Table 1 Antihepatitis C virus activities of oral antifungal agents in Huh7/Rep-Feo cells†

	EC_{50} (μM)	CC_{50} (μM)	SI
Griseofulvin	6.13 ± 0.17	217.93 ± 3.49	35.5
Fluconazole	135.6 ± 1.25	159.06 ± 1.07	1.2
Itraconazole	1.24 ± 0.21	3.35 ± 0.17	2.7

†All data represent means ± standard deviation for three separate experiments. CC_{50}, 50% cytotoxicity concentration based on the reduction of cell viability; EC_{50}, 50% effective concentration based on the inhibition of HCV replication; SI, selectivity index (CC_{50}/EC_{50}).

strated that griseofulvin treatment results in reduced levels of these viral proteins (Fig. 2b).

However, it remains to be clarified whether the griseofulvin inhibits firefly luciferase directly. To investigate this possibility, we examined the effect of griseofulvin on firefly luciferase activity using Huh7 cells expressing firefly luciferase constitutively. The treatment of these cells with griseofulvin resulted in no significant change in the firefly luciferase activity (Fig. 3). This result excludes the possibility that griseofulvin inhibits firefly luciferase activity directly.

Anti-HCV activity of griseofulvin is not mediated by the IFN signaling pathway

It has been reported that the HCV replicon is highly sensitive to IFN.[22,23] To determine whether the action of griseofulvin on the HCV subgenomic replicon involves the activation of IFN-stimulated gene responses, we analyzed the expression of IFN inducible genes in HCV replicon cells. The RT–PCR analysis showed that the messenger RNA for MxA and 2′,5′-OAS, which are both IFN inducible genes, were induced by IFNα-2b, but not by griseofulvin (Fig. 4). These results suggest that the action of griseofulvin on the intracellular replication of HCV replicon is independent of the IFN signaling pathway.

Synergistic inhibitory effect of griseofulvin and IFNα on HCV replicon

Whether a combination of griseofulvin and IFNα exhibits a synergistic, additive, or antagonistic effect was assessed using an isobologram method.[19] An isobologram analysis is an approach used in preclinical studies to quantify the extent of synergistic, additive, or antagonistic effects between drugs used in combination. For instance, a representation of an isobologram to evaluate a drug–drug interaction is shown in Figure 5a. It is understood that synergy, additivity, and antagonism are represented by concave, liner, and convex isoeffective curves (isoboles), respectively. The combined anti-HCV effects of griseofulvin and IFNα were evaluated. Prior to the combination experiments, the optimal concentration ratio of two compounds (combination ratio) had to be determined. After preliminary experiments, three different rations were chosen for each combination

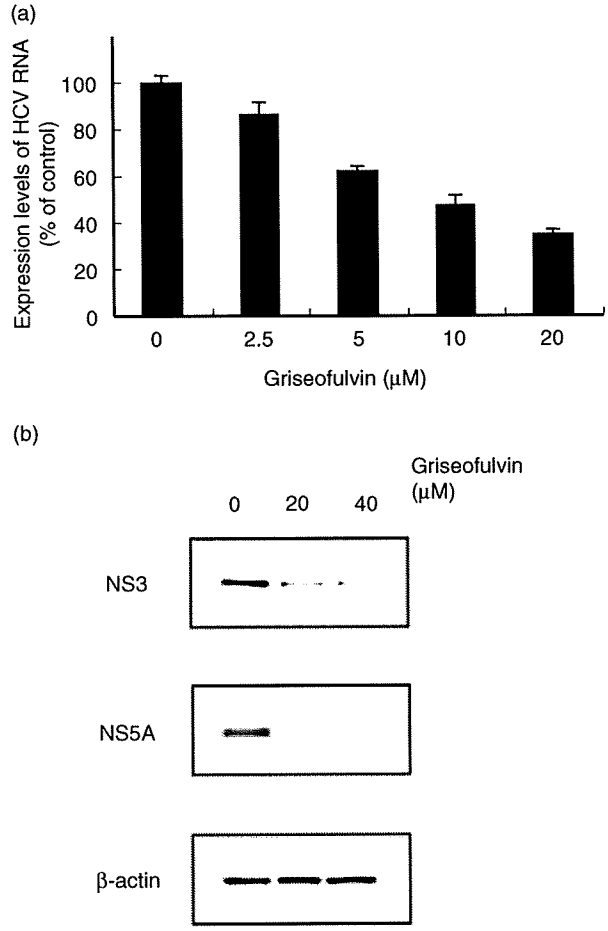

Figure 2 Suppressive effect of griseofulvin for hepatitis C virus (HCV) replicon was confirmed by real-time reverse transcription–polymerase chain reaction (RT–PCR) and Western blot analysis. (a) Incubation of Huh7/Rep-Feo cells with griseofulvin for 72 h resulted in dose-dependent antiviral effects. Real-time RT–PCR was performed on the extracted RNA. HCV-RNA levels are shown as relative percentages of untreated control. Error bars indicate mean ± SD. (b) Western blot analyses of NS3 and NS5A protein expressions were performed on protein extracts from cells that were treated for 72 h with varying dose of griseofulvin. β-Actin was used as a loading control.

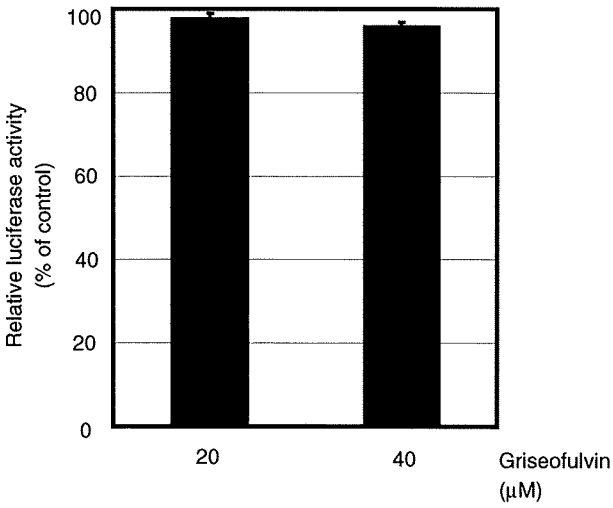

Figure 3 No inhibition of firefly luciferase activity by griseofulvin. pEF Fluc IN vector was stably transfected into Huh7 cells. Cells were cultured without (control) and with 20 μM or 40 μM griseofulvin for 72 h. Firefly luciferase assay was performed. Luciferase activity was normalized by the protein concentration. Error bars indicate mean ± SD.

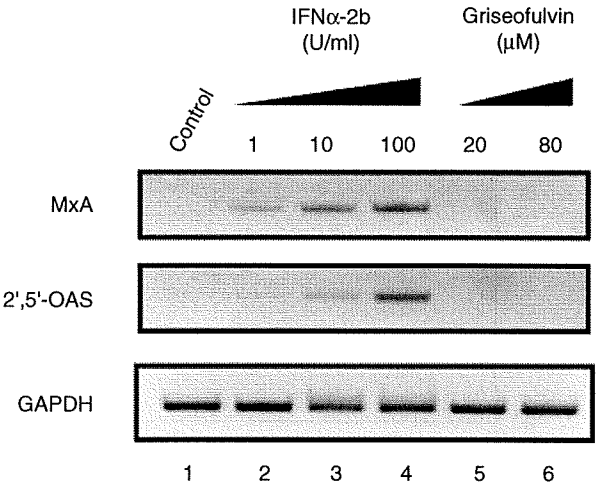

Figure 4 Griseofulvin elicited an interferon (IFN) response. Huh7/Rep-Feo cells were treated without (lane 1) or with 1, 10, or 100 U/mL IFNα-2b (lanes 2–4), and 20 (lane 5) or 80 μM griseofulvin (lane 6) for 72 h. Messenger RNA of human myxovirus resistance protein A (MxA), 2′,5′-oligoadenylate synthetase (2′,5′-OAS), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control were detected by reverse transcription–polymerase chain reaction analysis.

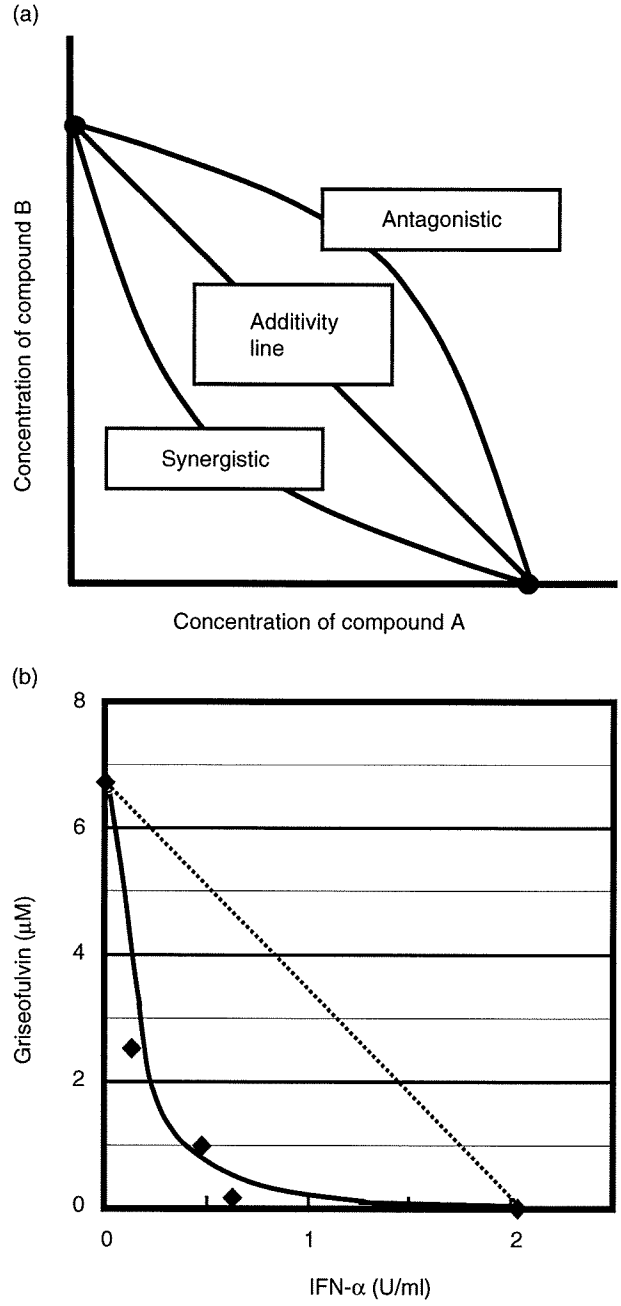

Figure 5 Effect of a combination of griseofulvin and interferon (IFN)α on intracellular hepatitis C virus (HCV)-RNA replication. (a) Representative isobologram for analyzing the interaction between two drugs. (b) Isobole plot of 50% inhibition of HCV-RNA replication. Huh7/Rep-Feo cells were treated with griseofulvin in combination with IFNα, and a luciferase assay was performed after 72 h of culture to obtain each isobole plot. Dotted line indicates an additive effect in the isobologram method used.

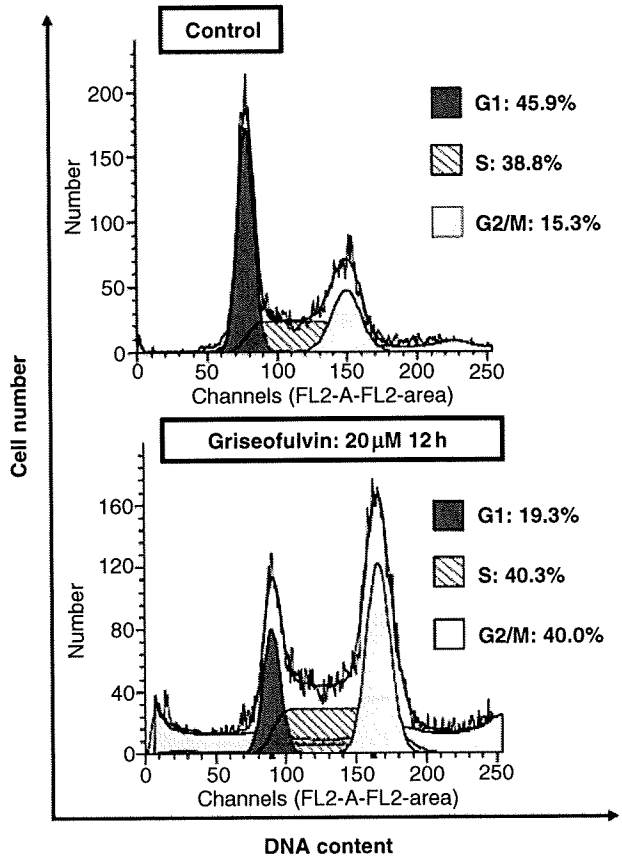

Figure 6 Griseofulvin induced G_2/M phase arrest in Huh7/Rep-Feo cells. Flow cytometry analysis of DNA content of untreated Huh7/Rep-Feo cells (control) and cells treated for 12 h with 20 μM of griseofulvin. [■ G_1: 45.9%, ▨ S: 38.8%, □ G_2/M: 15.3%; ■ G_1: 19.3%, ▨ S: 40.3%, □ G_2/M: 40.0%.]

