T-pIC treatment (Fig. 4C), but none of these NS3-4As could suppress the induction of IFN-β mRNA following M-pIC treatment (Fig. 4D).

We next examined the effects of NS3-4A on the phosphorylation and dimerization of IRF-3 in PH5CH8 cells. We observed that both T-pIC and M-pIC treatments induced the phosphorylation at Ser386 and Ser396 of IRF-3, and formed the dimerization of IRF-3 (Fig. 5A,B, lanes 1 and 2), and that NS3-4A remarkably inhibited the phosphorylation and dimerization of IRF-3 in the cells treated with T-pIC, depending on its protease activity (Fig. 5A). However, the phosphorylation and dimerization of IRF-3 induced by M-pIC treatment was not inhibited by NS3-4A (Fig. 5B). From these results, we concluded that, in PH5CH8 cells, NS3-4A could not block the TRIF-mediated signaling pathway, although it could block the Cardif-mediated signaling pathway.

NS3-4A blocks the Cardif-mediated pathway by cleaving Cardif

NS3-4A is able to cleave the Cardif [24,34,35] and TRIF [36] molecules, resulting in the blocking of dsRNA-induced antiviral signaling pathways. However, our finding that IFN-β production was not suppressed by NS3-4A in cells treated with M-pIC seemed to contradict the finding of a previous study [36] in which NS3-4A-mediated cleavage of TRIF inhibited dsRNA-activated signaling through the TLR3 pathway. Therefore, we evaluated whether or not NS3-4A could impair the functional ability of

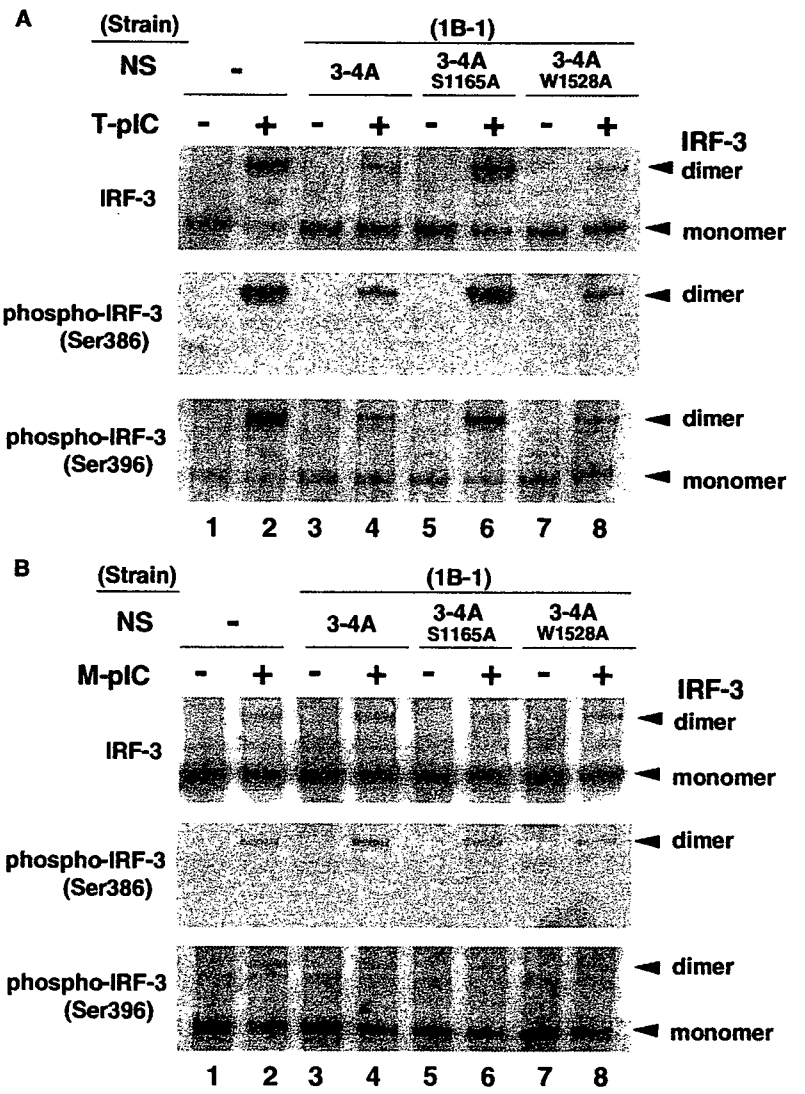

Fig. 5. Effect of NS3-4A on phosphorylation and dimerization of IRF-3 in PH5CH8 cells treated with intracellular or extracellular dsRNA. PH5CH8 cells that were the same as in Fig. 4C were used. The poly(I-C) treatment was performed as described in Fig. 1. (A) Effect of NS3-4A on phosphorylation and dimerization of IRF-3 in the PH5CH8 cells treated with T-pIC. The phosphorylation and dimerization analyses of IRF-3 were performed as described in Fig. 3C. Anti-phospho-IRF-3 (Ser396) serum was also used for the analysis. (B) Effects of NS3-4A on phosphorylation and dimerization of IRF-3 in the PH5CH8 cells treated with M-pIC. The phosphorylation and dimerization analyses of IRF-3 were performed as described in (A).

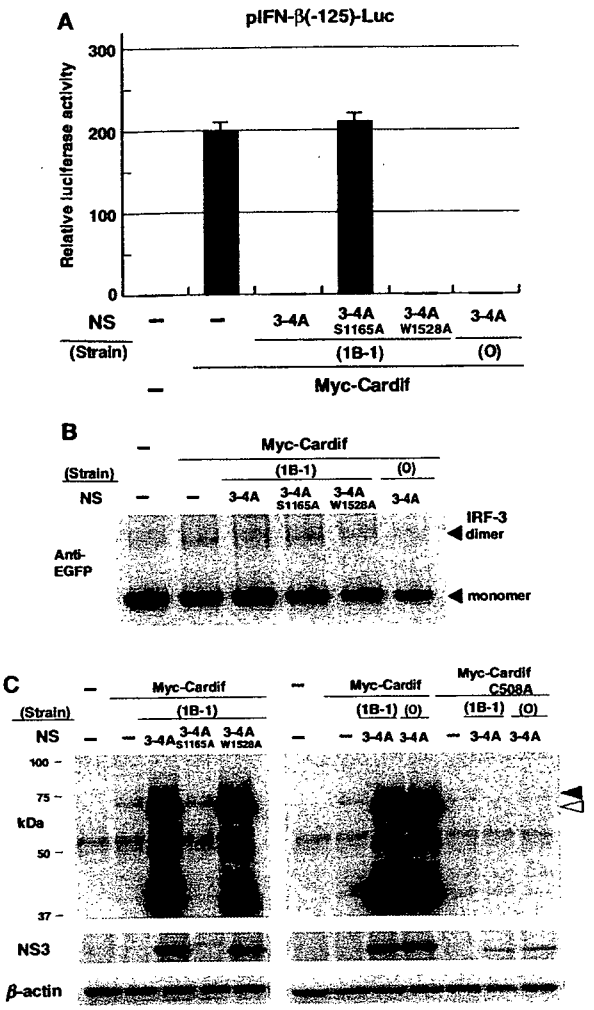

Fig. 6. NS3-4A blocks Cardif-mediated pathways by cleaving Cardif. (A) Effect of NS3-4A on the IFN-β gene promoter activated by the ectopic expression of Cardif in PH5CH8 cells. PH5CH8 cells were cotransfected with the pCX4bsr expression vector encoding NS3-4A or its mutant S1165A or W1528A, as described in Fig. 4, and the pCX4pur expression vector encoding myc-Cardif. The lysate of PH5CH8 cells transfected with the pCX4bsr and pCX4pur vectors was used as a control (NS–). The dual luciferase reporter assay was performed as described in Fig. 1A. (B) Effect of NS3-4A on IRF-3 dimerization induced by the ectopic expression of Cardif in PH5CH8 cells. The enhanced green fluorescent protein (EGFP)-IRF3 expression vector was used for the cotransfection in PH5CH8 cells with the myc-Cardif and NS3-4A (wild-type or its mutant S1165A or W1528A) expression vectors. The lysate of PH5CH8 cells transfected with the pCX4bsr and pCX4pur vectors was used as a control (NS–). The dimerization analysis of IRF-3 was preformed as described in Fig. 3C using anti-EGFP serum. (C) Cardif is cleaved by NS3-4A in PH5CH8 cells. PH5CH8 cells were cotransfected with the myc-Cardif (wild-type or its mutant C508A) and NS3-4A expression vectors (wild-type or its mutant S1165A or W1528A). Production of the myc-Cardif and NS3 in these cells was analyzed by immunoblotting using anti-myc and anti-NS3 sera, respectively. The PH5CH8 cells transfected with the pCX4bsr and pCX4pur vectors were used as a control (NS–). β-actin was used as a control for the amount of protein loaded per lane. The black and white arrowheads indicate Cardif and the cleaved Cardif, respectively.

TRIF as well as Cardif in PH5CH8 cells. First, we confirmed the effect of NS3-4A on the activation of the IFN-β gene promoter by the Cardif exogenously expressed in PH5CH8 cells. The results of the luciferase reporter assay revealed that NS3-4As (strains 1B-1 and HCV-O) completely suppressed the activation (200-fold induction) of the IFN-β gene promoter by Cardif, and that this suppression was dependent on the serine protease activity of NS3-4A (Fig. 6A). This result was supported by the results of the dimerization analysis of IRF-3 (Fig. 6B). Next, we confirmed that wild-type Cardif, but not the Cardif mutant (C508A located in the C-terminal region), was cleaved by the NS3-4As (strains 1B-1 and HCV-O), and that this cleavage was dependent on its serine protease activity (Fig. 6C). These results are in agreement with previous studies in which NS3-4A blocked the intracellular dsRNA signaling pathways through cleavage at the Cys508 residue of Cardif [24,34,35].

NS3-4A does not block the TRIF-mediated pathway because it lacks the ability to cleave TRIF

Because we demonstrated that NS3-4A blocked the intracellular dsRNA signaling pathways through cleavage of Cardif in PH5CH8 cells, we performed the same analysis regarding TRIF exogenously expressed in PH5CH8 cells. The results of the luciferase reporter assay using the IFN-β gene promoter revealed that NS3-4As (strains 1B-1 and HCV-O) could not suppress the activation (1000-fold induction) of the IFN-β gene promoter by TRIF (Fig. 7A). This result was also supported by the results of the dimerization analysis of IRF-3 (Fig. 7B). Furthermore, we demonstrated that the exogenously expressed TRIF was not cleaved by NS3-4As (strains 1B-1 and HCV-O) (Fig. 7C). These results indicate that NS3-4A could not block the TRIF-mediated signaling pathway, and suggest that NS3-4A did not suppress the M-pIC-induced production of IFN-β because NS3-4A did not have the ability to cleave TRIF.

