et al.*, 1995). This polyprotein is processed by a combination of the host and viral proteases into at least 10 proteins: the core, envelope 1 (E1), E2, p7, and non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B (Grakoui *et al.*, 1993; Hijikata *et al.*, 1991, 1993; Mizushima *et al.*, 1994). These HCV proteins not only function in virus replication but may also affect a variety of cellular functions, including gene expression, signal transduction and apoptosis (Bartenschlager & Lohmann, 2000; Kato, 2001).

The most characteristic feature of the HCV genome is its remarkable genetic diversity and variation. To date, more than 50 HCV genotypes have been identified worldwide (Bukh *et al.*, 1995; Simmonds, 1995; Tokita *et al.*, 1996). Each of these genotypes shows more than 20 % difference at the nucleotide level and more than 15 % difference at the amino acid level compared with any of the other genotypes, although the 5′ untranslated regions (5′ UTRs) and core protein-encoding regions are highly homologous among the 50 genotypes (homology of >90 %). Comparisons of HCV genomes that belong to a single genotype have revealed 5–8 % diversity in nucleotide sequences and 4–5 % diversity in amino acid sequences (Kato *et al.*, 1990b; Kato, 2001). An analysis of the genetic diversity among the HCV genomes in an individual revealed that the diversity in nucleotide sequences averaged 0·9 %, and distributed throughout

Supplementary material is available in JGV Online.

the genome except in the 5′ UTR (Tanaka *et al.*, 1992). This so-called 'quasispecies' nature of the HCV genome has generally been observed in a single patient with chronic hepatitis C (Kato *et al.*, 1992; Martell *et al.*, 1992). This remarkable genetic diversity of the HCV genome suggests that HCV frequently causes mutations of the viral genome.

To date, two groups have estimated the mutation rate of the HCV genome using specimens from a chimpanzee (interval of 8 years) and a patient (interval of 13 years) infected with HCV (Ogata *et al.*, 1991; Okamoto *et al.*, 1992). They estimated that the mutation rate of the HCV genome was $1{\cdot}4–1{\cdot}9 \times 10^{-3}$ base substitutions/site/year; however, it is not clear whether this value indicates the actual mutation rate of the HCV genome, because complicated quasispecies are generally observed in patients or chimpanzees infected with HCV *in vivo*. On the other hand, Major *et al.* (1999) used chimpanzees that received intrahepatic inoculation with a full-length HCV RNA, and they estimated that the mutation rate of the HCV genome was $1{\cdot}5 \times 10^{-3}$ base substitutions/site/year. However, such experiments on HCV replication in humans are ethically problematic. Thus, there have been few reports on the genetic variations of HCV in an experimental system of HCV replication because of the lack of reproducible and efficient HCV proliferation in cell culture (Kato & Shimotohno, 2000).

In 1999, an HCV replicon system carrying autonomously replicating HCV subgenomic RNA containing the NS3-NS5B regions derived from the strain Con-1 was first established by using a human hepatoma cell line, HuH-7 (Lohmann *et al.*, 1999). Since then, several additional replicon systems have been established (Ali *et al.*, 2004; Blight *et al.*, 2000, 2003; Ikeda *et al.*, 2002; Kato *et al.*, 2003a; Pietschmann *et al.*, 2002; Zhu *et al.*, 2003). In these systems, replicated HCV RNAs were detected by Northern blot analysis and the HCV proteins, which were produced, were detected by Western blot analysis. Therefore, HCV replicon systems are thought to be useful for the analysis of genetic variations and dynamics of HCV.

Recently, we also established two HCV replicons (50-1 and 1B-2R1) derived from two HCV strains, 1B-1 and 1B-2, respectively, using HuH-7 cells (Kato *et al.*, 2003b; Kishine *et al.*, 2002). The nucleotide sequences of the NS3-NS5B regions in the 50-1 replicon showed differences of 8·1% from those in the 1B-2R1 replicon (Kato *et al.*, 2003b), although both HCV strains belonged to genotype 1b. In order to understand the genetic variations and dynamics of HCV, we performed genetic analysis of HCV replicons obtained in long-term culture of 50-1 and 1B-2R1 replicon cells (termed 50-1 and 1B-2R1 cells, respectively). Here, we show that the accumulation of genetic mutations and the acquisition of the genetic diversity among HCV replicons are time dependent. In addition, we evaluated the effect of ribavirin and mizoribine on the genetic variations and dynamics of HCV replicons.