(data not shown). The ratios of griseofulvin and IFNα were 9:1, 1:1, and 1:9. Each concentration of griseofulvin and IFNα at 50% inhibition was plotted on the X- and Y-axes, respectively, to generate an isobologram (Fig. 5b). As shown in Figure 5b, each plot fell far below the line showing additivity, indicating that the effect of the griseofulvin and IFNα combination on HCV-RNA replication is strongly synergistic.

Griseofulvin induces G_2/M cell cycle arrest in HCV replicon cells

As described previously, griseofulvin blocks cell cycle progression at the G_2/M phase in several human cell lines.[24] Here, we examined the effect of griseofulvin on cell cycle progression in Huh7/Rep-Feo cells. As shown in Figure 6, the population of griseofulvin-treated Huh7/Rep-Feo cells in the G_2/M phase at 12 h was 40%, compared to 15.3% for the control cell populations. These data imply that griseofulvin might have the potential to arrest Huh7/Rep-Feo cells in the G_2/M phase.

As described earlier, the treatment of Huh7/Rep-Feo cells with 20 μM griseofulvin for 12 h results in G_2/M arrest (Fig. 6), while treatment for 72 h had no effect on cell growth (Fig. 1c). To explain this discrepancy, we examined the growth kinetics of griseofulvin-treated Huh7/Rep-Feo cells. The cells were cultured with 20 μM griseofulvin, and cell growth was monitored by MTS assay. The cell viability declined gradually until 48 h after treatment with 20 μM griseofulvin, but increased from 48 h to 72 h (Fig. 7). These data indicate that treatment with 20 μM griseofulvin arrests Huh7/Rep-Feo cells in the G_2/M phase, but does not inhibit cell growth completely.

Griseofulvin does not inhibit HCV IRES-dependent translation

Previous studies have shown that vinblastine sulfate and nocodazole, well-characterized inhibitors of microtubule polymerization and the cell cycle in G_2/M, inhibit

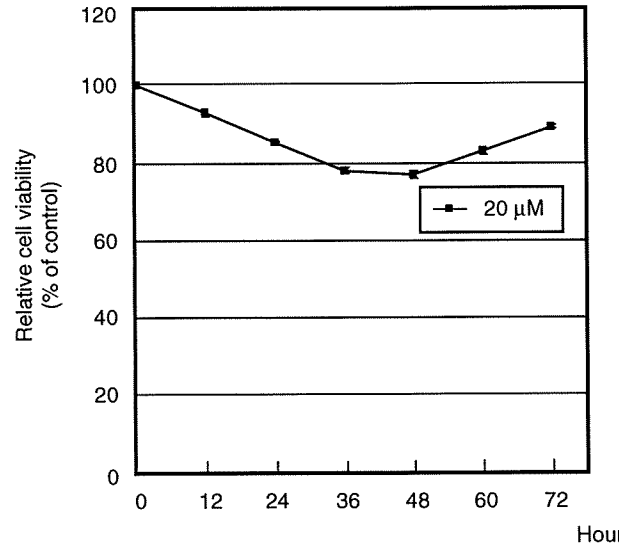

Figure 7 Growth kinetics of griseofulvin treatment of Huh7/Rep-Feo cells. Cells were cultured with [–•–] 20 μM griseofulvin, and cell viability was monitored by a 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium inner salt assay at the times indicated. Error bars indicate mean ± SD.

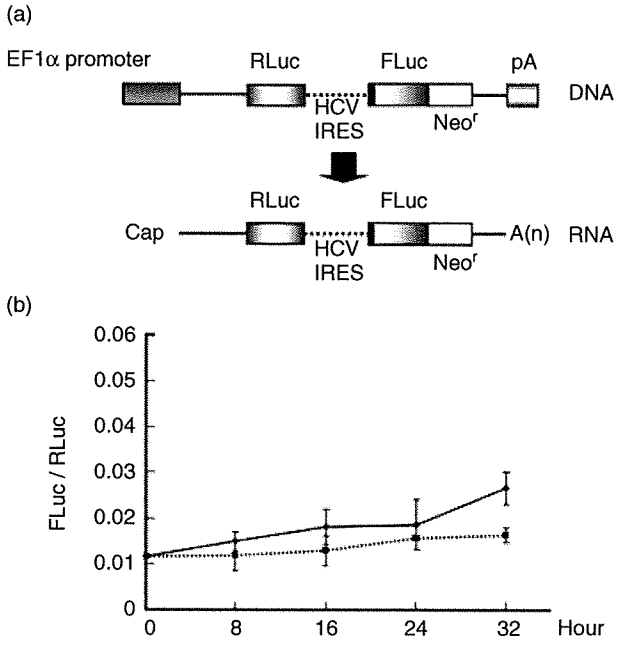

Figure 8 Griseofulvin does not influence hepatitis C virus (HCV) internal ribosomal entry site (IRES)-mediated translation. (a) Structure of the plasmid, pEF-Rluc-HCV IRES Feo. Transcription is initiated under the control of a composite elongation factor 1α (EF1α) promoter. Upstream cistron encodes Renilla luciferase (RLuc) and is translated by a cap-dependent mechanism in transfected cells, while the downstream cistron encodes a fusion (Feo) of the firefly luciferase (Fluc) and neomycin phosphotransferase (Neo^r) genes, translated under the control of the HCV IRES. (b) pEF-Rluc-HCV IRES Feo was stably transfected into Huh7 cells. Cells were treated without (control -◆-) and with 20 μM griseofulvin -■-. Dual luciferase activities were measured at the indicated time points after exposure to griseofulvin. Values are displayed as ratios of Fluc to Rluc. Error bars indicate mean ± SD.

HCV replication, but not HCV IRES-dependent translation.[25] Therefore, we determined whether G_2/M cell cycle arrest by griseofulvin affects HCV IRES-dependent translation using Huh7 cells transfected with pEF Rluc-HCV IRES Feo (Fig. 8a). The treatment of these cells with 20 μM griseofulvin resulted in no significant change of the internal luciferase activities, a concentration that suppressed the expression of the HCV replicon and arrested the HCV replicon cells in the G_2/M phase (Fig. 8b). These results suggested that cell cycle arrest by griseofulvin did not affect HCV IRES-dependent translation, as shown previously for vinblastine sulfate and nocodazole.

Griseofulvin suppressed JFH-1 HCV replication

The studies described thus far were carried out using the subgenomic HCV-1b replicon system. Recently, Wakita *et al.* established a cell culture model for HCV. This system, known as the JFH-1 system and based on genotype 2a HCV, allows the production of a virus that can be efficiently propagated in cell culture (HCVcc).[10] Therefore, we examined the effect of griseofulvin using the JFH-1 system. The Huh7.5.1/JFH-1 cells (cells persistently infected with HCV JFH-1) were cultured with 10 μM or 20 μM griseofulvin for 72 h. We detected the HCV NS3 protein in Huh7.5.1/JFH-1 HCV cells by immunostaining. As shown in Figure 9, in the absence of griseofulvin treatment, the NS3 protein was localized predominantly in the perinuclear region. After treatment of griseofulvin, the NS3 protein expression level was reduced substantially (Fig. 9). This result indicates that griseofulvin also suppresses HCV replication in the JFH-1 HCVcc system.

DISCUSSION

WE HAVE SHOWN here that griseofulvin inhibits the replication of HCV in the HCV subgenomic replicon cells, Huh7/Rep-Feo. In this reporter-based subgenomic replicon system, the EC_{50} of griseofulvin for the inhibition of HCV replication, determined by measurement of the luciferase activity, was approximately 6.13 μM. The real-time RT-PCR and Western blot analyses revealed that both RNA synthesis and its translation were inhibited by griseofulvin in a dose-dependent manner. The treatment of Huh7/Rep-Feo cells with griseofulvin did not activate the IFN inducible gene responses, suggesting that the inhibitory mechanism of griseofulvin in HCV replication is independent of the IFN signaling pathway. Moreover, we demonstrated that the combination treatment of griseofulvin and IFNα had a synergistic inhibitory effect in Huh7/Rep-Feo cells. We also demonstrated that griseofulvin suppressed replication of JFH-1 HCV.

A previous study demonstrated that griseofulvin induces G_2/M arrest in several human cell lines.[24] Here, we show that griseofulvin arrested the Huh7/Rep-Feo cells in the G_2/M phase. Recently, several studies have shown a correlation between HCV IRES-mediated translation and the cell cycle. Honda *et al.* reported that the HCV IRES activity was highest in the G_2/M phase.[26] In contrast, Venkatesan *et al.* reported that the HCV IRES activity was lowest in the G_2/M,[27] while other studies

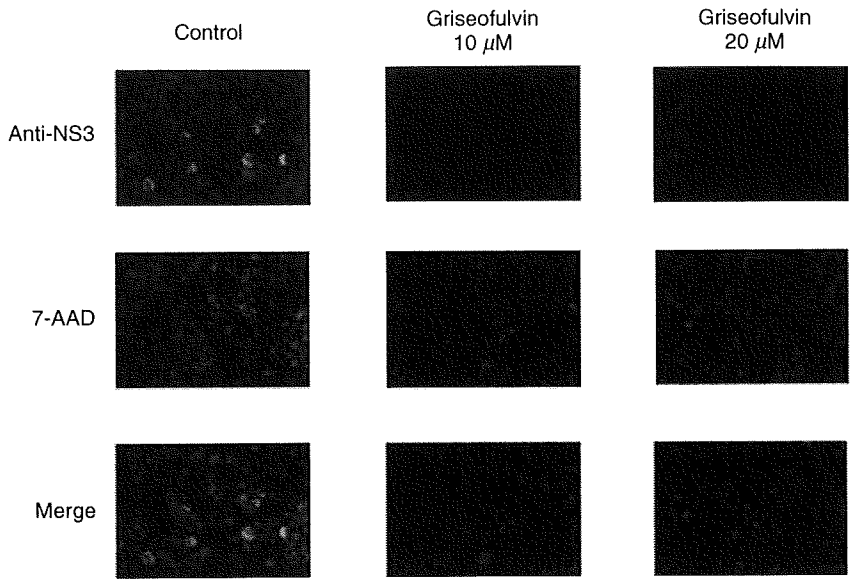

Figure 9 Griseofulvin suppresses JFH-1 replication. Immunofluorescent staining of Huh7.5.1/JFH-1 cells treated with various concentrations of griseofulvin. Hepatitis C virus NS3 protein is stained green and nuclei are stained with 7-aminoactinomycin D (7-AAD; red).

reported that the HCV IRES activity was independent of the stage of the cell cycle.[28,29] In addition, Bost *et al.* reported that several cell cycle inhibitors (vinblastine sulfate, colchicine, nocodazole, and cytochalasin D) did not affect HCV IRES-dependent translation.[25] We also have shown that cell cycle arrest by griseofulvin did not affect HCV IRES-dependent translation. Accordingly, our results support the hypothesis that the HCV IRES activity is independent of the cell cycle.

Previous studies have demonstrated that vinblastine sulfate and nocodazole, well-characterized inhibitors of microtubule polymerization, are able to inhibit HCV-RNA replication in HCV subgenomic replicon cells.[25] These findings indicate that microtubule polymerization is required for the formation of the HCV replication complex. Griseofulvin has been shown to arrest human cells in the G_2/M phase by acting on microtubule polymerization.[30] Thus it is speculated that the inhibition of microtubule polymerization by griseofulvin may influence the formation of the HCV-RNA replication complex. Further, defining the mechanism of action of griseofulvin against HCV replication may be important for defining a novel target for anti-HCV therapy.

Griseofulvin has been used for many years for the treatment of ringworm and other dermatophyte infections. Moreover, griseofulvin does not have significant toxicity for humans. Consequently, the development of derivatives of this compound may be a useful strategy for future therapeutic intervention in chronic hepatitis C.

ACKNOWLEDGMENTS

WE THANK DR Toshio Kitamura (University of Tokyo, Tokyo, Japan) for kindly providing the pGag-pol-IRES-bsr and pMXs-IN plasmids; Dr Francis V Chisari (The Scripps Research Institute, La Jolla, CA, USA) for kindly providing the Huh7.5.1 cell line; Dr Iyoko Katoh for helpful comments and discussion; and Mr Masashi Osano and Ms Ikuko Kayama (University of Yamanashi, Yamanashi, Japan) for technical assistance. This study was supported by a grant of Health Science from the Ministry of Health, Labor, and Welfare of Japan, and by a grant from the Human Sciences Foundation.

REFERENCES

1 Alter HJ, Purcell RH, Shih JW *et al.* Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. *N Engl J Med* 1989; 321: 1494–500.
2 Choo QL, Kuo G, Weiner AJ *et al.* Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 1989; 244: 359–62.
3 Alter MJ. Epidemiology of hepatitis C. *Hepatology* 1997; 26: 62S–5S.
4 Fried MW, Shiffman ML, Reddy KR *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; 347: 975–82.
5 Lohmann V, Korner F, Koch J *et al.* Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 1999; 285: 110–13.