To confirm the results obtained in PH5CH8 cells, we examined the status of Cardif and TRIF molecules expressed exogenously in the O cells replicating genome-length HCV-O RNA efficiently and their cured Oc cells. The results revealed that Cardif was cleaved in the O cells but not in the Oc cells (Fig. 8A,B), and that the cleavage of Cardif occurred

when NS3-4As (strains 1B-1 and HCV-O) were expressed in the Oc cells (Fig. 8B). From these results, we confirmed that NS3-4A could cleave Cardif in the O and Oc cells. In contrast, TRIF was not cleaved in either O or Oc cells (Fig. 8C). We further confirmed that TRIF was not cleaved in the O cells transfected with TLR3 siRNA, indicating that the resistance of TRIF to NS3-4A is not related to the presence of TLR3 (Fig. 8C). We also performed the same analysis using HeLa cells, and obtained results (supplementary Fig. S2) similar to those obtained in PH5CH8 cells (Figs 6C, 7C and 8). In addition, we observed that, like TRIF, exogenously expressed MDA5 and RIG-I were not cleaved by NS3-4A in PH5CH8 cells (data not shown). Taken together, the above results indicate that NS3-4A cleaves the Cardif molecule, resulting in interruption of the Cardif-mediated pathway, but NS3-4A is not able to cleave the TRIF molecule, and thus the TRIF-mediated pathway is not suppressed by NS3-4A.

Discussion

In the present study, we demonstrated that parental PH5CH cells and their clones retained both TRIF- and Cardif-mediated pathways as antiviral dsRNA signaling pathways, and confirmed that the PH5CH8 cell line was far more useful for the study of antiviral pathways than HuH-7 or the cell lines cloned from it. From the results of the present study and a previous study [41], we considered the possibility that immortalized hepatocyte cells possess the functional TRIF- and Cardif-mediated signaling pathways. Based on this assumption, we examined IFN-β production in three other immortalized human hepatocyte cell lines, NKNT-3 [52], IHH10.3 [53], and IHH12 [53], after treatment with poly(I-C). However, the results revealed that none of these immortalized cell lines responded to both M-pIC and T-pIC treatments. Therefore, we suggest that PH5CH and the cell lines cloned from it are uniquely suitable for the comprehensive study of antiviral TRIF- and Cardif-mediated signaling pathways.

We failed to obtain evidence that NS3-4A was able to cleave TRIF as reported by Li *et al.* [36]. In our study (Fig. 7C), there was no evidence of the cleavage of the TRIF molecule in NS3-4A-expressed PH5CH8

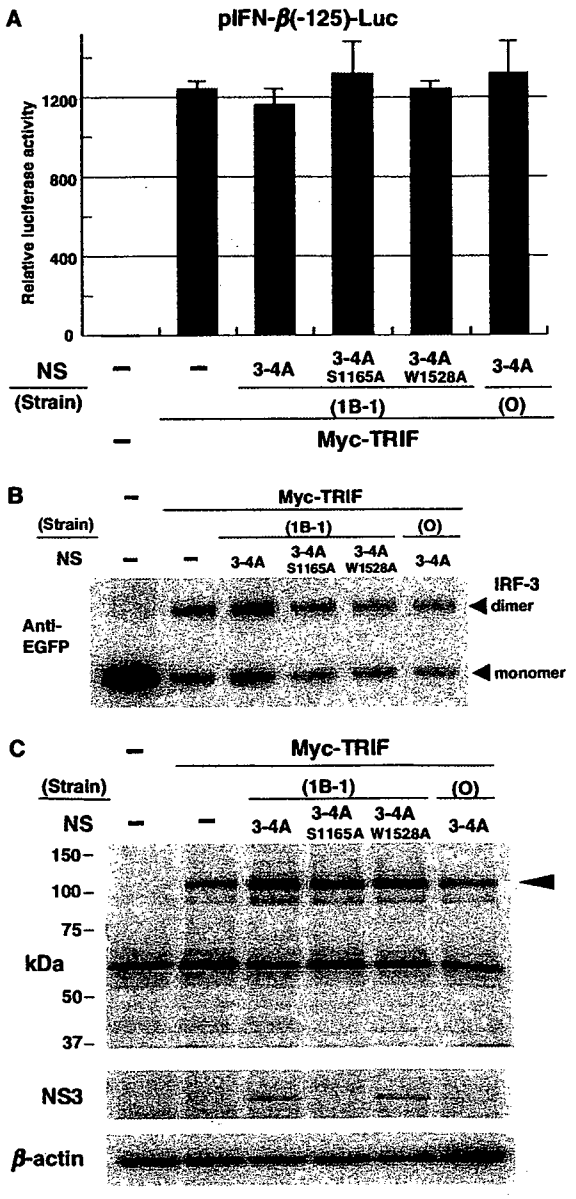

Fig. 7. NS3-4A does not block the TRIF-mediated pathway because it lacks the ability to cleave TRIF. (A) Effect of NS3-4A on the IFN-β gene promoter activated by the ectopic expression of TRIF in PH5CH8 cells. PH5CH8 cells were cotransfected with the pCX4bsr expression vector encoding NS3-4A or its mutant S1165A or W1528A, and the pCX4pur expression vector encoding myc-TRIF. The lysate of PH5CH8 cells transfected with the pCX4bsr and pCX4pur vectors was used as a control (NS–). The dual luciferase reporter assay was performed as described in Fig. 1A. (B) Effect of NS3-4A on IRF-3 dimerization induced by the ectopic expression of TRIF in PH5CH8 cells. The dimerization analysis of IRF-3 was performed as described in Fig. 6B except that the myc-TRIF expression vector was used in place of the myc-Cardif expression vector. (C) TRIF is not cleaved by NS3-4A in PH5CH8 cells. PH5CH8 cells were cotransfected with the myc-TRIF and NS3-4A (wild-type or its mutant S1165A or W1528A) expression vectors. Production of myc-TRIF and NS3 in these cells was analyzed by immunoblotting using anti-myc and anti-NS3 sera, respectively, as described in Fig. 6C. The black arrowhead indicates the noncleaved TRIF.

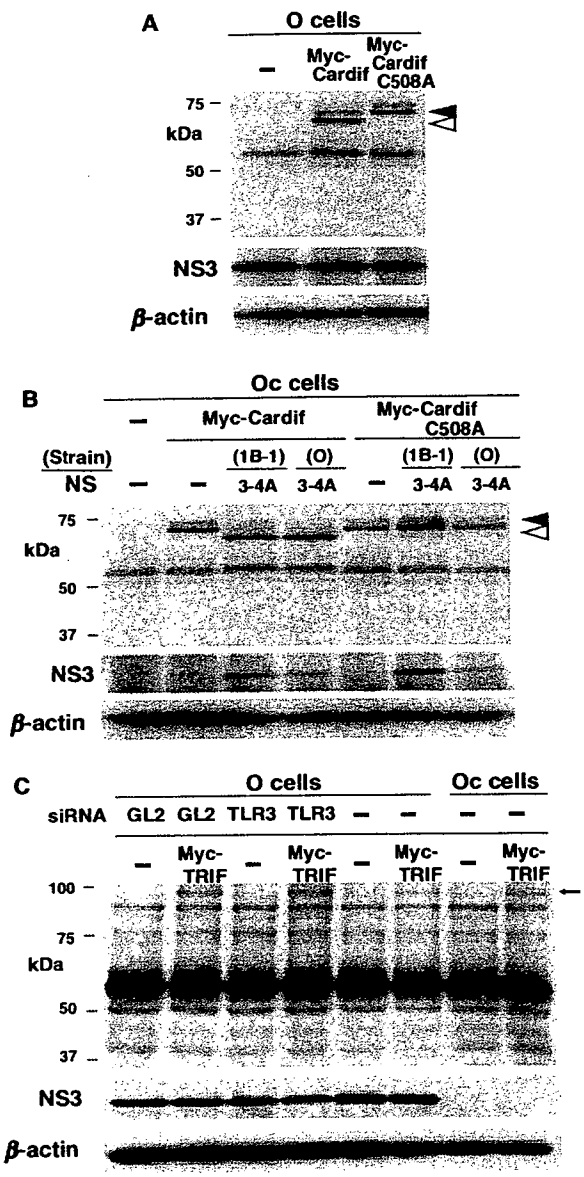

Fig. 8. TRIF is not cleaved in genome-length HCV RNA replicating cells. (A) Cardif is cleaved in the O cells replicating genome-length HCV-O RNA efficiently. The O cells were transfected with the myc-Cardif (wild-type or its mutant C508A) expression vector. Production of the myc-Cardif and NS3 in the O cells was analyzed by immunoblotting as described in Fig. 6C. The black and white arrowheads indicate Cardif and the cleaved Cardif, respectively. (B) Cardif is cleaved by NS3-4A in the cured Oc cells. The Oc cells were cotransfected with the myc-Cardif (wild-type or mutant C508A) and NS3-4A expression vectors. The production of the myc-Cardif and NS3 in these cells was analyzed by immunoblotting as described in Fig. 6C. The black and white arrowheads indicate Cardif and the cleaved Cardif, respectively. (C) TRIF is not cleaved in the O cells. The O and Oc cells were transfected with the myc-TRIF expression vector. The O cells transfected with GL2 or TLR3 siRNA were also used for the analysis. Production of myc-TRIF in these cells was analyzed by immunoblotting as described in Fig. 6C. The black arrowhead indicates the noncleaved TRIF.

cells. Nor did we observe any cleavage of TRIF by the NS3-4A expressed in the Oc cells, which exhibited almost no response to the T-pIC and M-pIC treatments (Figs 1 and 8C), or the HeLa cells, which exhibited a good response to the T-pIC and M-pIC treatments (supplementary Figs S1 and S2). We further observed that TRIF was not cleaved in the O cells, in which the HCV NS protein precursor was efficiently processed by NS3-4A (Fig. 8C). Regarding the cellular localization of NS3-4A, it has recently been reported that the localization of NS3-4A expressed transiently in HuH-7 cells was the same as that in genome-length HCV RNA replicating cells, and that NS3-4A was localized not only on the endoplasmic reticulum, but also on mitochondria [54]. From these findings, we suggest that NS3-4A is unable to cleave TRIF in cultured human cells.

Although amino acid sequences (PSSTPC/SAHLT, cleavage at Cys372; the P6 residue is underlined) surrounding the NS3-4A *trans*-cleavage site in TRIF [36] resemble those (DLEVVT/STWVL for NS3-4A; DEMEEC/ASHLP for NS4A/4B; DCSTPC/SGSWL for NS4B/5A; EDVVCC/SMSYS for NS5A/5B; the P6 residue is underlined) in the NS proteins from the 1B-1 and HCV-O strains and that (EREVPC/HRPSP, cleavage at Cys508; the P6 residue is underlined) in Cardif, only the TRIF site lacks the acidic P6 residue that is conserved and important in viral cleavage sites [55]. Accordingly, we examined whether or not a TRIF mutant (P to E at the P6 residue) is cleaved by NS3-4A in PH5CH8 cells. However, no cleavage of the TRIF mutant was observed (unpublished data). To clarify why TRIF is not cleaved by NS3-4A, further analysis will be necessary.

Although the results obtained in the present study suggest that the suppression of IFN-β production by NS3-4A is limited in human hepatocyte cells, it has recently been reported [56] that HCV can block the dsRNA-induced signaling pathway via the NS3-4A-independent pathway in addition to the NS3-4A-dependent pathway. However, because HuH-7 cells infected with the HCV genotype 2a clone (JFH1) were used in that study, it is not clear whether or not the TRIF-mediated pathway is also inhibited by the NS3-4A-independent pathway. To clarify this point, it will be necessary to study an HCV infection system using human hepatocyte cells in which both the TRIF- and

Cardif-mediated pathways are functional, such as PH5CH8 cells.