METHODS

Cell cultures. 50-1 and 1B-2R1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 300 μg G418 (Geneticine; Invitrogen) ml^{-1}. The HCV replicon cells were known to possess the G418-resistant phenotype, because neomycin phosphotransferase (Neo^R) was produced by the efficient replication of HCV replicon in the cells. Therefore, when an HCV replicon is excluded from the cells or its level is decreased, the cells are killed by the presence of G418. 50-1 cells were also cultured in the presence of 5 or 25 μM ribavirin (Sigma) or 25 μM mizoribine (Sigma). In general, these replicon cells were passaged every 4 days.

Northern blot analysis. Total RNA from the cultured cells were prepared using an RNeasy extraction kit (Qiagen). Total RNA (3 μg) was used to detect the HCV replicon RNA and β-actin mRNA. Northern blotting and hybridization were performed as described previously (Ikeda *et al.*, 2002; Kato *et al.*, 2003b). A digoxigenin-labelled, negative-sense RNA probe complementary to the NS5B region (positions 8935–9374 of the HCV genome) was used for the detection of the replicon RNA. A β-actin specific digoxigenin-labelled antisense RNA probe was used to check the amount of RNA. The synthetic RNA transcribed from pNSS1RZ2RU (Kato *et al.*, 2003b) (10^8 and 10^7 genome equivalents spiked into normal cellular RNA) was used to compare the level of replicon RNA. An RNA ladder (Invitrogen) was also used to mark the molecular length.

Western blot analysis. The preparation of cell lysates, SDS-PAGE and immunoblotting analysis with a PVDF membrane were performed as described previously (Hijikata *et al.*, 1993; Naganuma *et al.*, 2000). The antibodies used to examine the expression levels of HCV proteins were those against NS3 (Novocastra Laboratories) and NS5B (a generous gift from M. Kohara, Tokyo Metropolitan Institute of Medical Science, Japan). Anti-β-actin antibody (AC-15; Sigma) was also used to detect β-actin as an internal control. Immunocomplexes on the membranes were detected by enhanced chemiluminescence assay (Renaissance; Perkin-Elmer Life Sciences).

RT-PCR. To amplify HCV RNA RT-PCR was performed as described previously (Kato *et al.*, 2003b). Briefly, the total RNA (2 μg) obtained from the replicon cells was used as a template for reverse transcriptase using SuperScript II (Invitrogen). PCR using proofreading KOD-plus DNA polymerase (Toyobo) was performed separately in two parts; one part covered the 5′ UTR to the amino terminal of the NS3 region, and the other part covered the NS3 region to the NS5B region. The PCR yielded a 2033 bp fragment for the former part and a 6107 bp fragment for the latter part.

cDNA cloning and sequencing. The PCR products were subcloned into the *Xba*I site of pBR322MC (Kishine *et al.*, 2002), which was derived from pBR322 and contained the multiple cloning site of pUC19, as described previously (Kato *et al.*, 2003b). Plasmid inserts were sequenced in both the sense and antisense directions by using Big Dye terminator cycle sequencing on an ABI PRISM 310 genetic analyser (Applied Biosystems).

Molecular evolutionary analysis. Nucleotide sequences of the clones obtained by RT-PCRs from 50-1 and 1B-2R1 cells were analysed by the neighbour-joining analysis using the program GENETYX-MAC (Software Development).

RNA transfection and selection of G418-resistant cells. RNA transfection into Huh-7 cells was performed by electroporation as described previously (Lohmann *et al.*, 1999). Briefly, total RNA (80 μg) isolated from the replicon cells was electroporated into 5×10^6 HuH-7 cells, and then 1×10^5 or 3×10^5 cells were seeded into a 10 cm diameter dish. After 48 h, G418 was added to