6 Kim SS, Peng LF, Lin W *et al.* A cell-based, high-throughput screen for small molecule regulators of hepatitis C virus replication. *Gastroenterology* 2007; **132**: 311–20.
7 Tanabe Y, Sakamoto N, Enomoto N *et al.* Synergistic inhibition of intracellular hepatitis C virus replication by combination of ribavirin and interferon- alpha. *J Infect Dis* 2004; **189**: 1129–39.
8 Yokota T, Sakamoto N, Enomoto N *et al.* Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. *EMBO Rep* 2003; **4**: 602–8.
9 Lindenbach BD, Evans MJ, Syder AJ *et al.* Complete replication of hepatitis C virus in cell culture. *Science* 2005; **309**: 623–6.
10 Wakita T, Pietschmann T, Kato T *et al.* Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005; **11**: 791–6.
11 Zhong J, Gastaminza P, Cheng G *et al.* Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci USA* 2005; **102**: 9294–9.
12 Hinrichsen H, Benhamou Y, Wedemeyer H *et al.* Short-term antiviral efficacy of BILN 2061a hepatitis C virus serine protease inhibitor, in hepatitis C genotype 1 patients. *Gastroenterology* 2004; **127**: 1347–55.
13 Lamarre D, Anderson PC, Bailey M *et al.* An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus. *Nature* 2003; **426**: 186–9.
14 Reesink HW, Zeuzem S, Weegink CJ *et al.* Rapid decline of viral RNA in hepatitis C patients treated with VX-950: a phase Ib, placebo-controlled, randomized study. *Gastroenterology* 2006; **131**: 997–1002.
15 Sarrazin C, Rouzier R, Wagner F *et al.* SCH 503034, a novel hepatitis C virus protease inhibitor, plus pegylated interferon alpha-2b for genotype 1 nonresponders. *Gastroenterology* 2007; **132**: 1270–8.
16 Amemiya F, Maekawa S, Itakura Y *et al.* Targeting lipid metabolism in the treatment of hepatitis C. *J Infect Dis* 2008; **197**: 361–70.
17 Martell M, Gomez J, Esteban JI *et al.* High-throughput real-time reverse transcription-PCR quantitation of hepatitis C virus RNA. *J Clin Microbiol* 1999; **37**: 327–32.
18 Okada Y, Osada M, Kurata S *et al.* p53 gene family p51 (p63) -encoded, secondary transactivator p51B (TAp63alpha) occurs without forming an immunoprecipitable complex with MDM2, but responds to genotoxic stress by accumulation. *Exp Cell Res* 2002; **276**: 194–200.
19 Chou TC, Talaly P. A simple generalized equation for the analysis of multiple inhibitions of Michaelis-Menten kinetic systems. *J Biol Chem* 1977; **252**: 6438–42.
20 Kitamura T, Koshino Y, Shibata F *et al.* Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics. *Exp Hematol* 2003; **31**: 1007–14.
21 Morita S, Kojima T, Kitamura T. Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther* 2000; **7**: 1063–6.
22 Blight KJ, Kolykhalov AA, Rice CM. Efficient initiation of HCV RNA replication in cell culture. *Science* 2000; **290**: 1972–4.
23 Frese M, Pietschmann T, Moradpour D *et al.* Interferon-alpha inhibits hepatitis C virus subgenomic RNA replication by an MxA-independent pathway. *J Gen Virol* 2001; **82**: 723–3.
24 Ho YS, Duh JS, Jeng JH *et al.* Griseofulvin potentiates antitumorigenesis effects of nocodazole through induction of apoptosis and G2/M cell cycle arrest in human colorectal cancer cells. *Int J Cancer* 2001; **91**: 393–401.
25 Bost AG, Venable D, Liu L *et al.* Cytoskeletal requirements for hepatitis C virus (HCV) RNA synthesis in the HCV replicon cell culture system. *J Virol* 2003; **77**: 4401–8.
26 Honda M, Kaneko S, Matsushita E *et al.* Cell cycle regulation of hepatitis C virus internal ribosomal entry site-directed translation. *Gastroenterology* 2000; **118**: 152–62.
27 Venkatesan A, Sharma R, Dasgupta A. Cell cycle regulation of hepatitis C and encephalomyocarditis virus internal ribosome entry site-mediated translation in human embryonic kidney 293 cells. *Virus Res* 2003; **94**: 85–95.
28 Nelson HB, Tang H. Effect of cell growth on hepatitis C virus (HCV) replication and a mechanism of cell confluence-based inhibition of HCV RNA and protein expression. *J Virol* 2006; **80**: 1181–90.
29 Scholle F, Li K, Bodola F, Ikeda M *et al.* 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* 2004; **78**: 1513–24.
30 Panda D, Rathinasamy K, Santra MK *et al.* Kinetic suppression of microtubule dynamic instability by griseofulvin: implications for its possible use in the treatment of cancer. *Proc Natl Acad Sci USA* 2005; **102**: 9878–83.

Journal of General Virology (2007), **88**, 3323–3333 DOI 10.1099/vir.0.83056-0

Hepatitis C virus non-structural proteins responsible for suppression of the RIG-I/Cardif-induced interferon response

Megumi Tasaka,[1]† Naoya Sakamoto,[1,2]† Yoshie Itakura,[1,3] Mina Nakagawa,[1,2] Yasuhiro Itsui,[1] Yuko Sekine-Osajima,[1] Yuki Nishimura-Sakurai,[1] Cheng-Hsin Chen,[1] Mitsutoshi Yoneyama,[4] Takashi Fujita,[4] Takaji Wakita,[5] Shinya Maekawa,[3] Nobuyuki Enomoto[3] and Mamoru Watanabe[1]

Correspondence
Naoya Sakamoto
nsakamoto.gast@tmd.ac.jp

[1]Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan

[2]Department for Hepatitis Control, Tokyo Medical and Dental University, Tokyo, Japan

[3]First Department of Internal Medicine, University of Yamanashi, Yamanashi, Japan

[4]Laboratory of Molecular Genetics, Department of Genetics and Molecular Biology, Institute for Virus Research, Kyoto University, Kyoto, Japan

[5]Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan

Viral infections activate cellular expression of type I interferons (IFNs). These responses are partly triggered by RIG-I and mediated by Cardif, TBK1, IKKε and IRF-3. This study analysed the mechanisms of dsRNA-induced IFN responses in various cell lines that supported subgenomic hepatitis C virus (HCV) replication. Transfection of dsRNA into Huh7, HeLa and HEK293 cells induced an IFN expression response as shown by IRF-3 dimerization, whilst these responses were abolished in corresponding cell lines that expressed HCV replicons. Similarly, RIG-I-dependent activation of the IFN-stimulated response element (ISRE) was significantly suppressed by cells expressing the HCV replicon and restored in replicon-eliminated cells. Overexpression analyses of individual HCV non-structural proteins revealed that NS4B, as well as NS34A, significantly inhibited RIG-I-triggered ISRE activation. Taken together, HCV replication and protein expression substantially blocked the dsRNA-triggered, RIG-I-mediated IFN expression response and this blockade was partly mediated by HCV NS4B, as well as NS34A. These mechanisms may contribute to the clinical persistence of HCV infection and could constitute a novel antiviral therapeutic target.

Received 4 April 2007
Accepted 27 July 2007

INTRODUCTION

Type I interferon (IFN) plays a central role in eliminating virus, not only following clinical therapeutic application but also as a cellular immune response (Samuel, 2001; Taniguchi & Takaoka, 2002). Hepatitis C virus (HCV) infection is characterized by persistence and replication of the virus in the liver, despite an intact host immune system (Alter, 1997). Indeed, even after administration of the currently most potent IFN reagents, as many as half of the patients are refractory to the treatment and fail to eradicate the virus (Fried *et al.*, 2002). These features have led to speculation that HCV escapes from or attenuates the host antiviral response (Katze *et al.*, 2002).

†These authors contributed equally to this work.

Cellular antiviral responses are primarily mediated by IFN and IFN-stimulated genes (ISGs), including 2,5-oligoadenylate synthetase, dsRNA-dependent protein kinase R (PKR) and MxA proteins, as well as by as yet uncharacterized genes (Itsui *et al.*, 2006; Stark *et al.*, 1998). A study of experimental chimpanzee HCV infection has shown that various cytokines and chemokines are induced in the liver during the course of acute HCV infection and its clearance, and that a considerable proportion of the genes is induced by type I IFN (Bigger *et al.*, 2001).

Control of expression of ISGs is mediated by binding of type I IFNs to their receptors. Following receptor binding, STAT1 and STAT2 are phosphorylated to form ISGF-3, which translocates to the nucleus and binds the IFN-stimulated response element (ISRE), located in the promoter/enhancer region of ISGs, and activates transcription of ISGs (Samuel,

2001; Taniguchi *et al.*, 2001; Taniguchi & Takaoka, 2002). ISRE-dependent gene expression is also mediated by binding of the ISRE by molecules such as IRF-1, IRF-3 and IRF-7 (Kanazawa *et al.*, 2004). IRF-3 is a transducer of virus-mediated signalling and plays a critical role in the induction of cellular antiviral responses (Lin *et al.*, 1998; Sato *et al.*, 2000; Taniguchi *et al.*, 2001; Yoneyama *et al.*, 1998). Transcriptional activation and suppression of IRF-3 are inversely correlated with the level of HCV replication *in vitro* (Yamashiro *et al.*, 2006). Following virus infection, IRF-3 is phosphorylated by two cytoplasmic kinases, TBK1 and IKKε (Fitzgerald *et al.*, 2003; Sharma *et al.*, 2003). The phosphorylated IRF-3 forms a homodimer, translocates to the nucleus and predominantly activates expression of the IFN-β gene and certain ISGs (Doyle *et al.*, 2002; Nakaya *et al.*, 2001; Taniguchi & Takaoka, 2002).

RIG-I is a recently identified cytoplasmic DExD/H box RNA helicase that participates in recognition of virus-related dsRNA as a pathogen-related molecular pattern (Yoneyama *et al.*, 2005). RIG-I contains two caspase-recruitment domains (CARDs) in the N terminus and a DExD/H box RNA helicase in the C terminus. MDA5 has been identified as another CARD-containing DExD/H box RNA helicase (Andrejeva *et al.*, 2004). More recently, an adaptor molecule of RIG-I and MDA5, Cardif (also known as IPS-I, MAVS and VISA), has been identified by four independent groups (Kawai *et al.*, 2005; Meylan *et al.*, 2005; Seth *et al.*, 2005; Xu *et al.*, 2005). On association with dsRNA, RIG-I or MDA5 causes conformational changes and homo-oligomerization, and binds the CARD of Cardif (Saito *et al.*, 2007). Cardif subsequently recruits the kinases TBK1 and IKKε, which catalyse phosphorylation and activation of IRF-3 (Yoneyama *et al.*, 1998).

The IRF-3-mediated IFN-β induction pathway could be a target for viruses to counteract antiviral responses and promote their replication in host cells. Ebola virus, bovine viral diarrhea virus (BVDV) and influenza A virus interfere with the activation of IRF-3 through interactions of their virus-encoded proteins (Basler *et al.*, 2003; Schweizer & Peterhans, 2001; Talon *et al.*, 2000). There are several reports that HCV proteins interact with IFN-mediated antiviral systems. The NS5A and E2 proteins have been reported to interfere with the action of IFN by inhibiting the activity of PKR (He & Katze, 2002). It was reported recently that the HCV NS34A protease blocks virus-induced activation of IRF-3, possibly by proteolytic cleavage of Cardif (Foy *et al.*, 2003; Meylan *et al.*, 2005).

The HCV subgenomic replicon is an *in vitro* model that simulates autonomous cellular replication of HCV genomic RNA (Lohmann *et al.*, 1999). Expression of the HCV replicon can be abolished by treatment with small amounts of type I and type II IFNs (Blight *et al.*, 2000; Frese *et al.*, 2002; Guo *et al.*, 2001), suggesting intact IFN receptor-mediated cellular responses. In contrast, viral expression persists in the absence of the exogenous IFN. Baseline expression levels of ISG were substantially decreased in cells expressing the HCV replicon compared with parental Huh7 cells (Kanazawa *et al.*, 2004). These findings led us to speculate that intracellular virus-induced antiviral responses are attenuated or caused to malfunction by the expression of viral proteins.

In this study, we investigated cell lines that support subgenomic HCV replication and HCV cell culture for the dsRNA-induced cellular IFN expression pathway. Here, we report that RIG-I- and Cardif-mediated IFN gene activation is uniformly attenuated in several replicon-expressing cell lines of different lineages and, more importantly, that the HCV NS4B protein is involved in the suppression of antiviral IFN responses.