We clearly demonstrated that Cardif was cleaved by NS3-4As of 1B-1 and HCV-O strains obtained from healthy HCV carriers [57]. Although we observed that this cleavage was dependent on the protease activity of NS3-4A (Fig. 6), the correlation between the inhibitory effect of NS3-4A on the Cardif-mediated signaling pathway and the protease activity of NS3-4A remains unclear. Furthermore, we have no evidence that all NS3-4As derived from patients with HCV are able to cleave the Cardif molecule. To clarify these issues, further comparative analysis among HCV strains obtained from patients with different hepatic disease conditions will be needed. In addition, in the present study, we observed that the bands corresponding to the cleaved Myc-Cardif became extremely intense in PH5CH8 cells (Fig. 6C). This phenomenon has been observed in previous studies [24,34,49]. Although these previous studies did not explain what caused this phenomenon, we speculate that the cleaved Myc-Cardif is transferred to the cytosolic (soluble) fraction, although noncleaved Myc-Cardif remains in the membrane (insoluble) fraction. To clarify the reason for this phenomenon, several experiments may be needed.

In summary, we show that NS3-4A could not cleave TRIF, but could cleave Cardif, in PH5CH8 cells possessing functional TRIF- and Cardif-mediated antiviral signaling pathways, and suggest that the disruption of the IFN-β production system by NS3-4A is not sufficient in HCV-infected hepatocyte cells. This information will be useful for understanding the roles of NS3-4A in persistent HCV infection.

Experimental procedures

Cell culture

Non-neoplastic human hepatocyte PH5CH-derived cloned cells, including PH5CH8 cells, which are susceptible to HCV infection and supportive of HCV replication [45], were maintained as described previously [58]. HuH-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum. The O cells replicating genome-length HCV RNA were cultured in DMEM with 10% fetal bovine serum and G418 (300 $\mu g \cdot mL^{-1}$; Geneticin, Invitrogen) as described previously [43]. The Oc and OR6c cured cells, which were created by eliminating genome-length HCV RNA from O cells [43] and OR6 cells [44] by IFN treatment, respectively, were also cultured in DMEM with 10% fetal bovine serum.

Construction of expression vectors

Retroviral vectors pCX4bsr and pCX4pur [59], which contain the resistance gene for blasticidin and puromycin, respectively, were used to construct the various expression vectors. pCX4bsr/NS3-4A(1B-1), pCX4bsr/NS3(1B-1) and pCX4bsr/NS4A(1B-1) were constructed according to the previously described method [60]. The DNA fragments encoding NS3-4A, NS3, and NS4A derived from the HCV 1B-1 strain belonging to genotype 1b (accession no. AB0802999) [61] were subcloned into the *Eco*RI and *Not*I sites of pCX4bsr. To construct pCX4bsr/NS3-4A(O), the DNA fragment encoding NS3-4A derived from the HCV-O strain belonging to genotype 1b [43] were also subcloned into the *Eco*RI and *Not*I sites of pCX4bsr. pCX4bsr/NS3-4A (1B-1)/S1165A and pCX4bsr/NS3-4A(1B-1)/W1528A were constructed by PCR mutagenesis with primers containing base alterations according to the previously described method [62]. To construct pCX4pur/myc-Cardif, the DNA fragment encoding Cardif (IPS-1/MAVS/VISA, accession no. DQ181928) was amplified from cDNAs obtained from PH5CH8 cells by PCR using KOD-plus DNA polymerase (Toyobo, Osaka, Japan). The primer sequences containing the *Sph*I (forward) and *Not*I (reverse) recognition sites for Cardif were designed to enable expression of the Cardif ORF. The obtained DNA fragment was subcloned into the *Sph*I and *Not*I sites of pCX4pur/myc, which can express myc-tagged protein, according to the previously described method [39]. To construct pCX4pur/myc-TRIF, the *Eco*RI-*Not*I fragment of pCXpur/myc-TRIF encoding myc-TRIF ORF [39] was subcloned into the *Eco*RI and *Not*I sites of pCX4pur. To construct pEGFP-C1/IRF-3, the DNA fragment encoding IRF-3 (accession no. NM_001571) was amplified by PCR as described above. The primer sequences containing the *Xho*I (forward) and *Hin*dIII (reverse) recognition sites for IRF-3 were designed to enable expression of the IRF-3 ORF. The obtained DNA fragment was subcloned into the *Xho*I and *Hin*dIII sites of pEGFP-C1 (Clontech, Mountain View, CA, USA), and the obtained pEGFP-C1/IRF-3 was used for IRF-3 dimerization analysis. The nucleotide sequences of these constructed expression vectors were confirmed by Big Dye termination cycle sequencing using an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Poly(I-C) treatment

Poly(I-C) (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) was added to the medium at 50 $\mu g \cdot mL^{-1}$ (M-pIC), or 1 μg of poly(I-C) was complexed with Lipofectamine™ 2000 (Invitrogen) for transfection (T-pIC). Cells were assayed for poly(I-C)-induced responses 6 h after exposure by either route.

Luciferase reporter assay

For the dual luciferase assay, we used a firefly luciferase reporter vector, pIFN-β-(−125)-Luc [63], containing the IFN-β gene promoter region (−125 to +19). The reporter assay was carried out as previously described [40]. Briefly, a total of 0.3×10^5 cells were seeded in a 24-well plate, 24 h before transfection. Then, 0.1 μg firefly luciferase reporter vector, 0.2–0.4 μg HCV protein expression plasmid (pCX4bsr series), and 0.2 ng pRL-CMV (Promega, Madison, WI, USA) as an internal control reporter were transfected into the various cell lines. To maintain the efficiency of transfection, up to 0.4 μg of pCX4bsr was added instead of HCV protein expression vectors. In some cases, 20 ng of pCX4pur/myc-Cardif or pCX4pur/myc-TRIF were added as the effector plasmid. The cells were cultured for 48 h, and then a dual luciferase assay was performed according to the manufacturer's protocol (Promega). In some cases, the cells were cultured for 42 h and then poly(I-C) was added to the medium or transfected into the cells for 6 h before the reporter assay. Three independent triplicate transfection experiments were conduced to verify the reproducibility of the results. Relative luciferase activity was normalized to the activity of *Renilla* luciferase (internal control). A manual Lumat LB 9501/16 luminometer (EG & G Berthold, Bad Wildbad, Germany) was used to detect luciferase activity.

Western blot analysis

Preparation of cell lysates, SDS/PAGE, and immunoblotting were performed as described previously [64]. Anti-NS3 (Novocastra Laboratories, Newcastle, UK), anti-myc (PL14; Medical and Biological Laboratories, Nagoya, Japan) or anti-β-actin serum (AC-15; Sigma, St Louis, MO, USA) was used in this study as a primary antibody. Immunocomplexes were detected by a Renaissance enhanced chemiluminescence assay (Perkin-Elmer Life Sciences, Boston, MA, USA).

IRF-3 dimerization analysis

Preparation of cell lysates and native-polyacrylamide gel electrophoresis were performed as described previously [65]. After the separation of proteins, immunoblotting was performed as described above. Anti-IRF3 serum (FL-425; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for the detection of the endogenous IRF-3 dimerization. Anti-phospho-IRF-3 (Ser386) serum (IBL, Gunma, Japan) and anti-phospho-IRF-3 (Ser396) serum (Upstate Biotechnology, Lake Placid, NY, USA) were used for detection of the phosphorylated IRF-3. The dimerization of exogenous IRF-3 was detected by anti-EGFP monoclonal serum (JL-8; Clontech).

Preparation of PH5CH8 cells stably expressing HCV proteins

PH5CH8 cells were infected with retrovirus pCX4bsr encoding various HCV proteins, as described previously [64]. pCX4bsr/NS3-4A(1B-1), pCX4bsr/NS3-4A(1B-1)/S1165A, and pCX4bsr/NS3-4A(1B-1)/W1528A were used to obtain the PH5CH8 cells stably expressing NS3-4A(1B-1), the NS3-4A(1B-1)/S1165A mutant lacking the serine protease activity [51], and the NS3-4A(1B-1)/W1528A mutant lacking the helicase activity [66], respectively. At 2 days postinfection, PH5CH8 cells were changed with fresh medium containing blasticidin (20 $\mu g \cdot mL^{-1}$), and the culture was continued for 7 days to select the cells expressing HCV proteins.

Real-time LightCycler PCR

Total cellular RNA was extracted using an Isogen extraction kit (Nippon Gene, Toyama, Japan). Before reverse transcription, the RNA was treated with RNase-free DNase I (TaKaRa Bio, Ohtsu, Japan) to completely remove the genomic DNA as described previously [40]. Real-time LightCycler PCR was performed according to a method described previously [67]. The sequences of sense and antisense primers for TRIF (accession no. AB093555) were 5′-AAGCCATGATGAGCAACCTC-3′ and 5′-GTGTCC TGTTCCTTCCTCCAC-3′. The sequences of sense and antisense primers for RIG-I (accession no. NM_014314) were 5′-AATGAAAGATGCTCTGGATTACTTG-3′ and 5′-TTGTCTCTGGGTTTAAGTGGTACTC-3′. The sequences of sense and antisense primers for MDA5 (accession no. NM_022168) were 5′-AAGTCATTAGTAAA TTTCGCACTGG-3′ and 5′-TCATCTTCTCTCGGAAAT CATTAAC-3′. In addition, we used primer sets for IFN-β [40], TLR3 [39], TLR4 [39], Cardif [24] and GAPDH [40].

RNA interference

siRNA duplexes targeting the coding regions of human TLR3 [39], TLR4 (Dharmacon, Lafayette, CO, USA; catalog no. M-008088-00), TRIF (Dharmacon; catalog no. M-012833-00), and luciferase GL2 [68] (Dharmacon) as a control were chemically synthesized. PH5CH8 cells were transfected with the indicated siRNA duplex using OligofectAMINE (Invitrogen). Total RNA was extracted at 3 days after transfection, and real-time LightCycler PCR was performed to examine RNA-mediated interference efficiency as described above.

Acknowledgements

We are grateful to Dr Tsuyoshi Akagi (Osaka Bioscience Institute) for providing the pCX4bsr and

pCX4pur vectors. We thank Toshiko Maeta and Takashi Nakamura for their technical assistance. Drs Kazuhito Naka (Kanazawa University) and Yasuo Ariumi (Okayama University) are also thanked for their valuable input in this study. This work was supported by Grants-in-Aid for the Third-Term Comprehensive 10-Year Strategy for Cancer Control, and by a Grant-in-Aid for Research on Hepatitis, both from the Ministry of Health, Labor, and Welfare of Japan.

References

1 Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW & Houghton M (1989) Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244**, 359–362.

2 Kuo G, Choo QL, Alter HJ, Gitnick GL, Redeker AG, Purcell RH, Miyamura T, Dienstag JL, Alter MJ, Stevens CE, Tegtmeier GE, Bonino F, Colombo WS, Lee WS, Kuo C, Berger K, Shuster JR, Overby LR, Bradley DW & Houghton M (1989) An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. *Science* **244**, 362–364.

3 Ohkoshi S, Kojima H, Tawaraya H, Miyajima T, Kamimura T, Asakura H, Satoh A, Hirose S, Hijikata M, Kato N & Shimotohno K (1990) Prevalence of antibody against non-A, non-B hepatitis virus in Japanese patients with hepatocellular carcinoma. *Jpn J Cancer Res* **81**, 550–553.

4 Saito I, Miyamura T, Ohbayashi A, Harada H, Katayama T, Kikuchi Y, Watanabe S, Koi S, Onji M, Ohta Y, Choo QL, Houghton M & Kuo G (1990) Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc Natl Acad Sci USA* **87**, 6547–6549.