METHODS

Plasmids. Plasmids pEF-flagRIG-I and ΔRIG-I expressed full-length and C-terminally truncated RIG-I protein, respectively (Yoneyama *et al.*, 2004). The plasmid pER-flagRIG-IKA (RIG-IKA) has a point mutation in the putative ATP-binding site of the RIG-I helicase domain and was used as a negative control for ΔRIG-I and RIG-I full transfection assays. Expression plasmids for full-length Cardif (Cardif), Cardif CARD (CARD) and CARD-truncated Cardif (ΔCARD) were provided by Dr J. Tschopp (University of Lausanne, Switzerland) (Meylan *et al.*, 2005). Expression plasmids for toll-like receptor 3 (TLR3) and TIR domain-containing adaptor inducing IFN-β (TRIF), the transmembrane receptor of dsRNA and the adaptor molecule of TLR3, respectively, were provided by Dr S. Akira (Osaka University, Japan). Plasmids expressing HCV NS345, NS3, NS34A, NS4A, NS4B, NS5A and NS5B were amplified from HCV pCV-J4-L4S (Yanagi *et al.*, 1997) by PCR and subcloned. The DNA fragments were inserted into the vector pcDNA4/TO/*myc*-His (Invitrogen). Nucleotide sequences were confirmed by sequencing. Plasmids TOPO-NS34A (HCV N), TOPO-NS4B (HCV N) and pcDNA-NS4B (HCV JFH1) expressed Myc-tagged NS34A and NS4B proteins derived from the HCV N (Beard *et al.*, 1999) and HCV JFH1 (Wakita *et al.*, 2005) strains, as indicated. Plasmid pISRE-TA-Luc (Invitrogen) contained five copies of consensus ISRE motifs upstream of the firefly luciferase gene. Plasmid pIFNβ-Fluc was constructed by cloning the human IFN-β promoter region, spanning nt −110 to −36, upstream of the firefly luciferase gene of pGL3 Basic (Promega). Plasmid pcDNA3.1 (Invitrogen) was used as an empty vector for mock transfection. pRL-CMV (Promega), which expressed the *Renilla* luciferase protein, was used for correction of transfection efficiency.

Cell culture. HCV strain JFH1-infected Huh7.5.1, Huh7, Huh7.5.1 (kindly provided by Dr F. Chisari, The Scripps Institute, CA, USA; Zhong *et al.*, 2005), HeLa and HEK293 cells were maintained in Dulbecco's modified minimal essential medium (Sigma) supplemented with 2 mM L-glutamine and 10 % fetal calf serum at 37 °C with 5 % CO_2. Cells expressing the HCV replicon were cultured in medium containing 100 μg G418 (Wako) ml^{-1}.

HCV replicon constructs and transfected cell lines. An HCV subgenomic replicon plasmid, pHCVIbneo-delS (designated pRep-N), was derived from an HCV clone of strain N, genotype 1b, and pSGR-JFH1 was derived from HCV JFH1, genotype 2a (Guo *et al.*, 2001; Wakita *et al.*, 2005). These replicons were reconstructed by substituting the neomycin phosphotransferase gene with a fusion gene comprising *Renilla* luciferase and neomycin phosphotransferase to construct pRep-Reo-1b and pRep-Reo-2a, respectively (Tanabe *et al.*, 2004; Yokota *et al.*, 2003). RNA was synthesized from the replicons using T7 polymerase (Promega) and transfected into Huh7,

HeLa and HEK293 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established (Huh7/1bReo, Huh7/2aReo, HeLa/2aReo and 293/2aReo). We have previously reported that firefly luciferase activities of Feo-replicon-expressing cells correlate well with HCV NS3, NS4A and NS5A protein expression levels and with the levels of replicon RNA (Yokota *et al.*, 2003).

Transient transfection. Transient DNA transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. ISRE reporter assays were carried out as previously described (Nakagawa et al., 2004). To analyse IFN expression in HCV JFH1 cell cultures, a total of 1×10^5 Huh7.5.1, JFH-1 infected Huh7.5.1 and IFN-treated Huh7.5.1 cells were seeded into 24-well plates the day before transfection. Plasmids pISRE-TA-Luc and ΔRIG-I (200 ng each) were transfected using 1 μl Lipofectamine 2000. RIG-IKA was used as a control. Luciferase assays were performed on day 3 post-transfection.

For further study, 400 ng of each non-structural protein was added to 1×10^4 Huh7 or HEK293 cells that had been seeded into 96-well plates the day before transfection. pISRE-TA-Luc and ΔRIG-I (40 ng each) were transfected using 0.5 μl Lipofectamine 2000. RIG-IKA was used as a control.

Western blotting. Preparation of the cytoplasmic and nuclear fractions of cell lysates was carried out as described previously (Tanabe *et al.*, 2004). Protein (20 μg) was separated using NuPAGE 4–12 % Bis/Tris gels (Invitrogen) and blotted onto an Immobilon PVDF membrane (Roche). The membrane was immunoblotted with anti-IRF-3 (Santa Cruz) and detected by chemiluminescence (BM Chemiluminescence Blotting Substrate; Roche).

RT-PCR. Interleukin (IL)-8 mRNA was detected by RT-PCR as described previously (Itsui *et al.*, 2006). The primers used were IL8-S (5′-GCACAAACTTTCAGAGACAGCAGAGCACAC-3′) and IL8-AS (5′-CAGAGCTGCAGAAATCAGGAAGGCTGCCAA-3′).

Indirect immunofluorescence assay. Cells seeded onto tissue culture chamber slides were fixed with cold acetone. The cells were incubated with anti-protein disulphide isomerase (PDI) or anti-Myc antibodies and subsequently with Alexa 488- or Alexa 568-labelled secondary antibodies. Cells were mounted with VECTA SHIELD Mounting Medium and DAPI (Vector Laboratories) and visualized by fluorescence microscopy (BZ-8000; Keyence).

Luciferase reporter assays. Luciferase activity was measured using a 1420 Multilabel Counter (ARVO MX; PerkinElmer) using a Bright-Glo Luciferase Assay System (Promega) or a Dual Luciferase Assay System (Promega). Assays were carried out in triplicate and the results expressed as means ± SD.

MTS assay. To evaluate cell viabilities, dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assays were performed using a CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega) according to manufacturer's instructions.

Statistical analyses. Statistical analyses were performed using an unpaired, two-tailed Student's *t*-test. *P* values of less than 0.05 were considered to be statistically significant.

RESULTS

IRF-3 dimer formation is attenuated in cells expressing the HCV replicon

In the HCV replicon-expressing cell lines Huh7/Rep-Reo-2a, Hela/Rep-Reo-2a and 293/Rep-Reo-2a, replicon expression levels corresponded well to internal *Renilla* luciferase activities. Expression of the HCV replicon was suppressed by IFN in a dose-dependent manner (data not shown).

Activation of RIG-I or MDA5 induces phosphorylation and homodimerization of IRF-3. Following transfection of poly(I : C) into Huh7, HeLa or HEK293 cells, IRF-3 dimers were detected (Fig. 1). However, in cells supporting HCV replicons, IRF-3 dimer formation was almost completely abolished. These findings showed that expression of HCV proteins blocked activation of dsRNA-mediated IFN expression and that these effects were consistently found in several cell lines of different origin.

The HCV replicon suppresses RIG-I/Cardif-induced IFN responses

ISRE reporter activities did not increase in naïve Huh7, HeLa or HEK293 cells following transfection of poly(I : C), whilst overexpression of full-length RIG-I increased poly(I : C)-mediated ISRE reporter activity in Huh7 and HEK293 cells (data not shown). In RIG-I-overexpressing Huh7 cells, transduction with an HCV replicon abolished the poly(I : C)-induced ISRE activation, and elimination of the replicon by IFN treatment restored these ISRE responses (Fig. 2a). Consistent results were obtained by overexpression of ΔRIG-I, a constitutively active form. Transfection of ΔRIG-I in Huh7 and HEK293 cells induced ISRE activation, whilst these responses were abolished or significantly suppressed in cell lines expressing HCV replicons and were recovered by eliminating the replicon by IFN treatment (data not shown). Similarly, ISRE activation by overexpression of Cardif, an adaptor molecule of RIG-I, was almost completely blocked in replicon-expressing cells and was recovered by eliminating the replicon from the cells (data not shown). The RIG-I-mediated IFN response was

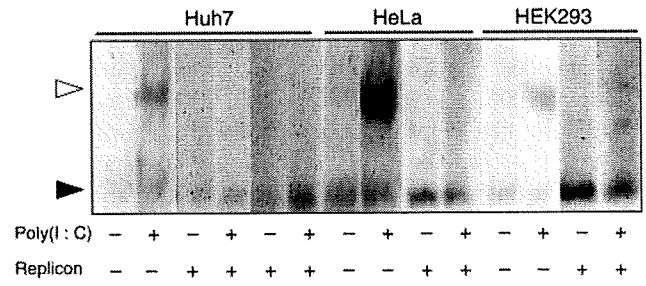

Fig. 1. Double-stranded RNA-induced IRF-3 dimer formation in cell lines that support HCV subgenomic replication. Poly(I : C) was transfected into naïve Huh7, HeLa and HEK293 cells, and into corresponding cell lines expressing the HCV replicon. Six hours after transfection, cell lysates were prepared, separated in polyacrylamide gels and blotted onto PVDF membrane. The membrane was immunoblotted with anti-IRF-3 and visualized by chemiluminescence (see Methods). The positions of the IRF-3 dimer (open arrowhead) and monomer (closed arrowhead) are indicated.

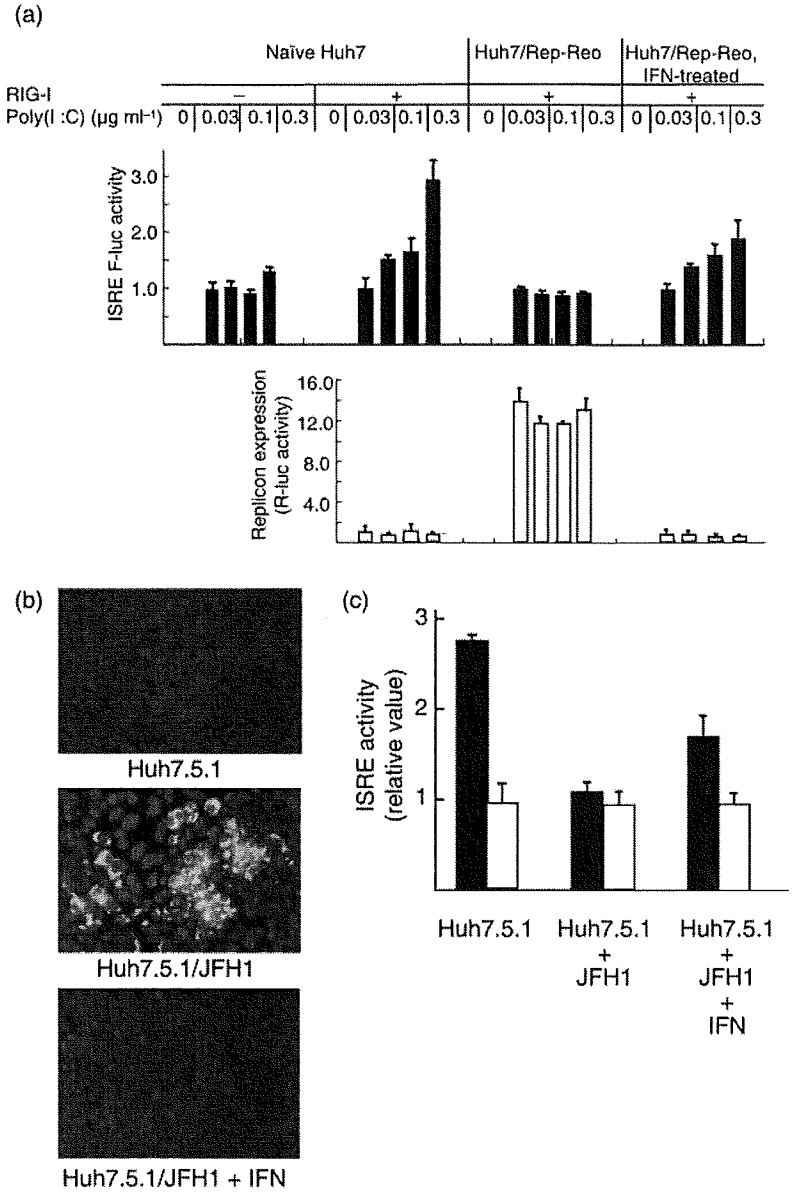

Fig. 2. Suppression of dsRNA-induced, RIG-I-mediated ISRE activation by HCV replication. (a) The HCV replicon suppresses transcriptional activation after poly(I : C) stimulation. The RIG-I expression plasmid and pISRE-TA-Luc were transiently transfected into the cell lines indicated. The following day, the amounts of poly(I : C) indicated were transfected into the corresponding cell lines and dual luciferase assays were carried out 8 h after transfection. Filled bars indicate ISRE-regulated firefly luciferase (F-luc) activities and open bars indicate *Renilla* luciferase (R-luc) activities representing replicon expression levels. In both graphs, scales for the *y*-axis are shown as relative values. Assays were carried out in triplicate and results are shown as means ± SD. (b) Immunofluorescence microscopy results. Huh7.5.1 cells infected with HCV JFH1 (Huh7.5.1/JFH1) and JFH1-infected cells from which the virus had been eliminated by IFN treatment (Huh7.5.1/JFH1 + IFN) were incubated with anti-core primary antibodies followed by Alexa Fluor-conjugated secondary antibody (green). Nuclei were stained with DAPI (blue). (c) ISRE activation by ΔRIG-I overexpression. The plasmid pISRE-TA-Luc was co-transfected with ΔRIG-I (filled bars) or RIG-IKA (empty bars) into naïve Huh7.5.1, Huh7.5.1/JFH1 or Huh7.5.1/JFH1 + IFN cells. Luciferase assays were carried out 8 h after transfection. The *y*-axis indicates ISRE-regulated luciferase activity shown as relative values. Assays were carried out in triplicate and results are shown as means ± SD.

also suppressed in HCV JFH1 virus cell culture. In JFH1-infected Huh7.5.1 cells, ΔRIG-I-induced ISRE reporter activation was significantly suppressed, but was recovered in IFN-treated, virus-eliminated cells (Fig. 2b and c). These results demonstrated that RIG-I- and Cardif-mediated antiviral responses were substantially suppressed by both subgenomic and genomic viral replication in both hepatocyte- and non-hepatocyte-derived host cells.