5 Thomas DL (2000) Hepatitis C epidemiology. *Curr Top Microbiol Immunol* **242**, 25–41.

6 Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugiyama 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 USA* **87**, 9524–9528.

7 Tanaka T, Kato N, Cho MJ & Shimotohno K (1995) A novel sequence found at the 3′ terminus of hepatitis C virus genome. *Biochem Biophys Res Commun* **215**, 744–749.

8 Hijikata M, Kato N, Ootsuyama Y, Nakagawa M & Shimotohno K (1991) Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. *Proc Natl Acad Sci USA* **88**, 5547–5551.

9 Hijikata M, Mizushima H, Tanji Y, Komada Y, Hirowatari Y, Akagi T, Kato N, Kimura K & Shimotohno K (1993) Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. *Proc Natl Acad Sci USA* **90**, 10773–10777.

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

11 Kawai T & Akira S (2006) Innate immune recognition of viral infection. *Nat Immunol* **7**, 131–137.

12 Meylan E & Tschopp J (2006) Toll-like receptors and RNA helicases: two parallel ways to trigger antiviral responses. *Mol Cell* **22**, 561–569.

13 Alexopoulou L, Holt AC, Medzhitov R & Flavell RA (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* **413**, 732–738.

14 Honda K & Taniguchi T (2006) IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. *Nat Rev Immunol* **6**, 644–658.

15 Kang DC, Gopalkrishnan RV, Wu Q, Jankowsky E, Pyle AM & Fisher PB (2002) mda-5: an interferon-inducible putative RNA helicase with double-stranded RNA-dependent ATPase activity and melanoma growth-suppressive properties. *Proc Natl Acad Sci USA* **99**, 637–642.

16 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.

17 Yoneyama M, Kikuchi M, Matsumoto K, Imaizumi T, Miyagishi M, Taira K, Foy E, Loo YM, Gale M Jr, Akira S, Yonehara S, Kato A & Fujita T (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.

18 Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ, Yamaguchi O, Otsu K, Tsujimura T, Koh CS, Reis e Sousa C, Matsuura Y, Fujita T & Akira S (2006) Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* **441**, 101–105.

19 Hornung V, Ellegast J, Kim S, Brzozka K, Jung A, Kato H, Poeck H, Akira S, Conzelmann KK, Schlee M, Endres S & Hartmann G (2006) 5′-Triphosphate RNA is the ligand for RIG-I. *Science* **314**, 994–997.

20 Pichlmair A, Schulz O, Tan CP, Naslund TI, Liljestrom P, Weber F & Reis e Sousa C (2006) RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. *Science* **314**, 997–1001.

21 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.

22 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.
23 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.
24 Meylan E, Curran J, Hofman 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.
25 Kawai T, Takahashi K, Sato S, Coban C, Kumar H, Kato H, Ishii KJ, Takeuchi O & Akira S (2005) IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat Immunol* **6**, 981–988.
26 Seth RB, Sun L, Ea CK & Chen ZJ (2005) Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* **122**, 669–682.
27 Xu LG, Wang YY, Han KJ, Li LY, Zhai Z & Shu HB (2005) VISA is an adapter protein required for virus-triggered IFN-beta signaling. *Mol Cell* **19**, 727–740.
28 Yoneyama M, Suhara W, Fukuhara Y, Fukada 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.
29 Lin R, Heylbroeck C, Pitha PM & 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.
30 Sharma S, tenOever BR, Grandvaux N, Zhou GP, Lin R & Hiscott J (2003) Triggering the interferon antiviral response through an IKK-related pathway. *Science* **300**, 1148–1151.
31 Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, Coyle AJ, Liao SM & Maniatis T (2003) IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol* **4**, 491–496.
32 Talon J, Horvath CM, Polley R, Basler CF, Muster T, Palese P & Garcia-Sastre A (2000) Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein. *J Virol* **74**, 7989–7996.
33 Foy E, Li K, Wang C, Sumpter R Jr, Ikeda M, Lemon SM & Gale M Jr (2003) Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. *Science* **300**, 1145–1148.
34 Li XD, Sun L, Seth RB, Pineda G & Chen ZJ (2005) Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. *Proc Natl Acad Sci USA* **102**, 17717–17722.
35 Foy E, Li K, Sumpter R Jr, Loo YM, Johnson CL, Wang C, Fish PM, Yoneyama M, Fujita T, Lemon SM & Gale M Jr (2005) Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signaling. *Proc Natl Acad Sci USA* **102**, 2986–2991.
36 Li K, Foy E, Ferreon JC, Nakamura M, Ferreon AC, Ikeda M, Ray SC, Gale M Jr & Lemon SM (2005) Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc Natl Acad Sci USA* **102**, 2992–2997.
37 Otsuka M, Kato N, Moriyama M, Taniguchi H, Wang Y, Dharel N, Kawabe T & Omata M (2005) Interaction between the HCV NS3 protein and the host TBK1 protein leads to inhibition of cellular antiviral responses. *Hepatology* **41**, 1004–1012.
38 Breiman A, Grandvaux N, Lin R, Ottone C, Akira S, Yoneyama M, Fujita T, Hiscott J & Meurs EF (2005) Inhibition of RIG-I-dependent signaling to the interferon pathway during hepatitis C virus expression and restoration of signaling by IKKepsilon. *J Virol* **79**, 3969–3397.
39 Naka K, Dansako H, Kobayashi N, Ikeda M & Kato N (2005) Hepatitis C virus NS5B delays cell cycle progression by inducing interferon-beta via Toll-like receptor 3 signaling pathway without replicating viral genomes. *Virology* **346**, 348–362.
40 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**, 458–468.
41 Li K, Chen Z, Kato N, Gale M Jr & Lemon SM (2005) Distinct poly(I-C) and virus-activated signaling pathways leading to interferon-beta production in hepatocytes. *J Biol Chem* **280**, 16739–16747.
42 Lanford RE, Guerra B, Lee H, Averett DR, 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.
43 Ikeda M, Abe K, Dansako H, Nakamura T, Naka K & Kato N (2005) 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* **329**, 1350–1359.
44 Naka K, Ikeda M, Abe K, Dansako H & Kato N (2005) Mizoribine inhibits hepatitis C virus RNA replication: effect of combination with interferon-alpha. *Biochem Biophys Res Commun* **330**, 871–879.
45 Ikeda M, Sugiyama K, Mizutani T, Tanaka T, Tanaka K, Sekihara H, Shimotohno K & Kato N (1998) Human hepatocyte clonal cell lines that support persist-

ent replication of hepatitis C virus. *Virus Res* **56**, 157–167.
46 Kawai T, Takeuchi O, Fujita T, Inoue J, Muhlradt PF, Sato S, Hoshino K & Akira S (2001) Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J Immunol* **167**, 5887–5894.
47 Doyle S, Vaidya S, O'Connell R, Dadgostar H, Dempsey P, Wu T, Rao G, Sun R, Haberland M, Modlin R & Cheng G (2002) IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity* **17**, 251–263.
48 Li XD, Sun L, Seth RB, Pineda G & Chen ZJ (2005) Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. *Proc Natl Acad Sci USA* **102**, 17717–17722.
49 Lin R, Lacoste J, Nakhaei P, Sun Q, Yang L, Paz S, Wilkinson P, Julkunen I, Vitour D, Meurs E & Hiscott J (2006) Dissociation of a MAVS/IPS-1/VISA/Cardif-IKKepsilon molecular complex from the mitochondrial outer membrane by hepatitis C virus NS3-4A proteolytic cleavage. *J Virol* **80**, 6072–6083.
50 Loo YM, Owen DM, Li K, Erickson AK, Johnson CL, Fish PM, Carney DS, Wang T, Ishida H, Yoneyama M, Fujita T, Saito T, Lee WM, Hagedorn CH, Lau DT, Weinman SA, Lemon SM & Gale M Jr (2006) Viral and therapeutic control of IFN-beta promoter stimulator 1 during hepatitis C virus infection. *Proc Natl Acad Sci USA* **103**, 6001–6006.
51 Hijikata M, Mizushima H, Akagi T, Mori S, Kakiuchi N, Kato N, Tanaka T, Kimura K & Shimotohno K (1993) Two distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. *J Virol* **67**, 4665–4675.
52 Kobayashi N, Fujiwara T, Westerman KA, Inoue Y, Sakaguchi M, Noguchi H, Miyazaki M, Cai J, Tanaka N, Fox IJ & Leboulch P (2000) Prevention of acute liver failure in rats with reversibly immortalized human hepatocytes. *Science* **287**, 1258–1262.
53 Nguyen TH, Mai G, Villiger P, Oberholzer J, Salmon P, Morel P, Buhler L & Trono D (2005) Treatment of acetaminophen-induced acute liver failure in the mouse with conditionally immortalized human hepatocytes. *J Hepatol* **43**, 1031–1037.
54 Nomura-Takigawa Y, Nagano-Fujii M, Deng L, Kitazawa S, Ishido S, Sada K & Hotta H (2006) Nonstructural protein 4A of hepatitis C virus accumulates on mitochondria and renders the cells prone to undergoing mitochondria-mediated apoptosis. *J Gen Virol* **87**, 1935–1945.
55 De Francesco R & Steinkuhler C (2000) Structure and function of the hepatitis C virus NS3-NS4A serine proteinase. *Curr Top Microbiol Immunol* **242**, 149–169.
56 Cheng G, Zhong J & Chisari FV (2006) Inhibition of dsRNA-induced signaling in hepatitis C virus-infected cells by NS3 protease-dependent and -independent mechanisms. *Proc Natl Acad Sci USA* **103**, 8499–8504.
57 Ikeda M, Kato N, Mizutani T, Sugiyama K, Tanaka K & Shimotohno K (1997) Analysis of the cell tropism of HCV by using in vitro HCV-infected human lymphocytes and hepatocytes. *J Hepatol* **27**, 445–454.
58 Noguchi M & Hirohashi S (1996) Cell lines from non-neoplastic liver and hepatocellular carcinoma tissue from a single patient. *In Vitro Cell Dev Biol Anim* **32**, 135–137.
59 Akagi T, Sasai K & Hanafusa H (2003) Refractory nature of normal human diploid fibroblasts with respect to oncogene-mediated transformation. *Proc Natl Acad Sci USA* **100**, 13567–13572.
60 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.
61 Sugiyama K, Kato N, Mizutani T, Ikeda M, Tanaka T & Shimotohno K (1997) Genetic analysis of the hepatitis C virus (HCV) genome from HCV-infected human T cells. *J Gen Virol* **78**, 329–336.
62 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.
63 Benech P, Vigneron M, Peretz D, Revel M & Chebath J (1987) Interferon-responsive regulatory elements in the promoter of the human 2′,5′-oligo(A) synthetase gene. *Mol Cell Biol* **7**, 4498–4504.
64 Naganuma A, Dansako H, Nakamura T, Nozaki A & Kato N (2004) Promotion of microsatellite instability by hepatitis C virus core protein in human non-neoplastic hepatocyte cells. *Cancer Res* **64**, 1307–1314.
65 Iwamura T, Yoneyama M, Yamaguchi K, Suhara W, Mori W, Shiota K, Okabe Y, Namiki H & Fujita T (2001) Induction of IRF-3/-7 kinase and NF-kappaB in response to double-stranded RNA and virus infection: common and unique pathways. *Genes Cells* **6**, 375–388.
66 Tai CL, Pan WC, Liaw SH, Yang UC, Hwang LH & Chen DS (2001) Structure-based mutational analysis of the hepatitis C virus NS3 helicase. *J Virol* **75**, 8289–8297.
67 Abe K, Ikeda M, Dansako H, Naka K, Shimotohno K & Kato N (2005) cDNA microarray analysis to compare HCV subgenomic replicon cells with their cured cells. *Virus Res* **107**, 73–81.
68 Elbashir SM, 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.