NS34A and NS4B are responsible for suppressing RIG-I-mediated IFN responses

We next sought to define which HCV proteins were responsible for inhibition of the RIG-I- and IRF-3-mediated IFN induction pathway. We constructed expression plasmids that expressed the non-structural proteins NS345, NS3, NS34A, NS4A, NS4B, NS5A and NS5B (Fig. 3a). We transfected each expression plasmid with simultaneous activation of the RIG-I pathway by overexpression of ΔRIG-I, Cardif, TBK1 and IKKε (Fig. 3b–e). Expression of full-length non-structural (NS345) and NS34A proteins inhibited ISRE activation mediated by expression of RIG-I and Cardif but not that mediated by TBK1 and IKKε. Interestingly, it was found that NS4B also inhibited ISRE activation mediated by expression of RIG-I and Cardif, but not by TBK1 and IKKε. Consistent with Fig. 3(b), overexpression of NS4B significantly suppressed ΔRIG-I-induced activation of the authentic IFN-β promoter (Fig. 3f).

Another group has studied IFN antagonism of flavivirus non-structural proteins and has reported that HCV NS4B did not affect IFN responses (Muñoz-Jordán *et al.*, 2005).

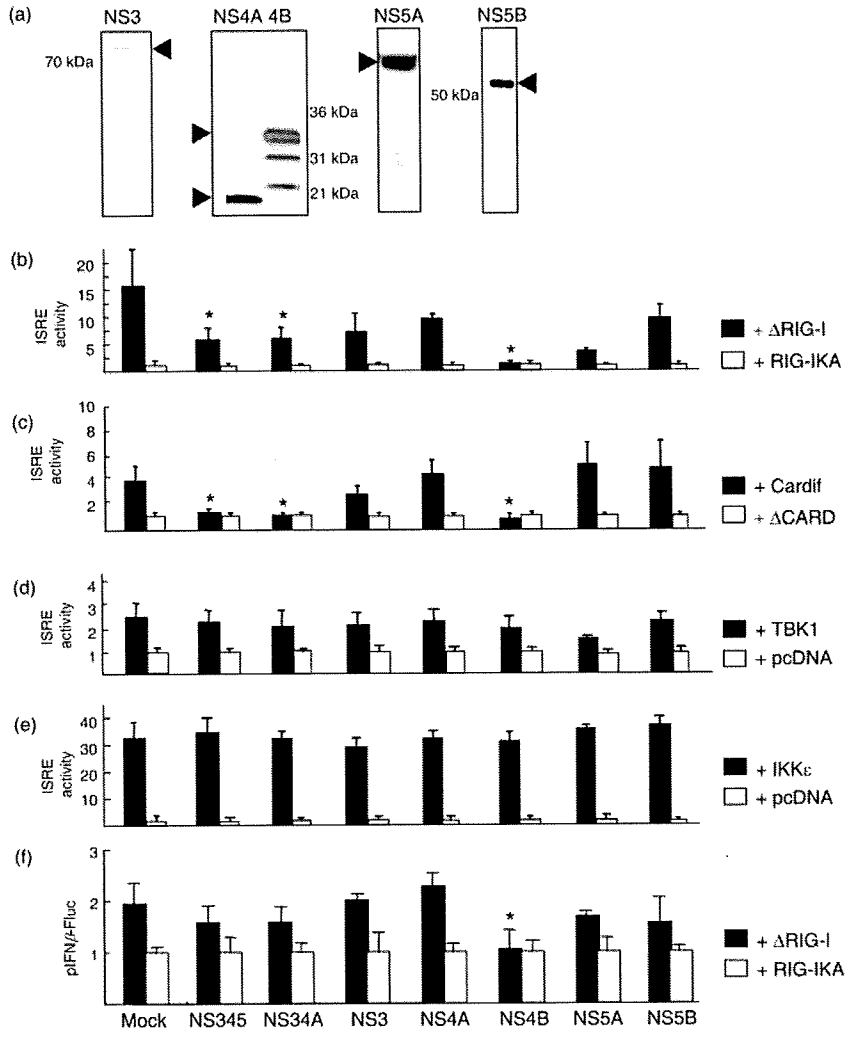

Fig. 3. Co-transfection analyses using plasmids that express individual HCV non-structural proteins. (a) Western blotting. Plasmids expressing the indicated Myc-tagged HCV proteins were transfected into Huh7 cells. Western blotting was carried out using anti-Myc antibody. (b–e) The following plasmids were co-transfected into Huh7 cells: pISRE-TA-Luc, pRL-CMV, the indicated plasmids expressing ΔRIG-I (b), Cardif (c), TBK1 (d) and IKKε (e), and the indicated plasmids expressing individual HCV non-structural proteins. Plasmids RIG-IKA, ΔCARD or pcDNA were used as negative controls as indicated. Twenty-four hours after transfection, luciferase activities were measured. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD. *, $P<0.05$. (f) pIFN-β and pRL-CMV were co-transfected into Huh7 cells, with plasmids expressing individual HCV non-structural proteins and plasmid expressing ΔRIG-I. Luciferase activities were measured 24 h after transfection. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD. *, $P<0.05$. Plasmid RIG-IKA was used as a negative control.

To investigate strain-specific differences in the characteristics of NS4B proteins, we performed co-transfection assays using NS4B expression constructs from HCV N (Beard *et al.*, 1999) and JFH1 (Wakita *et al.*, 2005) strains, as well as HCV strain J4 (Fig. 4a and b). All NS4B constructs suppressed ΔRIG-I- or Cardif-mediated ISRE activation. These results suggested that the above-described effects of NS4B were independent of HCV strain.

NS4B has been reported to induce an unfolded protein response or endoplasmic reticulum (ER) stress through ATF6 or IRE1-X box protein (XBP1) pathways (Zheng *et al.*, 2005). The ER stress induces production of IL-8, which has been reported to interfere with the IFN system (Polyak *et al.*, 2001). Therefore, we detected expression of IL-8 using RT-PCR in cells with and without overexpression of NS4B. As shown in Fig. 4(c), no significant difference was observed in IL-8 mRNA levels among mock-, NS34A- and NS4B-transfected cells. These results showed that NS4B overexpression in the present study did not induce expression of IL-8 and that the IFN-antagonizing effects of NS4B were independent of IL-8.

It has been reported that NS34A suppresses the TLR3-mediated IFN response (Breiman *et al.*, 2005; Ferreon *et al.*, 2005). However, overexpression of HCV non-structural proteins did not suppress ISRE activation that was induced by overexpression of TLR-3 or TRIF (Fig. 5a and b), nor did NS34A from two different HCV strains, J4 and N, show significant suppression of TRIF-mediated ISRE activation (Fig. 5c). Although strain-specific differences might be involved, these data suggest that neither NS34A nor NS4B affect the TLR3-triggered, TRIF-mediated IFN expression signalling pathway.

The NS4B N terminus is involved in inhibition of the RIG-I-mediated pathway

Given the result that NS4B suppressed the RIG-I-mediated IFN expression pathway, we next investigated which domain of NS4B was responsible. We constructed plasmids that expressed truncated NS4B in which the protein-coding frame was truncated at four positions corresponding to the five transmembrane domains (Lundin *et al.*, 2003) (Fig. 6a).

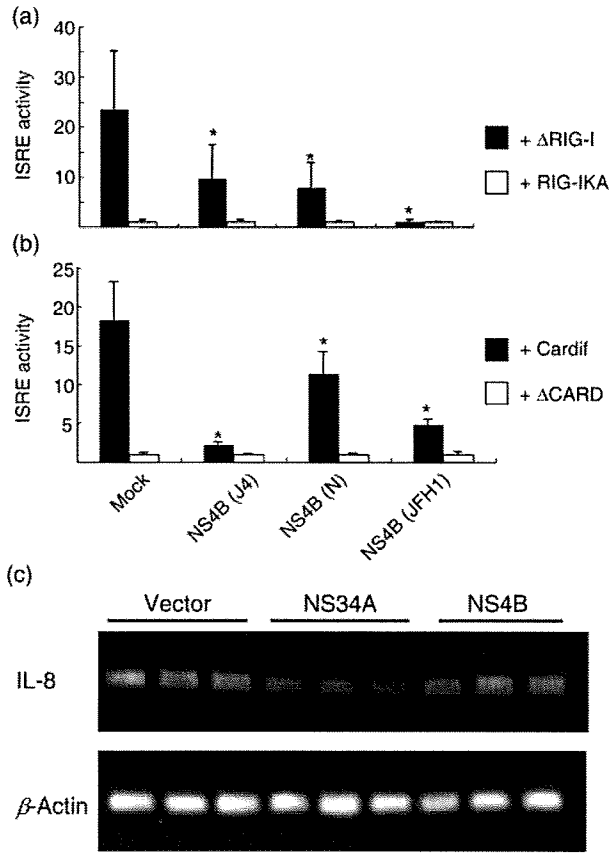

Fig. 4. Co-transfection analyses of HCV NS4B proteins of different origins. NS4B (J4), NS4B (N) and NS4B (JFH1) denote HCV NS4B proteins that were cloned from HCV strains J4, N and JFH1, respectively. The NS4B plasmids indicated were co-transfected with plasmids expressing ΔRIG-I (a) or Cardif (b). Luciferase activities were measured 24 h after transfection. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD. *, $P<0.05$. (c) Semi-quantitative detection of IL-8 mRNA by RT-PCR. cDNA was prepared from Huh7 cells transfected with empty vector or with NS34A or NS4B expression plasmid.

Expression and subcellular localization of NS4B truncated proteins were visualized by indirect immunofluorescence assays (Fig. 7). Each of the NS4B truncated proteins was localized predominantly to the perinuclear rim as dense spots. Some of the spots were similar to the staining of the ER-resident host protein PDI, consistent with previous reports (Lindström *et al.*, 2006; Lundin *et al.*, 2006). These truncated expression plasmids were co-transfected with Cardif expression plasmids into Huh7 cells. As shown in Fig. 6(b), Cardif-mediated ISRE activation was significantly suppressed by co-transfection of NS4Bt1–156 and NS4Bt1–186, as well as full-length NS4B, whilst transfection of NS4Bt90–260 and NS4Bt110–260 did not significantly suppress Cardif-mediated ISRE activation. The shortest construct, NS4Bt131–260, partially retained the ability to reduce ISRE activity. These results suggested that the N-terminal domain of NS4B, which includes aa 1–110, might function directly to suppress RIG-I-mediated IFN expression responses.

DISCUSSION

The recent discovery of cytoplasmic dsRNA sensor molecules has resulted in rapid expansion of knowledge about the IFN-mediated virus defence pathway (Yoneyama *et al.*, 2004). Several reports suggest that viruses target the IFN system to establish replication in the host cells (Kato *et al.*, 2006). We have confirmed that the dsRNA-triggered, IRF-3-mediated IFN activation pathway was blocked in several replicon-supporting cell lines (Fig. 1). Similarly, the dsRNA responses were substantially suppressed in HCV JFH-1 cell culture compared with parental Huh7 cells (Fig. 2b and c). Overexpression analyses showed that RIG-I- and Cardif-mediated ISRE activation was significantly suppressed in HCV replicon-expressing cells, which recovered after elimination of the replicon by IFN treatment (Fig. 2a). In contrast, TBK1- or IKKε-mediated ISRE activation was not suppressed in replicon-expressing cells. Overexpression of individual HCV non-structural proteins revealed that not only NS34A but also NS4B inhibited the ISRE activation signal (Figs 3, 4 and 5). These results suggested that HCV non-structural proteins suppress the IFN induction pathway and that the target host molecule could be Cardif or an unknown adaptor molecule acting between Cardif and TBK1/IKKε.

NS4B protein is a 27 kDa hydrophobic integral membrane protein that is localized in the ER with other non-structural proteins. Studies on other flaviviruses such as Kunjin virus and BVDV support the notion that NS4B may indeed be an essential part of the replication mechanism (Grassmann *et al.*, 2001; Khromykh *et al.*, 2000; Li & McNally, 2001; Qu *et al.*, 2001). These systems have demonstrated that intact NS4B is necessary in a *cis* configuration in the polyprotein for maintaining viral replication (Grassmann *et al.*, 2001; Khromykh *et al.*, 2000). Furthermore, single mutations in NS4B alter the cytopathic effects of BVDV and even mediate changes in the cellular tropism of Dengue virus (Hanley *et al.*, 2003; Qu *et al.*, 2001). In HCV, the search for cell-culture-adaptive mutations in HCV subgenomic replicons has led to the generation of mutations in the NS4B region that confer higher replication levels and resistance to IFNs, as well as broadening the tropism for different cell lines (Lohmann *et al.*, 2003; Sumpter *et al.*, 2004; Zhu *et al.*, 2003). These pieces of evidence may imply that NS4B is not only part of the replication machinery but may also have other functions that enable establishment of viral replication.