Supplementary material

The following supplementary material is available online:

Fig. S1. NS3-4A blocked the Cardif-mediated signaling pathway, but not the TRIF-mediated signaling pathway in HeLa cells.

Fig. S2. NS3-4A is capable of cleaving Cardif, but not TRIF in HeLa cells.

Table S1. Quantitative RT-PCR analysis of mRNA expression of several factors involving in innate immune response in the various cell lines.

This material is available as part of the online article from http://www.blackwell-synergy.com

Please note: Blackwell Publishing is not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

J Pharmacol Sci 105, 145 – 150 (2007)

Journal of Pharmacological Sciences
©2007 The Japanese Pharmacological Society

Forum Minireview

Life Style-Related Diseases of the Digestive System: Cell Culture System for the Screening of Anti-Hepatitis C Virus (HCV) Reagents: Suppression of HCV Replication by Statins and Synergistic Action With Interferon

Masanori Ikeda[1,*] and Nobuyuki Kato[1]

[1]*Department of Molecular Biology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan*

Received July 10, 2007; Accepted September 10, 2007

Abstract. Hepatitis C virus (HCV) infection causes chronic hepatitis and leads to liver fibrosis and hepatocellular carcinoma. Pegylated-interferon and ribavirin is the current standard therapy for chronic hepatitis C. However, the therapy is only effective in 50% of the patients. To overcome this problem, we recently developed the HCV cell culture system (OR6 system) for the screening of anti-HCV reagents. In this OR6 system, the luciferase gene was introduced into the upstream portion of the HCV genome to facilitate the monitoring of HCV RNA replication. Recently lipid metabolism is reported to be involved in HCV RNA replication. Cholesterol and sphingolipid are the major components in lipid rafts, which seem to be the scaffold for HCV RNA replication. Statins inhibit cholesterol biosynthesis and also have the pleiotropic effects by the inhibition of prenylation. We demonstrated different anti-HCV effects of statins (atorvastatin, simvastatin, fluvastatin, lovastatin, and pitavastatin) using the OR6 system. Surprisingly, in contrast to the other statins, pravastatin exhibited no anti-HCV effect. Furthermore, statins enhanced the anti-HCV effect of interferon in combination. Statins may be a promising candidate for the adjuvant in interferon therapy and may improve the efficiency of the current interferon and ribavirin therapy.

Keywords: life style-related disease, hepatitis C virus (HCV), statin, interferon, cell culture system

Introduction

Approximately 170 million people worldwide are infected with the hepatitis C virus (HCV). HCV infection causes chronic hepatitis C (CH-C) and leads to liver-related death by liver cirrhosis and/or hepatocellular carcinoma. To prevent the progress of fatal liver disease after HCV infection, the elimination of the virus seems to be the most effective strategy. However, the current pegylated-interferon (PEG-IFN) and ribavirin therapy was only effective in 50% of the patients (1). Therefore, the development of more effective anti-HCV reagents is an urgent concern. When HCV replicates in hepatocytes, some of the cellular factors are essential for HCV RNA replication. These cellular factors are the targets for antiviral as well as viral proteins such as NS3 protease or NS5B RNA-dependent RNA polymerase. Inhibition of cellular factors may cause side effects by the inhibition of their primary roles. However, one of the advantages of this strategy is that it could overcome the viral mutation leading to the resistance to the reagent against the viral proteins. Lipid metabolism is one of the candidates in the context of this strategy. To explore the best partner of IFN, we examined different six statins, which are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, using our recently developed OR6 system (2). In the OR6 system, genome-length HCV RNAs (HCV-O strain of genotype 1b) replicate efficiently and the HCV RNA level can be monitored by luciferase activities (3, 4). Statins exhibited various anti-HCV activities except for pravastatin that was not active against HCV (2). We also

*Corresponding author. maikeda@md.okayama-u.ac.jp
Published online in J-STAGE: October 6, 2007
doi: 10.1254/jphs.FM0070050

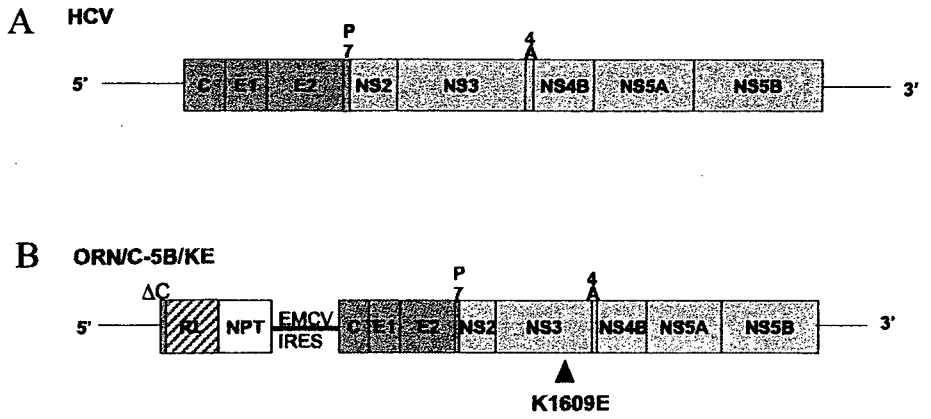

Fig. 1. HCV RNA with reporter gene. Schematic gene organization of genome-length HCV RNA. A: The authentic HCV RNA was composed of the N-terminal part of the structural region and C-terminal part of the nonstructural region. B: The genome-length HCV RNA with reporter gene was constructed based on the authentic HCV RNA. EMCV IRES was introduced for the translation of HCV proteins. Renilla luciferase was expressed as a fusion protein with NPT. The position of the adaptive mutation, K1609E, is indicated by a black triangle.

investigated whether or not statins could enhance the inhibitory effect of IFN on HCV RNA replication. In this review, we would like to summarize our recent findings and the literature regarding lipid metabolism as the target of anti-HCV with a focus on statins.

Cell culture system for HCV RNA replication

Cell culture systems for HCV have been developed since the first breakthrough of the establishment of the subgenomic replicon by Lohmann et al. (5). The replicon system has provided the information concerning the mechanism of the replication machinery of HCV and has revealed the cellular factors essential for HCV RNA replication. After the development of the subgenomic replicon, genome-length HCV RNA replication systems using different HCV strains (H, N, Con1, and O) were developed by several groups since the subgenomic replicon did not possess the structural region in the genome (4, 6 – 8). For the screening of anti-HCV reagents, the replicon system has also been improved by the introduction of reporter genes (9). The introduction of the reporter gene into the HCV genome facilitated the monitoring of HCV RNA replication. For this purpose, we developed a cell culture system (OR6 system) in which genome-length HCV RNA containing renilla luciferase (RL) replicate efficiently under the selection by G418 (4). As shown in Fig. 1, RL, neomycin phosphotransferase (NPT), and encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES) genes were introduced between the 5' untranslated region and Core (C) of HCV. This genome-length HCV RNA robustly replicated in the hepatoma cell line HuH-7 after the electroporation and one of the colonies designated OR6 was selected by G418 and used for the studies including determining the anti-HCV effect of statins. A recent milestone was the development of an HCV infection system using a genotype 2a HCV strain, JFH-1 (10 – 12). This system could reconstruct the HCV life cycle in cell culture. The future issue of the cell culture system is the development of a robust genotype 1 HCV virus production system because the efficiency of PEG-IFN and ribavirin therapy in patients with genotype 1 HCV remained lower than that in patients with genotype 2 HCV: the sustained virological responses were approximately 50% versus 80% – 90%, respectively (13). More recently, pioneering studies have been reported by several groups using genotype 1 HCV strains for virus production (14, 15). However, the genotype 1 HCV virus production systems could not allow re-infection with the supernatant from the HCV-infected cells. These ongoing studies will lead to the development of a robust genotype 1 HCV infection system like genotype 2a HCV in the near future.

HCV and lipid metabolism

Lipid metabolism is involved in the life cycle of many viruses. The resulting metabolites work as physiologically active molecules such as eicosanoids and so on, and some of them are incorporated into the lipid raft membrane. A lipid raft is distinct from other lipid membranes. It is enriched in cholesterols and sphingolipids and is detergent-resistant. Lipid rafts play an important role in virus entry, replication, and assembly. HCV also forms a replication complex on the lipid raft membrane structure (16). Therefore, the depletion of the cholesterol and sphingolipid from the lipid raft leads to the inhibition of HCV RNA replication. Aizaki et al. (17) reported that lovastatin inhibited HCV RNA replication in HCV replicon-harboring cells. Statins are inhibitors for HMG-CoA reductase in the cholesterol biosynthesis pathway (Fig. 2). Statins also possess the cholesterol-independent action (pleiotropic effect) (18). Many of these pleiotropic effects are mediated by the isoprenoid. Farnesyl pyrophosphate (FPP) and gera-

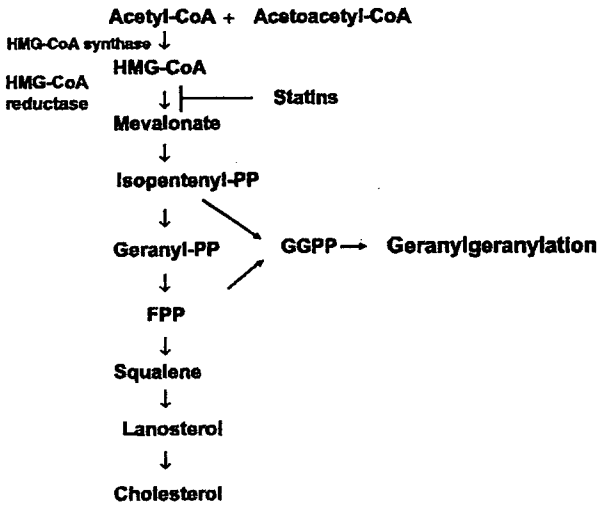

Fig. 2. Cholesterol biosynthesis pathway and statins. In the cholesterol biosynthesis pathway, the production of mevalonate by HMG-CoA reductase is the rate-limiting step. Statins inhibit HMG-CoA reductase, resulting in the inhibition of the production of isoprenoids as well as cholesterol. Geranyl-PP: geranylpyrophosphate and GGPP: geranylgeranylpyrophosphate.

nylgeranyl pyrophosphate (GGPP) are mevalonate-derived isoprenoids (Fig. 2). The attachment of isoprenoid to the cellular proteins is called prenylation. Prenylation regulates a variety of cellular functions, including growth, differentiation, and oncogenesis. From the aspect of the pleiotropic effect of the statins, Wang et al. (19) recently identified FBL2 as geranylgeranylated cellular protein required for HCV RNA replication. FBL2 belongs to the FBL family of proteins, all of which contain an F box and a multiple leucine-rich repeat. These two possible inhibitory mechanisms are proposed for the anti-HCV effect of statins. The low-density lipoprotein receptor (LDLR) is reported as one of the potential HCV receptors (20). However, the precise role of LDLR for HCV is still controversial (21). It will be worth trying to examine the effect of statins in the JFH-1 infection system since statins enhance the expression of LDLR.

Sphingolipid is another major component of lipid rafts and thereby is also the antiviral target for HCV. Serine palmitoyltransferase (SPT) is the enzyme responsible for the condensation of L-serine with palmitoyl-CoA to produce 3-ketodihydrosphingosine in the first step of sphingolipid biosynthesis. Sakamoto et al. (22) and Umehara et al. (23) reported that myriocin, a selective inhibitor of SPT, inhibited the HCV RNA replication in replicon-harboring cells and in HCV-infected chimeric mice with humanized livers, respectively. These results further support the significance of lipid metabolism in HCV RNA replication.

Other than cholesterol and sphingolipid biosynthesis, fatty acids are reported to be metabolites that affect HCV RNA replication. Leu et al. (24) reported that polyunsaturated fatty acids (PUFAs) possessed an anti-HCV effect using HCV-replicon harboring cells. Arachidonic acid, docosahexaenoic acid, linoleic acid, and eicosapentaenoic acid belonging to PUFAs possessed anti-HCV activity. On the other hand, saturated fatty acids enhanced HCV RNA replication. The precise mechanisms of fatty acids regarding HCV RNA replication have remained unclear. Very recently, we examined the effect of ordinary nutrients on HCV RNA replication using the OR6 system (25). Interestingly, we found that vitamin E negated the anti-HCV effect of linoleic acid (25). Given that linoleic acid and vitamin E are an oxidant and antioxidant, respectively, oxidative stress may be involved in HCV RNA replication. Further study in this field will provide clues for developing anti-HCV reagents.

Different anti-HCV effects of statins

Statins are one of the most worldwide used reagents for the treatment of hypercholesterolemia and they are beneficial in the prevention of coronary heart disease. In the cholesterol biosynthesis pathway, the production of mevalonate by HMG-CoA reductase is the rate-limiting step. Statins inhibit mevalonate synthesis by inhibiting HMG-CoA reductase, resulting in decreased production of isoprenoids as well as cholesterol. The activities of some cellular proteins are regulated by the attachment of isoprenoids (prenylation). For example, statins inhibited the function of small G proteins, Ras and Rho. Ras and Rho are major substrates for prenylation with FPP and GGPP, respectively. So far, among the statins, lovastatin is the only one with a well-characterized inhibitory effect against HCV RNA replication in cell culture (17, 26, 27). Recently, FBL2 was identified as one of the of geranylgeranylated cellular proteins required for HCV RNA replication (19). Geranylgeranyrated FBL2 binds to NS5A of HCV and the resulting complex is required for HCV RNA replication (19).

The anti-HCV effect of the statins other than lovastatin remains to be clarified. Therefore, we used the OR6 system to test anti-HCV effect of five statins: lovastatin, simvastatin, atorvastatin, fluvastatin, and pravastatin (2). More recently, we also added pitavastatin to this list, so that finally six statins were tested for their effects on HCV RNA replication. None of the statins exhibited cytotoxicity at the concentrations tested. The 50% effective concentrations (EC_{50}) of statins are summarized in Table 1. The anti-HCV effects of simvastatin, atorvastatin, fluvastatin, and pitavastatin

Table 1. EC_{50} of statins on HCV RNA replication

Statins	EC_{50} (μM)
Lovastatin	2.16
Simvastatin	1.57
Atorvastatin	1.39
Fluvastatin	0.90
Pitavastatin	0.45

were stronger than that previously reported for lovastatin. The EC_{50} of lovastatin, simvastatin, atorvastatin, fluvastatin, and pitavastatin were 2.16, 1.57, 1.39, 0.90, and 0.45 μM, respectively. Pitavastatin possessed the strongest anti-HCV activity among the statins tested and its EC_{90} was calculated as 1.25 μM (Fig. 3A). In contrast, pravastatin exhibited no anti-HCV effect. Pravastatin is the only hydrophilic statin among the statins tested and does not cross the cellular membrane passively. It has been reported that a human liver-specific organic anion transporter, LST-1, mediates the uptake of pravastatin in human hepatocytes (28). Therefore, we examined the expression levels of LST-1 in OR6 cells. OR6 cells expressed the mRNA of LST-1 at levels equivalent to that in normal human liver (2). We ruled out the possibility that pravastatin didn't actually work as the inhibitor for HMG-CoA reductase in the cells. We confirmed that pravastatin induced HMG-CoA reductase by a positive feedback mechanism in response to the decrease of cholesterol by the inhibition of HMG-CoA reductase by pravastatin (2). These results suggest that there may be another mechanism underlying the depletion of GGPP and cholesterol by statins. One of the clues for resolving this puzzle is that pravastatin has a different effect on P450 induction compared with the other statins (29). However, further study will be needed to clarify this issue.

Statins in combination with IFN

The combination therapy of PEG-IFN and ribavirin is a current standard therapy for patients with CH-C. Ribavirin by itself possessed no anti-HCV effect for the patients. However, ribavirin alone exhibited an anti-HCV effect in the OR6 cell culture system when it was used at a concentration higher than that in the serum of patients undergoing ribavirin treatment. The EC_{50} of ribavirin is calculated as 76 μM in the OR6 system and this is approximately 5 – 7 times higher concentration than that in serum from the patients with ribavirin treatment (3). Furthermore, the synergistic effect of ribavirin at the low concentration with IFN was also confirmed in different cell culture systems, including the OR6 system (3, 30, 31). These results suggest that ribavirin works as a kind of the adjuvant for IFN at the low concentration.

To test the effect of statins in combination with IFN-α on HCV RNA replication, we treated the OR6 cells with

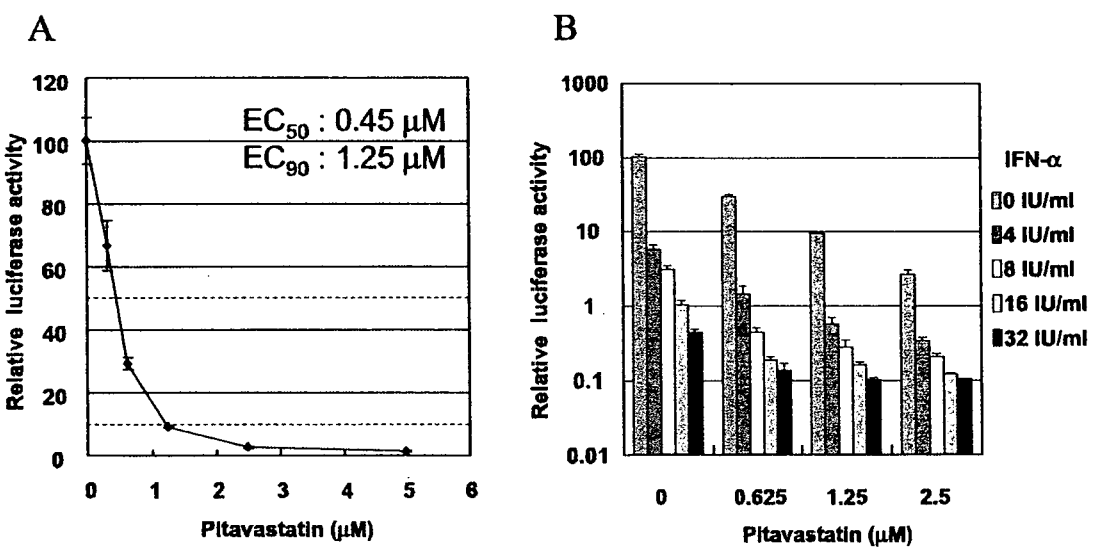

Fig. 3. Anti-HCV effect of pitavastatin in combination with IFN-α. A: OR6 cells were treated with pitavastatin at concentrations of 0, 0.625, 1.25, 2.5, and 5 μM for 72 h. The EC_{50} and EC_{90} were calculated from the result. Shown here is the relative luciferase activity (%) calculated when the luciferase activity of untreated cells was assigned as 100%. B: The effect of pitavastatin in combination with IFN-α. OR6 cells were treated with pitavastatin (0, 0.625, 1.25, and 2.5 μM) and IFN-α (0, 4, 8, 16, and 32 IU/ml) for 72 h. The relative luciferase activity was calculated as shown above.

pitavastatin (0, 0.625, 1.25, and 2.0 μM) and IFN-α (0, 4, 8, 16, and 32 IU/ml) (Fig. 3B). Pitavastatin enhanced the anti-HCV effect of IFN-α in a dose-dependent manner for a fixed concentration of IFN-α, 0, 4, 8, 16, or 32 IU/ml (Fig. 3B). Furthermore, we observed the decrease of luciferase activity to almost the background level in the OR6 reporter assay when OR6 cells were co-treated with 32 IU/ml of IFN-α and pitavastatin at the concentration of 1.25 or 2.5 μM (Fig. 3B). The concentrations of the statins tested in the cell culture were higher than that in the sera from patients with statin administration. However, the statins may enhance the anti-HCV effect of IFN for patients with CH-C at a lower concentration than the EC_{50} in cell culture. Recently O'Leary et al. (32) reported that the monotherapy of atorvastatin does not exhibit anti-HCV activity in a pilot clinical trial. Although the monotherapy of statin seems to be insufficient for patients with CH-C, statin may be a candidate for the adjuvant of IFN therapy like ribavirin.

Conclusions

The OR6 system was developed for the precise and quantitative assay of HCV RNA replication in cell culture. The statins were compared for their anti-HCV effects using the OR6 system and were found to possess different effects on HCV RNA replication. Lovastatin, simvastatin, atorvastatin, fluvastatin, and pitavastatin had different anti-HCV profiles in cell culture. However, pravastatin had no anti-HCV effect, although it worked as inhibitor for HMG-CoA reductase. Pitavastatin exhibited the strongest anti-HCV effect (EC_{50}: 0.45 μM) among the statins tested and enhanced the effect of IFN-α. It may be difficult to achieve the cell culture based EC_{50} of statins in patients with CH-C. However, statins at lower concentration than the EC_{50} in cell culture may enhance the anti-HCV effect of IFN-α in patients with CH-C. Therefore, statins may be suitable as an adjuvant of IFN-α like ribavirin rather than for monotherapy. Lipid metabolism including cholesterol, sphingolipid, and fatty acid biosynthesis seems to be an attractive field for the development of antiviral reagents for HCV.

References

1 Hayashi N, Takehara T. Antiviral therapy for chronic hepatitis C: past, present, and future. J Gastroenterol. 2006;41:17–27.

2 Ikeda M, Abe K, Yamada M, Dansako H, Naka K, Kato N. Different anti-HCV profiles of statins and their potential for combination therapy with interferon. Hepatology. 2006;44:117–125.

3 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.

4 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.

5 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.

6 Pietschmann T, Lohmann V, Kaul A, Krieger N, Rinck G, Rutter G, et al. Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. J Virol. 2002;76:4008–4021.

7 Blight KJ, McKeating JA, Marcotrigiano J, Rice CM. Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. J Virol. 2003;77:3181–3190.

8 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.

9 Yokota T, Sakamoto N, Enomoto N, Tanabe Y, Miyagishi M, Maekawa S, et al. Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. EMBO Rep. 2003;4:602–608.

10 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.

11 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.

12 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.

13 Kasahara A, Hayashi N, Hiramatsu N, Oshita M, Hagiwara H, Katayama K, et al. Ability of prolonged interferon treatment to suppress relapse after cessation of therapy in patients with chronic hepatitis C: a multicenter randomized controlled trial. Hepatology. 1995;21:291–297.