NS4B truncation assays showed that RIG-I/Cardif-mediated ISRE activation was significantly suppressed by expression of N terminus-containing constructs (Fig. 6). These results imply that the N-terminal domain of NS4B, which is located between positions 1 and 110, may be essential for suppressing IFN expression responses in host

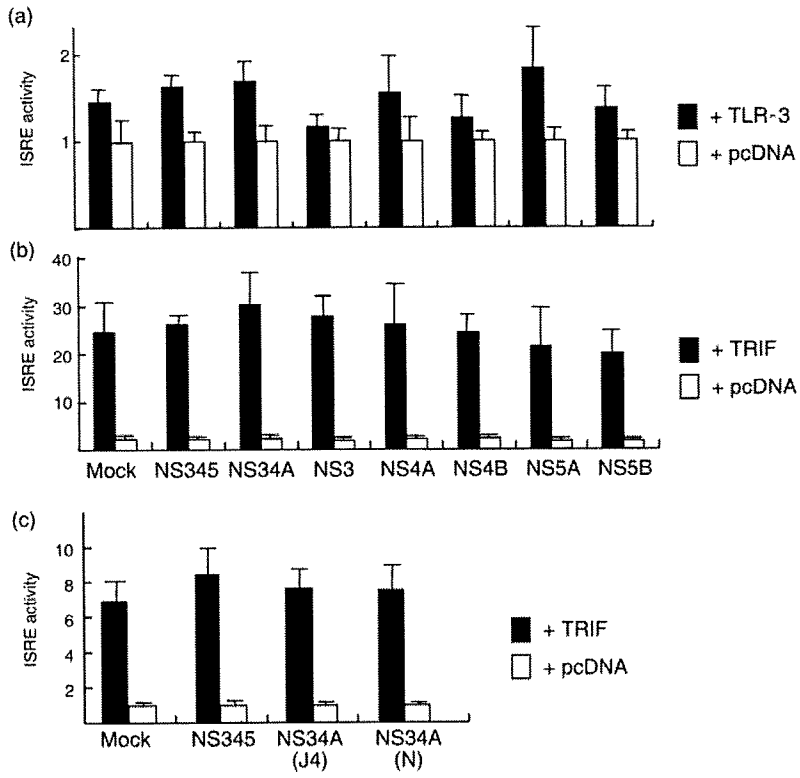

Fig. 5. Co-transfection analyses of HCV non-structural proteins and plasmid expressing TLR-3 or TRIF. (a, b). The following plasmids were co-transfected into Huh7 cells: pISRE-TA-Luc, pRL-CMV, plasmids expressing TLR-3 or TRIF and plasmids expressing individual HCV non-structural proteins, as indicated. Luciferase activities were measured 24 h after transfection. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD. (c) NS34A (J4) and NS34A (N) denote plasmids expressing HCV NS34A derived from HCV strains J4 and N, respectively. The NS4B plasmids indicated were co-transfected with pISRE-TA-Luc, pRL-CMV and plasmids expressing TRIF or pcDNA. Luciferase activities were measured 24 h after transfection. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD.

cells. Lindström *et al.*, (2006) investigated single point mutations in NS4B that negatively affected expression efficiency of the HCV replicon and reported that most of the active mutations were located around the N-terminal domain. A distinctive feature of NS4B is that it requires membrane rearrangement to form its native structure

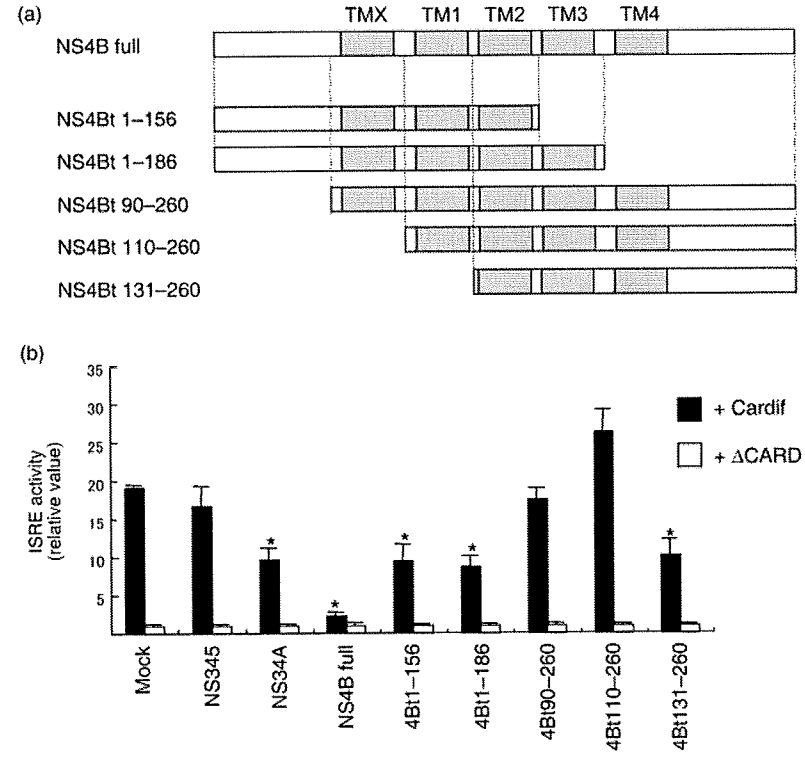

Fig. 6. Co-transfection analyses using truncated NS4B expression constructs. (a) Truncated constructs of NS4B. The protein-coding frame of NS4B was truncated in five constructs corresponding to the five transmembrane domains, as reported by Lundin *et al.* (2003). (b) The truncated NS4B plasmids, pISRE-TA-Luc and the Cardif- or ΔCARD-expressing plasmids indicated were co-transfected into Huh7 cells. Luciferase activities were measured 24 h after transfection. Results are given as means ± SD.

(Lindström *et al.*, 2006; Lundin *et al.*, 2006). The HCV polyprotein is translated from a single reading frame and subjected to proteolytic cleavage by the host signal peptidase and two viral proteases (Grakoui *et al.*, 1993). The mature form of NS4B is localized in the ER and constitutes a subcellular structure called the membrane-associated focus (MAF) (Gretton *et al.*, 2005). Once the NS4B is cleaved, the N-terminal peptide of NS4B is translocated from the cytoplasmic to the luminal side, giving it a fifth transmembrane region (Lundin *et al.*, 2006). The N-terminal amphipathic helix (AH) 1 of NS4B is necessary for this translocation and for MAF formation; NS4B molecules that were truncated at the AH1 lacked the ability to create the MAF, to translocate and to replicate (Elazar *et al.*, 2004; Lindström *et al.*, 2006).

In our assay, NS4Bt131–260 regained the ability to reduce ISRE activity. As we confirmed that all mutants co-localized with the ER, there may be some effect of the N-terminal localization of this mutant. The precise mechanism of NS4B suppression is still not clear and further experiments are needed.

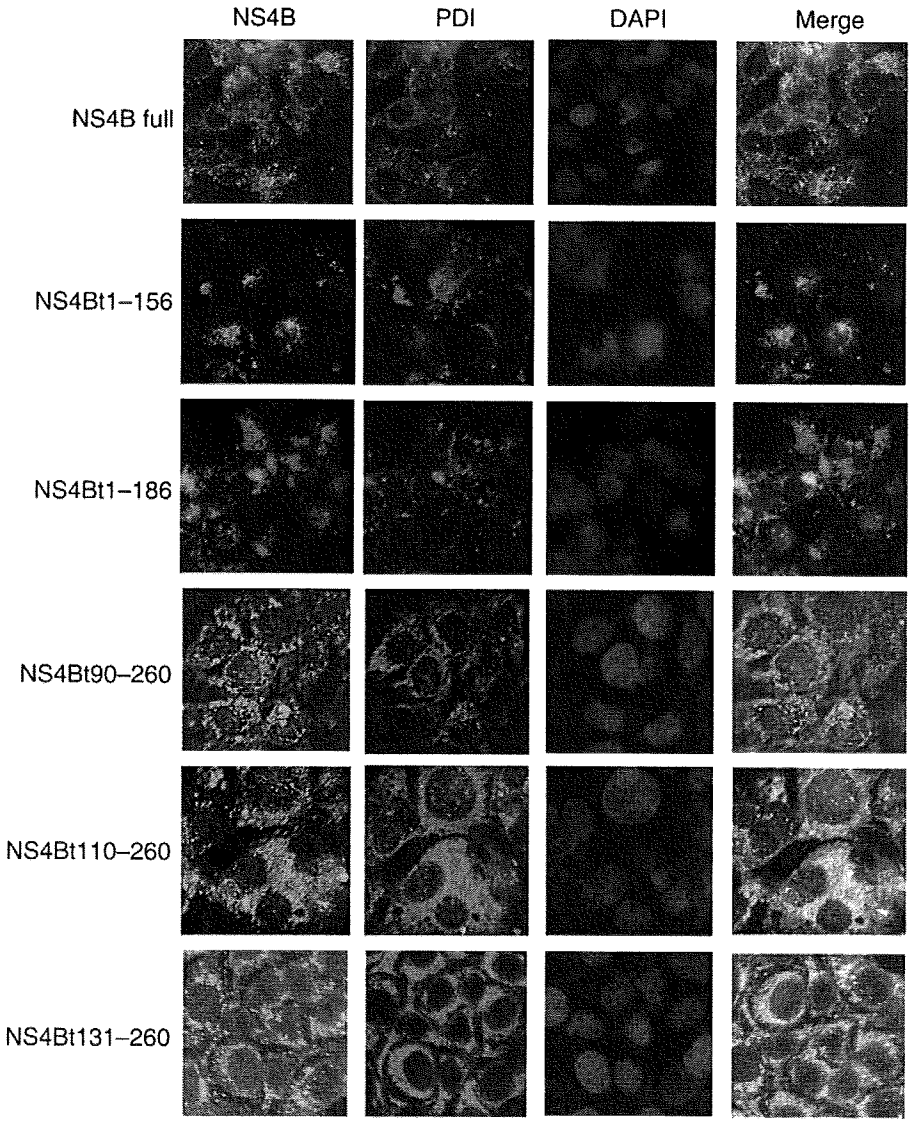

Fig. 7. Indirect immunofluorescence analysis of truncated NS4B proteins. The NS4B constructs indicated were transiently transfected into Huh7 cells. After 48 h, cells were labelled with anti-Myc or anti-PDI antibody. NS4B proteins were immunostained with Alexa Fluor 488-labelled goat anti-mouse IgG, whilst PDI was stained with Alexa Fluor 568-labelled goat anti-rabbit IgG. DAPI staining revealed the nuclear chromatin. Representative immunofluorescence images derived from a number of experiments are shown as four images of a single focal plane of Huh7 cells, showing NS4B proteins (green), PDI (red), DAPI staining (blue) and the superimposed images (merge).

NS4B has been reported to induce an unfolded protein response or ER stress (Zheng *et al.*, 2005). Accumulation of unfolded or misfolded proteins in the ER is detected by three ER sensor proteins, ATF6, IRE-1 and PKR-like ER kinase (PERK), and triggers the unfolded protein response as a stress response and induces expression of molecular chaperon proteins, global shut-off of protein translation and apoptotic cell death (Mori, 2000). Therefore, it may be possible that transgenic overexpression of NS4B induces ER stress and suppresses overall protein synthesis, including that of IFNs. In our experiments, however, NS4B suppressed the ISRE-mediated IFN gene activation but did not suppress non-specific protein expression, as demonstrated by *Renilla* luciferase activity in the control plasmid driven by the herpes simplex thymidine kinase promoter. In addition, the growth and viability of cells that overexpressed NS4B did not differ from untransfected cells or from those transfected with the other HCV proteins. IL-8 overproduction induced by ER stress was not observed in our NS4B-overexpressing cells (Fig. 4c). These findings indicated that the inhibitory effect of NS4B is specific to the IFN induction pathway and is not a non-specific effect through ER stress.

In conclusion, we have shown that dsRNA-induced IFN expression was suppressed by NS4B. These virus–host interactions probably contribute to HCV persistence and to the pathogenesis of HCV-associated liver disease.

ACKNOWLEDGEMENTS

We are indebted to Dr Jürg Tschopp for providing Cardif, ΔCARD and CARD, and to Dr Frank Chisari for providing the Huh7.5.1 cell line. This study was supported by grants from the Japan Society for the Promotion of Science, Miyakawa Memorial Research Foundation and Viral Hepatitis Research Foundation of Japan.

REFERENCES

Alter, M. J. (1997). Epidemiology of hepatitis C. *Hepatology* **26**, 62S–65S.