14 Yi M, Villanueva RA, Thomas DL, Wakita T, Lemon SM. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. Proc Natl Acad Sci U S A. 2006;103:2310–2315.

15 Heller T, Saito S, Auerbach J, Williams T, Moreen TR, Jazwinski A, et al. An in vitro model of hepatitis C virion production. Proc Natl Acad Sci U S A. 2005;102:2579–2583.

16 Shi ST, Lee KJ, Aizaki H, Hwang SB, Lai MM. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. J Virol. 2003;77:4160–4168.

17 Aizaki H, Lee KJ, Sung VM, Ishiko H, Lai MM. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. Virology. 2004;324:450–461.

18 Liao JK, Laufs U. Pleiotropic effects of statins. Annu Rev Pharmacol Toxicol. 2005;45:89–118.

19 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.

20 Molina S, Castet V, Fournier-Wirth C, Pichard-Garcia L, Avner R, Harats D, et al. The low-density lipoprotein receptor plays a role in the infection of primary human hepatocytes by hepatitis C virus. J Hepatol. 2007;46:411–419.
21 von Hahn T, McKeating JA. In vitro veritas? The challenges of studying hepatitis C virus infectivity in a test tube. J Hepatol. 2007;46:355–358.
22 Sakamoto H, Okamoto K, Aoki M, Kato H, Katsume A, Ohta A, et al. Host sphingolipid biosynthesis as a target for hepatitis C virus therapy. Nat Chem Biol. 2005;1:333–337.
23 Umehara T, Sudoh M, Yasui F, Matsuda C, Hayashi Y, Chayama K. Serine palmitoyltransferase inhibitor suppresses HCV replication in a mouse model. Biochem Biophys Res Commun. 2006;346:67–73.
24 Leu GZ, Lin TY, Hsu JT. Anti-HCV activities of selective polyunsaturated fatty acids. Biochem Biophys Res Commun. 2004;318:275–280.
25 Yano M, Ikeda M, Abe K, Dansako H, Ohkoshi S, Aoyagi Y, et al. Comprehensive analysis of the effects of ordinary nutrients on hepatitis C virus RNA replication in cell culture. Antimicrob Agents Chemother. 2007;51:2016–2027.
26 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.
27 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.
28 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.
29 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.
30 Tanabe Y, Sakamoto N, Enomoto N, Kurosaki M, Ueda E, Maekawa S, et al. Synergistic inhibition of intracellular hepatitis C virus replication by combination of ribavirin and interferon-alpha. J Infect Dis. 2004;189:1129–1139.
31 Kim SS, Peng LF, Lin W, Choe WH, Sakamoto N, Schreiber SL, et al. A cell-based, high-throughput screen for small molecule regulators of hepatitis C virus replication. Gastroenterology. 2007;132:311–320.
32 O'Leary JG, Chan JL, McMahon CM, Chung RT. Atorvastatin does not exhibit antiviral activity against HCV at conventional doses: a pilot clinical trial. Hepatology. 2007;45:895–898.

Available online at www.sciencedirect.com
ScienceDirect
Advanced Drug Delivery Reviews 59 (2007) 1277–1289
Advanced DRUG DELIVERY Reviews
www.elsevier.com/locate/addr

Modulation of host metabolism as a target of new antivirals ☆

Masanori Ikeda *, Nobuyuki Kato

Department of Molecular Biology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1, Shikata-cho, Okayama 700-8558, Japan

Received 28 March 2007; accepted 30 March 2007
Available online 11 August 2007

Abstract

The therapy for chronic hepatitis C (CH–C) started with interferon (IFN) monotherapy in the early 1990s and this therapy was considered effective in about 10% of cases. The present standard therapy of pegylated IFN with ribavirin achieves a sustained virologic response in about 50% of patients. However, about half of the CH–C patients are still at risk of fatal liver cirrhosis and hepatocellular carcinoma. The other significant event in hepatitis C virus (HCV) research has been the development of a cell culture system. The subgenomic replicon system enables robust HCV RNA replication in hepatoma cells. And recently, the complete life cycle of HCV has been achieved using a genotype 2a strain, JFH1. These hallmarks have provided much information about the mechanisms of HCV replication, including information on the host molecules required for the replication. Anti-HCV reagents targeting HCV proteins have been developed, and some of them are now in clinical trials. However, the RNA-dependent RNA polymerase frequently causes mutations in the HCV genome, which lead to the emergence of drug-resistant HCV mutants. Some of the cellular proteins essential for HCV RNA replication have already been discovered using the HCV cell culture system. These host molecules are also candidate targets for antivirals. Here, we describe the recent progress regarding the anti-HCV reagents targeting host metabolism.
© 2007 Elsevier B.V. All rights reserved.

Keywords: Hepatitis C virus; Replicon; Antiviral; Interferon; Host metabolism; Statin

Contents

Abbreviations: HCV, hepatitis C virus; CH, chronic hepatitis; HCC, hepatocellular carcinoma; IFN, interferon; SVR, sustained virological response; PEG-IFN, pegylated-IFN; GBV-B, GB virus B; uPA-SCID, urokinase plasminogen activator-severe combined immunodeficiency; NS, nonstructural; RdRp, RNA dependent RNA polymerase; CyPB, cyclophilin B; CsA, cyclosporine A; HSP90, heat shock protein 90; La, La auto antigen; PTB, polypyrimidine tract-binding protein; ALT, alanine aminotransferase; Neo, neomycin phosphotransferase; EMCV, encephalomyocarditis virus; IRES, internal ribosome entry site; ORF, open reading frame; FKBP8, FK-506-binding protein 8; GGPP, geranylgeranyl pyrophosphate; FPP, farnesyl pyrophosphate; FTase, farnesyltransferase; GGTase-I, geranylgeranyltransferase type I; GGTI, GGTase-I inhibitor; HMG–CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LOV, lovastatin; ATV, atorvastatin; FLV, fluvastatin; PRV, pravastatin; SMV, simvastatin; EC_{50}, 50%; effective concentration to inhibit HCV RNA replication; PTV, pitavastatin; RSV, respiratory syncytial virus; CMV, cytomegarovirus; HIV, human immunodeficiency virus; ICAM-1, integrin intercellular adhesion molecule 1; LFA-1, lymphocyte function associated antigen-1; DRM, detergent resistant membrane; SPT, serine palmitoyltransferase; ER, endoplasmic reticulum; GSL, glycosphingolipid; SBD, sphingolipid-binding domain; IMPDH, inosine monophosphate dehydrogenase; XMP, xanthosine 5′ ; monophosphate; MPA, mycophenolic acid; RMP, ribavirin monophosphate; RDP, ribavirin diphosphate; RTP, ribavirin triphosphate; GTP, guanosine triphosphate; SARS, severe acute respiratory syndrome; HBV, hepatitis B virus; VLP, virus-like particle; PIAS1, protein inhibitor of activated STAT1; PRMT1, protein arginine methyltransferase 1; PP2Ac, catalytic subunit of protein phosphatase 2A; AdoMet, S-adenosyl-L-methionine; PUFAs, polyunsaturated fatty acids; AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid.

☆ This review is part of the *Advanced Drug Delivery Reviews* theme issue on "Toward Evidence Based Control of Hepatitis C Virus Infection".

* Corresponding author. Tel.: +81 86 235 7386; fax: +81 86 235 7392.
E-mail address: maikeda@md.okayama-u.ac.jp (M. Ikeda).

0169-409X/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.addr.2007.03.021

1. Introduction

Hepatitis C virus (HCV) was discovered in 1989 [1] as the causative agent of chronic hepatitis C (CH–C), liver cirrhosis and hepatocellular carcinoma (HCC) [2]. It is estimated that 170 million people worldwide are infected with HCV [3]. The ultimate goal of both clinical and basic HCV studies is the suppression of liver-related death caused by HCV infection. With respect to clinical studies, interferon (IFN) has played a major role in the treatment of patients with CH–C. IFN therapy started with IFN monotherapy in the early 1990s, and a sustained virologic response (SVR) was obtained in about 10% of patients [4]. IFN therapy was developed by the hepatologists, and the current therapy of pegylated IFN (PEG-IFN) with ribavirin has improved the SVR to about 50% [4]. Therefore, the next stage of the therapy for CH–C is to develop new anti-HCV reagents to improve the SVR.

During the development of IFN therapy, the most striking discovery in the basic research was the development of a cell culture system for robust HCV RNA replication. In 1999, Lohmann et al. [5] achieved subgenomic HCV RNA replication in a human hepatoma cell line, HuH-7. The advantages of this novel system (known as the replicon system) were that it provided not only a way to screen for anti-HCV reagents but also information about the mechanism of HCV RNA replication. This cell culture system has been further improved, and recently the complete life cycle of HCV was achieved using a genotype 2a HCV strain, JFH1 [6–8]. This newest system has extended the targets of the anti-HCV therapy to the virus infection and release.

The effects of anti-HCV reagents selected from the cell culture-based screening should be evaluated using an animal model system for HCV infection before they can be released to clinical trial. Chimpanzees were the only animal model in the early HCV studies [9]. However, the use of chimpanzees is limited for ethical and financial reasons. In addition to chimpanzees, a study using tree shrews (*Tupaia belangeri chinensis*) has been reported [10]. A different approach to the study of HCV using animal models was achieved using the related GB virus B (GBV-B). GBV-B belongs to the *Flaviviridae* family and can be transmitted to tamarins and marmosets [11,12]. These animal models may be valuable surrogate models for HCV study. Another approach was demonstrated in a study using urokinase plasminogen activator-severe combined immunodeficiency (uPA-SCID) mice transplanted with human hepatocytes [13]. This chimeric mouse model can support chronic HCV viremia under the circumstance without immune system. Mass screening for anti-HCV reagents using cell culture systems will become a more powerful tool when combined with small animal model systems to evaluate the antiviral effects of selected reagents before clinical trial.

In considering a new strategy for CH–C to be used in place of or in combination with IFN, the main targets are HCV proteins and HCV RNA. With respect to the HCV proteins, two of these, nonstructural (NS) 3-4A and NS5B, have been well-characterized as protease and RNA-dependent RNA polymerase (RdRp), respectively [14,15]. Several reagents have been reported to be inhibitors of NS3-4A serine protease, including SCH6 [16,17], SCH503034 [18], VX-950 [19,20], and BILN-2061 [21]. Valopicitabine (NM283) was reported to inhibit NS5B RdRp [22]. HCV RNA itself is also a target of antivirals, and recent RNA interference technologies using siRNA or shRNA have targeted HCV RNA [23–25]. As RdRp lacks proofreading activity, the high mutation rate of RdRp allows the virus to escape from the reagents targeting HCV proteins and HCV RNA. These anti-HCV reagent-targeting viral proteins and genome will be reviewed in another section.

Other targets are the cellular proteins essential for HCV RNA replication and infection. The expression of HCV proteins is thought to affect the host cells' gene expression profiles and vise versa [26]. The interaction of the specific cellular proteins with HCV proteins is essential for HCV replication (Table 1). Cyclosporine A (CsA) is one of the best characterized inhibitors targeting the cellular proteins required for HCV replication [27–36]. The interaction of cyclophilin B (CyPB) with NS5B is required for HCV RNA replication [28]. CsA inhibits HCV RNA replication by interrupting the interaction between NS5B and CyPB. Heat shock protein 90 (HSP90) has also been reported to be an essential cellular protein for HCV RNA replication [37–39]. Knockdown or inhibition of HSP90 has been shown to result in the anti-HCV activity in cell culture and in uPA-SCID mouse systems [37].

Table 1
Host molecules as targets of anti-HCV

Target molecules	Reagents	References
HMG–CoA reductase	Statin	[68–71]
Serine palmitoyltransferase	NA255	[81]
	Myriocin	[82]
IMP dehydrogenase	VX-497	[98]
	Ribavirin	[74,86]
	Mizoribin	[74]
	MPA	[97]
Protein arginin methyltransferase	AdoMet, Betaine	[102]
α-Glucosidase	Deoxynojirimycin	[101]
Cyclophilins	CsA	[27–36]
	NIM811	[27,33]
	DEBIO-025	[34]
HSP90	Geldanamycin, Radicicol	[37–39]
FKBP8		[38]
Unknown	PUFAs	[70,103,108]

FKBP8, a member of the FK506-binding protein family, specifically interacts with NS5A and forms a complex with HSP90 [38]. The La autoantigen (La) and polypyrimidine tract-binding protein (PTB) are also candidate cellular proteins for the inhibition of HCV RNA replication [40], although no inhibitors for these proteins have been reported to date. Thus, inhibition of the metabolism has recently been reported as a target of the new antivirals. Here, we survey the recent progress on enzyme inhibitors of the cholesterol, sphingolipid, and guanosine triphosphate (GTP) synthesis pathways, as well as other metabolic pathways.

2. Current standard therapy for chronic hepatitis C

HCV was discovered to be the causative agent of non-A, non-B hepatitis by the Chiron Corporation in 1989 [1]. However, a treatment for patients with non-A, non-B hepatitis was established before the discovery of HCV. In 1986, Hoofnagle et al. reported that IFN-α treatment normalized the serum alanine aminotransferase (ALT) levels in patients with non-A, non-B hepatitis [41]. Since the initial discovery of its anti-HCV activity, IFN-α has become the major reagent for CH–C treatment [4]. The replication of HCV RNA itself seems to stimulate IFN production signaling, and our recent results have suggested that core and/or NS5B induce IFN-stimulated genes [42–44]. However, viral NS3-4A protease inhibits the IFN production, although it does not completely shut it off. Therefore, exogenous IFN administration is needed for patients with CH–C. The SVR is affected by multiple factors, such as genotype, viral load and duration of therapy. IFN-α monotherapy was begun in the early 1990s, but an SVR was achieved in only about 10% of patients. In the early 2000s, IFN-α and ribavirin combination therapy was developed and the SVR was improved to about 30-40%. Furthermore, IFN itself has been modified by the attachment of PEG, thereby enhancing its stability in the blood. The SVR of the current standard therapy by PEG-IFN and ribavirin is as high as 50% [4]. In the current PEG-IFN and ribavirin combination therapy, the genotype of HCV is one of the major determinants of the SVR. HCV genotypes are classified into 6 groups, and genotype 1 is currently considered a problem due to its IFN resistance [45]. For example, in genotype 1 HCV, 12 months of treatment resulted in an SVR in 50% of patients, while in genotype 2, 6 months of treatment achieved an SVR of 80-90% [46]. The precise mechanisms of the IFN resistance remain unclear. However, the recently developed IFN-resistant HCV replicon-harboring cells will be useful for studies examining ways to improve the SVR [47–49]. Therefore, the focus in the treatment of patients with CH–C has shifted to increasing the SVR in genotype 1 HCV.

3. Cell culture-based HCV RNA-replication system

Before the development of an HCV replicon system, screening of anti-HCV reagents was rather difficult. The HCV replicon system developed by Lohmann et al. [5] was the first milestone in HCV study using a cell culture system. The replicon system has provided a wealth of information concerning the replication machinery of HCV. We can make strategies for the Achilles' heel of HCV based on the information regarding HCV RNA replication. The HCV replicon has been improved to be a suitable system for the screening of anti-HCV reagents by the introduction of reporter genes such as luciferase [50]. However, this system does not contain a structural region. Therefore, selectable genome-length HCV RNA-replicating cell culture systems have been developed [51–54]. The second milestone was the infectious virus production system established by the three groups using a genotype 2a HCV strain, JFH1 [6–8]. This system has extended the range of the HCV study to the viral entry and release. Therefore, the life cycle of HCV in the cells has been reconstructed *in vitro*. Since the development of the HCV replicon and infectious HCV production systems, many cellular proteins have been identified as essential host molecules for HCV RNA replication.

3.1. From HCV replicon to infectious HCV production

The HCV replicon reported by Lohmann et al. contained neomycin phosphotransferase (Neo) and encephalomyocarditis virus (EMCV) internal ribosome entry sites (IRES) instead of the HCV structural regions (Fig. 1) [5]. This HCV replicon consists of 2 cistrons. In the first cistron, Neo is translated by HCV-IRES and in the second cistron NS3-NS5B is translated by EMCV-IRES introduced in the region upstream of the NS region (Fig. 1). After the development of the HCV replicon system [52,53,55–58], genome-length HCV RNA replication systems using different HCV strains (H, N, Con1, and O) were developed by several groups [51–54]. In these genome-length HCV RNA replication systems, a complete open reading frame (ORF) of HCV was introduced into the second cistron instead of the NS region (Fig. 1).

For the mass screening for anti-HCV reagents, evaluation of the levels of HCV RNA or HCV proteins requires time and complicated procedures. To facilitate the monitoring of the replication level of HCV RNA, the reporter gene (Renilla luciferase) was fused to the Neo gene. In this system, anti-HCV activity was evaluated by the value of the reporter instead of the

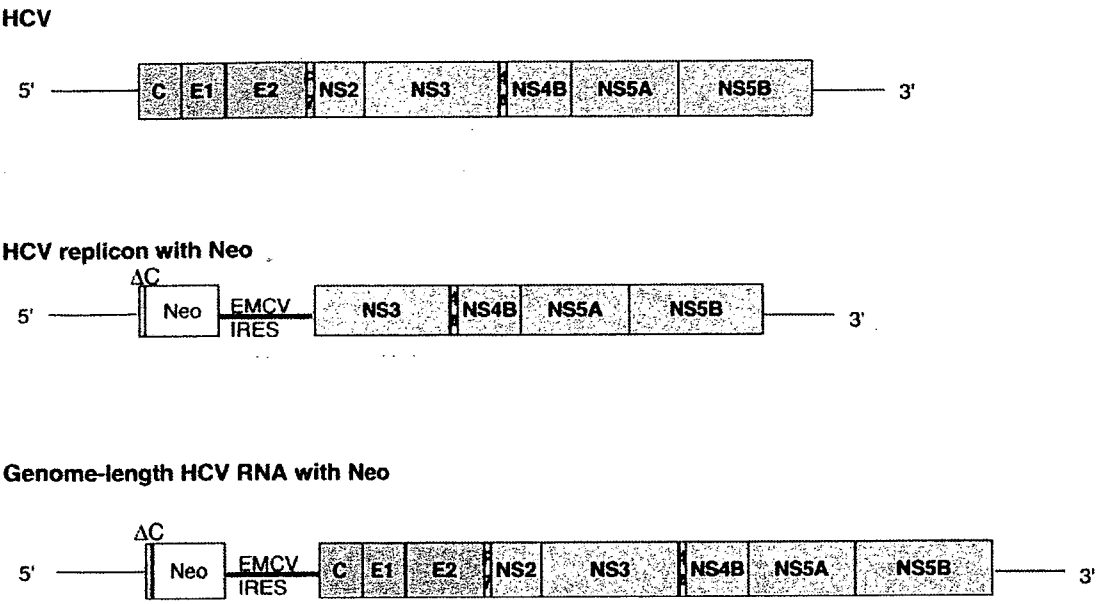

Fig. 1. Gene organizations of HCV and selectable HCVs. HCV ORF, untranslated regions, EMCV IRES, and Neo are depicted as shaded boxes, thin lines, thick lines, and open boxes, respectively. ΔC indicates the 12 N-terminal amino acid residues of the core as a part of IRES.

quantification of HCV RNA or HCV proteins. As shown in Fig. 2A, ORN/C-5B/KE contains the fused Renilla luciferase and Neo genes in the first cistron [51]. One of the cloned cell lines, OR6, was established by the G418 selection after introduction of ORN/C-5B/KE RNA into HuH-7 cells. HCV RNA and HCV proteins were stably expressed in the OR6 cells, and the Renilla luciferase activity was correlated well with the level of HCV RNA [51]. Therefore, the antiviral effect of the reagents on HCV RNA replication could be monitored by the activity of Renilla luciferase. The OR6 assay system

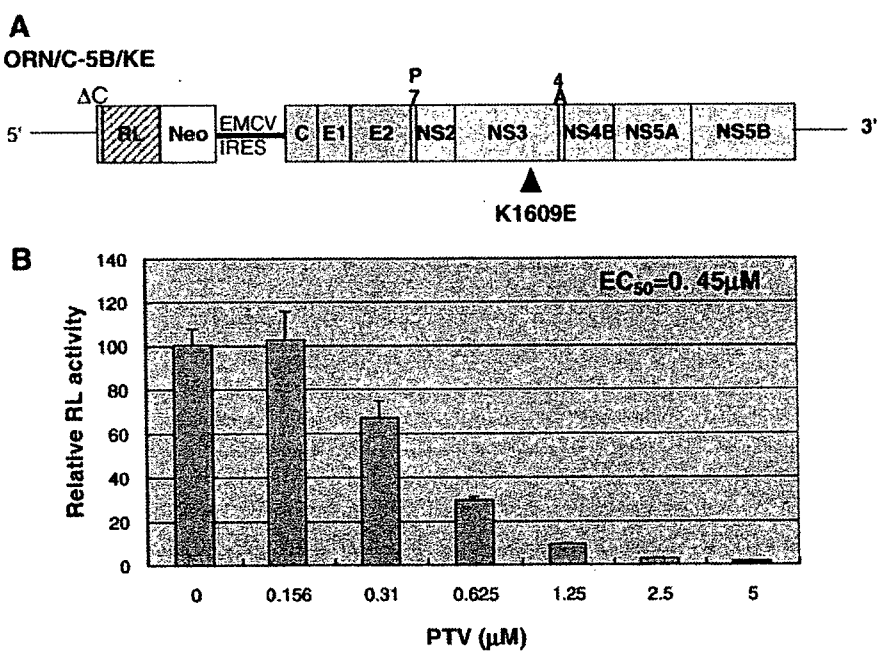

Fig. 2. Inhibitory effect of statin on HCV RNA replication in OR6 cells. (A) Schematic gene organization of genome-length HCV RNA (ORN/C-5B/KE) derived from genotype 1b, strain O. The Renilla luciferase gene, which is symbolized as RL, is depicted as a striped box and is expressed as a fusion protein with Neo. The adaptive mutation from lysine (K) to glutamine (E) at amino acids position 1609 was previously reported [51] and introduced into the genome-length HCV RNA. (B) Inhibition of HCV RNA by PTV. OR6 cells were cloned cell line selected by G418 [51]. OR6 cells were treated with PTV at a concentration of 0, 0.156, 0.31, 0.625, 1.25, 2.5, or 5 μM. After 72 hours of treatment the RL activities were determined. Shown here is the relative RL activity (%) calculated when the RL activity of untreated cells was assigned as 100%. The data indicate the means ± standard deviation from three independent experiments. The EC_{50} of PTV was determined as 0.45 μM.