Andrejeva, J., Childs, K. S., Young, D. F., Carlos, T. S., Stock, N., Goodbourn, S. & Randall, R. E. (2004). The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the *IFN-β* promoter. *Proc Natl Acad Sci U S A* **101**, 17264–17269.

Basler, C. F., Mikulasova, A., Martinez-Sobrido, L., Paragas, J., Muhlberger, E., Bray, M., Klenk, H. D., Palese, P. & García-Sastre, A. (2003). The Ebola virus VP35 protein inhibits activation of interferon regulatory factor 3. *J Virol* **77**, 7945–7956.

Beard, M. R., Abell, G., Honda, M., Carroll, A., Gartland, M., Clarke, B., Suzuki, K., Lanford, R., Sangar, D. V. & Lemon, S. M. (1999). An infectious molecular clone of a Japanese genotype 1b hepatitis C virus. *Hepatology* **30**, 316–324.

Bigger, C. B., Brasky, K. M. & Lanford, R. E. (2001). DNA microarray analysis of chimpanzee liver during acute resolving hepatitis C virus infection. *J Virol* **75**, 7059–7066.

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

Breiman, A., Grandvaux, N., Lin, R., Ottone, C., Akira, S., Yoneyama, M., Fujita, T., Hiscott, J. & Meurs, E. F. (2005). Inhibition of RIG-I-dependent signaling to the interferon pathway during hepatitis C virus expression and restoration of signaling by IKKε. *J Virol* **79**, 3969–3978.

Doyle, S., Vaidya, S., O'Connell, R., Dadgostar, H., Dempsey, P., Wu, T., Rao, G., Sun, R., Haberland, M. & other authors (2002). IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity* **17**, 251–263.

Elazar, M., Liu, P., Rice, C. M. & Glenn, J. S. (2004). An N-terminal amphipathic helix in hepatitis C virus (HCV) NS4B mediates membrane association, correct localization of replication complex proteins, and HCV RNA replication. *J Virol* **78**, 11393–11400.

Ferreon, J. C., Ferreon, A. C., Li, K. & Lemon, S. M. (2005). Molecular determinants of TRIF proteolysis mediated by the hepatitis C virus NS3/4A protease. *J Biol Chem* **280**, 20483–20492.

Fitzgerald, K. A., McWhirter, S. M., Faia, K. L., Rowe, D. C., Latz, E., Golenbock, D. T., Coyle, A. J., Liao, S. M. & Maniatis, T. (2003). IKKε and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol* **4**, 491–496.

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

Frese, M., Schwärzle, V., Barth, K., Krieger, N., Lohmann, V., Mihm, S., Haller, O. & Bartenschlager, R. (2002). Interferon-γ inhibits replication of subgenomic and genomic hepatitis C virus RNAs. *Hepatology* **35**, 694–703.

Fried, M. W., Shiffman, M. L., Reddy, K. R., Smith, C., Marionos, G., Goncales, F. L., Häussinger, D., Diago, M., Garosi, G. & other authors (2002). Peginterferon α-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* **347**, 975–982.

Grakoui, A., Wychowski, C., Lin, C., Feinstone, S. M. & Rice, C. M. (1993). Expression and identification of hepatitis C virus polyprotein cleavage products. *J Virol* **67**, 1385–1395.

Grassmann, C. W., Isken, O., Tautz, N. & Behrens, S. E. (2001). Genetic analysis of the pestivirus nonstructural coding region: defects in the NS5A unit can be complemented in *trans*. *J Virol* **75**, 7791–7802.

Gretton, S. N., Taylor, A. I. & McLauchlan, J. (2005). Mobility of the hepatitis C virus NS4B protein on the endoplasmic reticulum membrane and membrane-associated foci. *J Gen Virol* **86**, 1415–1421.

Guo, J. T., Bichko, V. V. & Seeger, C. (2001). Effect of alpha interferon on the hepatitis C virus replicon. *J Virol* **75**, 8516–8523.

Hanley, K. A., Manlucu, L. R., Gilmore, L. E., Blaney, J. E., Jr, Hanson, C. T., Murphy, B. R. & Whitehead, S. S. (2003). A trade-off in replication in mosquito versus mammalian systems conferred by a point mutation in the NS4B protein of dengue virus type 4. *Virology* **312**, 222–232.

He, Y. & Katze, M. G. (2002). To interfere and to anti-interfere: the interplay between hepatitis C virus and interferon. *Viral Immunol* **15**, 95–119.

Itsui, Y., Sakamoto, N., Kurosaki, M., Kanazawa, N., Tanabe, Y., Koyama, T., Takeda, Y., Nakagawa, M., Kakinuma, S. & other authors (2006). Expressional screening of interferon-stimulated genes for antiviral activity against hepatitis C virus replication. *J Viral Hepat* **13**, 690–700.

Kanazawa, N., Kurosaki, M., Sakamoto, N., Enomoto, N., Itsui, Y., Yamashiro, T., Tanabe, Y., Maekawa, S., Nakagawa, M. & other authors (2004). Regulation of hepatitis C virus replication by interferon regulatory factor 1. *J Virol* **78**, 9713–9720.

Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T. & other authors (2006). Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* **441**, 101–105.

Katze, M. G., He, Y. & Gale, M. (2002). Viruses and interferon: a fight for supremacy. *Nature Reviews* **2**, 675–687.

Kawai, T., Takahashi, K., Sato, S., Coban, C., Kumar, H., Kato, H., Ishii, K. J., Takeuchi, O. & Akira, S. (2005). IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat Immunol* **6**, 981–988.

Khromykh, A. A., Sedlak, P. L. & Westaway, E. G. (2000). *cis*- and *trans*-acting elements in flavivirus RNA replication. *J Virol* **74**, 3253–3263.

Li, Y. & McNally, J. (2001). Characterization of RNA synthesis and translation of bovine viral diarrhea virus (BVDV). *Virus Genes* **23**, 149–155.

Lin, R., Heylbroeck, C., Pitha, P. M. & Hiscott, J. (1998). Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. *Mol Cell Biol* **18**, 2986–2996.

Lindström, H., Lundin, M., Häggström, S. & Persson, M. A. (2006). Mutations of the hepatitis C virus protein NS4B on either side of the ER membrane affect the efficiency of subgenomic replicons. *Virus Res* **121**, 169–178.

Lohmann, V., Körner, 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.

Lohmann, V., Hoffmann, S., Herian, U., Penin, F. & Bartenschlager, R. (2003). Viral and cellular determinants of hepatitis C virus RNA replication in cell culture. *J Virol* **77**, 3007–3019.

Lundin, M., Monné, M., Widell, A., Von Heijne, G. & Persson, M. A. (2003). Topology of the membrane-associated hepatitis C virus protein NS4B. *J Virol* **77**, 5428–5438.

Lundin, M., Lindström, H., Grönwall, C. & Persson, M. A. (2006). Dual topology of the processed hepatitis C virus protein NS4B is influenced by the NS5A protein. *J Gen Virol* **87**, 3263–3272.

Meylan, E., Curran, J., Hofmann, K., Moradpour, D., Binder, M., Bartenschlager, R. & Tschopp, J. (2005). Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* **437**, 1167–1172.

Mori, K. (2000). Tripartite management of unfolded proteins in the endoplasmic reticulum. *Cell* **101**, 451–454.

Muñoz-Jordán, J. L., Laurent-Rolle, M., Ashour, J., Martínez-Sobrido, L., Ashok, M., Lipkin, W. I. & García-Sastre, A. (2005). Inhibition of alpha/beta interferon signaling by the NS4B protein of flaviviruses. *J Virol* **79**, 8004–8013.

Nakagawa, M., Sakamoto, N., Enomoto, N., Tanabe, Y., Kanazawa, N., Koyama, T., Kurosaki, M., Maekawa, S., Yamashiro, T. & other authors (2004). Specific inhibition of hepatitis C virus replication by cyclosporin A. *Biochem Biophys Res Commun* **313**, 42–47.

Nakaya, T., Sato, M., Hata, N., Asagiri, M., Suemori, H., Noguchi, S., Tanaka, N. & Taniguchi, T. (2001). Gene induction pathways mediated by distinct IRFs during viral infection. *Biochem Biophys Res Commun* **283**, 1150–1156.

Polyak, S. J., Khabar, K. S., Paschal, D. M., Ezelle, H. J., Duverlie, G., Barber, G. N., Levy, D. E., Mukaida, N. & Gretch, D. R. (2001). Hepatitis C virus nonstructural 5A protein induces interleukin-8, leading to partial inhibition of the interferon-induced antiviral response. *J Virol* **75**, 6095–6106.

Qu, L., McMullan, L. K. & Rice, C. M. (2001). Isolation and characterization of noncytopathic pestivirus mutants reveals a role for nonstructural protein NS4B in viral cytopathogenicity. *J Virol* **75**, 10651–10662.

Saito, T., Hirai, R., Loo, Y. M., Owen, D., Johnson, C. L., Sinha, S. C., Akira, S., Fujita, T. & Gale, M., Jr (2007). Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2. *Proc Natl Acad Sci U S A* **104**, 582–587.

Samuel, C. E. (2001). Antiviral actions of interferons. *Clin Microbiol Rev* **14**, 778–809 (Table of Contents).

Sato, M., Suemori, H., Hata, N., Asagiri, M., Ogasawara, K., Nakao, K., Nakaya, T., Katsuki, M., Noguchi, S. & other authors (2000). Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-α/β gene induction. *Immunity* **13**, 539–548.

Schweizer, M. & Peterhans, E. (2001). Noncytopathic bovine viral diarrhea virus inhibits double-stranded RNA-induced apoptosis and interferon synthesis. *J Virol* **75**, 4692–4698.

Seth, R. B., Sun, L., Ea, C. K. & Chen, Z. J. (2005). Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-κB and IRF 3. *Cell* **122**, 669–682.

Sharma, S., tenOever, B. R., Grandvaux, N., Zhou, G. P., Lin, R. & Hiscott, J. (2003). Triggering the interferon antiviral response through an IKK-related pathway. *Science* **300**, 1148–1151.

Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H. & Schreiber, R. D. (1998). How cells respond to interferons. *Annu Rev Biochem* **67**, 227–264.

Sumpter, R., Jr, Wang, C., Foy, E., Loo, Y. M. & Gale, M., Jr (2004). Viral evolution and interferon resistance of hepatitis C virus RNA replication in a cell culture model. *J Virol* **78**, 11591–11604.

Talon, J., Horvath, C. M., Polley, R., Basler, C. F., Muster, T., Palese, P. & García-Sastre, A. (2000). Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein. *J Virol* **74**, 7989–7996.

Tanabe, Y., Sakamoto, N., Enomoto, N., Kurosaki, M., Ueda, E., Maekawa, S., Yamashiro, T., Nakagawa, M., Chen, C. H. & other authors (2004). Synergistic inhibition of intracellular hepatitis C virus replication by combination of ribavirin and interferon-α. *J Infect Dis* **189**, 1129–1139.

Taniguchi, T. & Takaoka, A. (2002). The interferon-α/β system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors. *Curr Opin Immunol* **14**, 111–116.

Taniguchi, T., Ogasawara, K., Takaoka, A. & Tanaka, N. (2001). IRF family of transcription factors as regulators of host defense. *Annu Rev Immunol* **19**, 623–655.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H. G. & other authors (2005). Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* **11**, 791–796.

Xu, L. G., Wang, Y. Y., Han, K. J., Li, L. Y., Zhai, Z. & Shu, H. B. (2005). VISA is an adapter protein required for virus-triggered IFN-β signaling. *Mol Cell* **19**, 727–740.

Yamashiro, T., Sakamoto, N., Kurosaki, M., Kanazawa, N., Tanabe, Y., Nakagawa, M., Chen, C. H., Itsui, Y., Koyama, T. & other authors (2006). Negative regulation of intracellular hepatitis C virus replication by interferon regulatory factor 3. *J Gastroenterol* **41**, 750–757.

Yanagi, M., Purcell, R. H., Emerson, S. U. & Bukh, J. (1997). Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. *Proc Natl Acad Sci U S A* **94**, 8738–8743.

Yokota, T., Sakamoto, N., Enomoto, N., Tanabe, Y., Miyagishi, M., Maekawa, S., Yi, L., Kurosaki, M., Taira, K. & other authors (2003). Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. *EMBO Rep* **4**, 602–608.

Yoneyama, M., Suhara, W., Fukuhara, Y., Fukuda, M., Nishida, E. & Fujita, T. (1998). Direct triggering of the type I interferon system by

virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. *EMBO J* **17**, 1087–1095.

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.

Yoneyama, M., Kikuchi, M., Matsumoto, K., Imaizumi, T., Miyagishi, M., Taira, K., Foy, E., Loo, Y. M., Gale, M., Jr & other authors (2005). Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J Immunol* **175**, 2851–2858.

Zheng, Y., Gao, B., Ye, L., Kong, L., Jing, W., Yang, X., Wu, Z. & Ye, L. (2005). Hepatitis C virus non-structural protein NS4B can modulate an unfolded protein response. *J Microbiol* **43**, 529–536.

Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D. R., Wieland, S. F., Uprichard, S. L., Wakita, T. & Chisari, F. V. (2005). Robust hepatitis C virus infection *in vitro*. *Proc Natl Acad Sci U S A* **102**, 9294–9299.

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.

Hepatology Research 2009; 39: 648–656 doi: 10.1111/j.1872-034X.2009.00519.x

Original Article

Case–control study for the identification of virological factors associated with fulminant hepatitis B

Atsunori Kusakabe,[1,2] Yasuhito Tanaka,[1] Satoshi Mochida,[3] Nobuaki Nakayama,[3] Kazuaki Inoue,[4] Michio Sata,[5] Norio Isoda,[6] Jong-Hon Kang,[7] Yasukiyo Sumino,[8] Hiroshi Yatsuhashi,[9] Yasuhiro Takikawa,[10] Shuichi Kaneko,[11] Gotaro Yamada,[12] Yoshiyasu Karino,[13] Eiji Tanaka,[14] Junji Kato,[15] Isao Sakaida,[16] Namiki Izumi,[17] Fuminaka Sugauchi,[2] Shunsuke Nojiri,[2] Takashi Joh,[2] Yuzo Miyakawa[18] and Masashi Mizokami[1,19]

[1]Departments of Clinical Molecular Informative Medicine and [2]Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya, [3]Division of Gastroenterology and Hepatology, Internal Medicine, Saitama Medical University, Saitama, [4]Showa University Fujigaoka Hospital, Yokohama, [5]Kurume University School of Medicine, Kurume, [6]Jichi Medical University, Tochigi, [7]Center for Gastroenterology, Teinekeijinkai Hospital, Sapporo, [8]Toho University Omori Medical Center, Tokyo, [9]National Hospital Organization Nagasaki Medical Center, Nagasaki, [10]Iwate Medical University, Morioka, [11]Department of Signal Transduction, Cancer Research Institute, Kanazawa University, Kanazawa, [12]Kawasaki Hospital, Okayama, [13]Sapporo-Kosei General Hospital, Sapporo, [14]Shinsyu University Graduate School of Medicine, Matsumoto, [15]Sapporo Medical University Hospital, Sapporo, [16]Yamaguchi University Hospital, Ube, [17]Musashino Red Cross Hospital, Musashino, [18]Miyakawa Memorial Research Foundation, Tokyo, and [19]Research Center for Hepatitis and Immunology, International Medical Center of Japan, Kohnodai Hospital, Ichikawa, Japan

Background: Host and viral factors can promote the development of fulminant hepatitis B (FHB), but there have been no case–control studies for figuring out virological parameters that can distinguish FHB.

Methods: In a case–control study, virological factors associated with the development of FHB were sought in 50 patients with FH developed by transient hepatitis B virus (HBV) infection (FH-T) and 50 with acute self-limited hepatitis B (AHB) who were matched for sex and age. In addition, 12 patients with FH developed by acute exacerbation (AE) of asymptomatic HBV carrier (ASC) (FH-C) were also compared with 12 patients without FH by AE of chronic hepatitis B (AE-C).

Results: Higher HBV DNA levels, subgenotype B1/Bj, A1762T/G1764A, G1896A, G1899A and A2339G mutation were significantly more frequent ($P < 0.05$), while hepatitis B e-antigen was less frequent in the FH-T patients than AHB. In multivariate analysis, G1896A mutation (odds ratio [OR], 13.53; 95% confidence interval [CI], 2.75–66.64), serum HBV DNA more than 5.23 log copies/mL (OR, 5.14; 95% CI, 1.10–24.15) and total bilirubin more than 10.35 mg/mL (OR, 7.81; 95% CI, 1.77–34.51) were independently associated with a fulminant outcome by transient HBV infection. On the other hand, in comparison with the patients between FH-C and AE-C groups, there was no significant difference of virological factors associated with the development of FHB.

Conclusion: A number of virological factors have been defined that may distinguish FH-T from AHB in a case–control study. The pathogenic mechanism of FHB between transient HBV infection and AE of ASC would be different.

Key words: acute exacerbation of asymptomatic hepatitis B virus carrier, fulminant hepatitis, genotypes, transient hepatitis B virus infection

INTRODUCTION

IN JAPAN, 634 patients with fulminant hepatitis (FH) were registered from 1998–2003. Of them, 41.8% were infected with hepatitis B virus (HBV) that is the most frequent cause of FH there.[1] HBV is classified into eight genotypes (A–H) based on a sequence divergence of more than 8% in the entire genome of approximately

Correspondence: Dr Yasuhito Tanaka, Department of Clinical Molecular Informative Medicine, Nagoya, City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan. Email: ytanaka@med.nagoya-cu.ac.jp
Received 22 August 2008; revision 26 January 2009; accepted 24 February 2009.

3200 nucleotides.[2–5] They have distinct geographical distributions and are associated with the severity of liver disease.[6,7] Furthermore, subgenotypes have been reported for HBV/A, B and C, and they are named A1/Aa (Asian/African type) and A2/Ae (European type),[8] B1/Bj (Japanese type) and B2/Ba (Asian type),[9] and C1/Cs (Southeast Asian type) and C2/Ce (East Asian type).[10,11] HBV genotypes/subgenotypes and mutations in the pre-core region and the core promoter can influence the viral replication and expression of hepatitis B e-antigen (HBeAg).[6,12]

Acute HBV infection in adulthood resolves in the most cases by far, but can induce FH or go on to become chronic in some. It has been reported that host and viral factors may influence the development of fulminant hepatitis B (FHB), but the pathogenesis of FHB remains unclear. As for virological factors associated with FHB, mutations in the core promoter (A1762T/G1764A)[13] and the pre-core region (G1896A)[14–16] have been reported in association with the development of FHB in Asia and the Middle East. Additional mutations, including T1753V, T1754V and A2339G in the core gene are implicated, also.[17,18] In regard of HBV genotypes, subgenotype B1/Bj is highly associated with the development of FHB in Japan.[15] In contrast, an association of HBV genotypes with the fulminant outcome has not been reproduced in patients from the USA and Europe.[19–22] Such a discrepancy would be attributed, at least in part, to distinct geographical distributions of HBV genotypes/subgenotypes over the world.

The original definition by Trey *et al.*[23] about fulminant hepatic failure is widely used all over the world. On the other hand, in Japan, the diagnosis of FH was contingent on a slight modification of Trey's original definition by the Inuyama Symposium (Aichi, Japan in 1981). Furthermore, the Intractable Liver Diseases Study Group of Japan modified the criteria for the etiology of FH and late-onset hepatic failure in 2002. According to the criteria of the Intractable Liver Diseases Study Group of Japan, there are two clinical entities of FHB that are induced, respectively, by transient HBV infection and acute exacerbation (AE) of an asymptomatic HBV carrier (ASC).[1]

Recently, FH developing in ASC who undergo AE is increasing in Japan.[1] In patients with hematological malignancy, in particular, rituximab and/or glucocorticoid, can reactivate HBV for the development of FHB.[24] The outcome is poor for FHB precipitating in ASC who undergo acute exacerbation,[1] but it has been difficult to identify it by clinical examinations.

As there have been no case–control studies for figuring out virological parameters that can distinguish FHB, a case–control study was conducted on the patients with FH by transient HBV infection and acute self-limited hepatitis B (AHB) in this study, for the identification of virological factors that influence a fulminant outcome. In addition, the patients with FH by AE of ASC, which is assumed as a different clinical condition from transient HBV infection, were also compared with the patients without FH by AE of chronic hepatitis B (CHB) in a case–control study.

METHODS

Patients

DURING 9 YEARS from 1998 to 2006, in twenty-six hospitals all over Japan, sera were obtained from the 50 FH patients by transient HBV infection (the FH-T group) and the 50 patients with AHB (the AHB group) who were controlled for age and sex. As the elder patients with FHB were enrolled in this study (mean age, 42.8 years), the mean age of AHB patients became relatively high (42.9 years, Table 1). Furthermore, the 12 FH patients developed by AE of ASC (the FH-C group) were also compared with the 12 patients without FH by AE of CHB who were matched by age and sex (the AE-C group).

All the serum samples tested for this study were collected at hospitalization. All 124 patients had hepatitis B surface antigen (HBsAg) in serum. Infection with hepatitis A virus and hepatitis C virus, as well as alcoholic hepatitis, were excluded in them.

The diagnosis of acute hepatitis B was based on sudden manifestation of clinical symptoms of hepatitis and detection of high-titered immunoglobulin (Ig)M anti-hepatitis B core (HBC). Patients with initial high-titered anti-HBC (>90% inhibition by a 1:200 diluted serum) were excluded. The diagnosis of FH was contingent on a slight modification by Inuyama Symposium (Aichi, Japan in 1981) of the original definition by Trey *et al.*:[23] (i) coma of grade II or higher; and (ii) a prothorombin time less than 40% developing within 8 weeks after the onset of hepatitis. To exclude AE of ASC in FH-T and AHB groups, we confirmed the negativity of HBsAg before onset of FHB or AHB and no family histories of hepatitis were found among all the patients. Furthermore, serum HBsAg in all patients with FH-T or AHB became naturally seronegative within 24 weeks. AE of ASC or CHB was defined as the elevation of alanine aminotransferase (ALT >300 IU/L) or total bilirubin (T.bil >3.0 mg/dL).[25] All 24 patients with AE of ASC or CHB could be confirmed positive for serum HBsAg before the onset of acute liver injury.

Table 1 Baseline characteristics between fulminant hepatitis B patients by transient infection (FH-T) and acute self-limited hepatitis B (AHB) patients

Features	FH-T ($n = 50$)	AHB ($n = 50$)	Differences *P*-value
Age (years)	42.8 ± 16.1	42.9 ± 14.6	Matched
Men	25 (50%)	25 (50%)	Matched
ALT (IU/L)	3788 ± 2856	2170 ± 1350	<0.001
AST (IU/L)	3131 ± 3673	1676 ± 1851	<0.05
Total bilirubin (mg/dL)	14.8 ± 8.6	9.5 ± 9.8	<0.01
Prothrombin time (%)	16.9 ± 11.2	72.8 ± 26.0	<0.001
HBeAg positive	15 (30%)	28 (56%)	<0.01
Core protein (log U/mL)	3.21 ± 1.28	3.01 ± 1.00	NS
HBcrAg (log U/mL)	5.30 ± 1.32	5.95 ± 1.13	<0.01
HBV DNA (log copies/mL)	5.97 ± 1.87	4.98 ± 1.17	<0.005
Deceased	19 (38%)	0 (0%)	<0.001

AHB, acute self-limited hepatitis B; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FH-T, fulminant hepatitis B by transient HBV infection; HBcrAg, hepatitis B core related antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; NS, not significant.

Serological markers of HBV infection

Hepatitis B surface antigen, HBeAg and the corresponding antibody (anti-HBe) were determined by enzyme immunoassay (EIA) (AxSYM; Abbott Japan, Tokyo, Japan) or chemiluminescence enzyme immunoassay (CLEIA) (Fujirebio, Tokyo, Japan). Anti-HBC of IgM and IgG classes were determined by radioimmunoassay (Abbott Japan). Core protein constituting the viral nucleocapsid and HBV core-related antigen (HBcrAg), both of which correlate with HBV DNA in serum, were measured by CLEIA as described elsewhere.[26,27]

Quantification of serum HBV DNA

Hepatitis B virus DNA sequences spanning the S gene were amplified by real-time detection polymerase chain reaction (RTD-PCR) in accordance with the previously described protocol[28] with a slight modification;[8] it has a detection limit of 100 copies/mL.

Sequencing and molecular evolutionary analysis of HBV

Nucleic acids were extracted from serum samples (100 μL) using the QIAamp DNA extraction kit (Qiagen, Hilden, Germany) and subjected to PCR for amplifying genomic areas bearing enhancer II/core promoter/pre-core/core regions [nt 1628–2364], as described previously.[29] The target of PCR covered several mutations which were associated with FHB. Amplicons were sequenced directly with use of the ABI Prism Big Dye ver. 3.0 kit in the AMI 3100 DNA automated sequencer (Applied Biosystems, Foster City, CA, USA). All sequences were analyzed in both forward and backward directions.

Hepatitis B virus genotypes were determined by molecular evolutionary analysis. Reference HBV sequences were retrieved from the DDBJ/EMBL/GenBank database and aligned by CLUSTAL X, then genetic distances were estimated with the 6-parameter method in the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp/).[30] Based on obtained distances, phylogenetic trees were constructed by the neighbor-joining (NJ) method with the mid-point rooting option. To confirm the reliability of the phylogenetic trees, bootstrap resampling tests were performed 1000 times.

Statistical analysis

Statistical differences were evaluated by the Mann–Whitney *U*-test, Fisher's exact probability test and χ^2-test, where appropriate. Differences were considered to be statistically significant at $P < 0.05$. Multivariate analyses with logistic regression were utilized to sort out independent risk factors for FHB. STATA Software ver. 8.0 was employed for all analyses.

RESULTS

Baseline characteristics of the patients with FHB by transient HBV infection and AHB

TABLE 1 COMPARES baseline clinical characteristics of the 50 FH-T patients and the 50 AHB who