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Suppression of hepatitis C virus replicon by TGF-β

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Abstract

Hepatitis C virus (HCV) is one of the major causative agents of liver diseases, such as liver inflammation, fibrosis, cirrhosis, and hepatocellular carcinoma. Using an efficient HCV subgenomic replicon system, we demonstrate that transforming growth factor-beta (TGF-β) suppresses viral RNA replication and protein expression from the HCV replicon. We further show that the anti-viral effect of this cytokine is associated with cellular growth arrest in a manner dependent on Smad signaling, not mitogen-activated protein kinase (MAPK) signaling. These results suggest a novel insight into the mechanisms of liver diseases caused by HCV.

Keywords: TGF-β; Hepatitis C virus; Replicon; Smad; MAPK

Introduction

Hepatitis C virus (HCV), a member of the *Flaviviridae* family, is an enveloped virus with a positive single-stranded 9.6-kb RNA genome (Murphy et al., 1995). The virus has been identified as the major causative agent of non-A, non-B hepatitis (Choo et al., 1989) that persistently infects several millions of people throughout the world. Although acute phase HCV infection is asymptomatic in most cases, the virus frequently establishes a persistent infection. This condition is associated with serious clinical diseases, including chronic hepatitis and liver fibrosis, which can lead to liver cirrhosis and eventually hepatocellular carcinoma (Goodman and Ishak, 1995).

Despite the clinical significance, molecular investigation of the virus has been hampered due to the lack of cell culture systems that efficiently support HCV replication. In 1999, the establishment of an HCV subgenomic replicon cell culture system (Lohmann et al., 1999) improved the situation. The subgenomic replicon RNA is composed of the HCV 5′-untranslated region (UTR) containing an internal ribosomal entry site (IRES), a neomycin phosphotransferase (*neo*) gene, the HCV nonstructural (NS) proteins 3 through 5B under the control of an encephalomyocarditis virus (EMCV) IRES, followed by the HCV 3′-UTR. The *neo* gene is expressed under the control of the HCV IRES, and thereby, gives the resistance to the cells in which replicon RNA exists. Instead of the *neo* gene, the luciferase gene can be used as a marker. Using the luciferase gene is beneficial in that it offers easy, speedy, and reliable detection. As the RNA replicates autonomously in cultured cells, this replicon system provides a unique tool for the analysis of the molecular mechanisms of HCV replication and the screening of anti-HCV compounds.

Transforming growth factor-beta (TGF-β) promotes the development of liver fibrosis and cirrhosis (Gressner et al., 2002); serum cytokine levels are associated with the severity of liver fibrosis in patients with chronic HCV (Nelson et al., 1997; Neuman et al., 2001; Tsushima et al., 1999). As high levels of TGF-β expression correlate with chronic hepatitis and cirrhosis (Calabrese et al., 2003; Shirai et al., 1994), cytokine serum concentrations serve as useful serologic markers for hepatitis, cirrhosis, and carcinoma (Song et al., 2002). Despite accumulating clinical observations, the direct effect of TGF-β on HCV replication remains unknown.

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Molecular biological analyses have revealed that the cytokine is a multifunctional cytokine that regulates multiple biological functions, including cellular growth inhibition, extracellular matrix (ECM) formation, apoptosis, and cell differentiation (reviewed in Derynck and Zhang, 2003; Miyazono et al., 2000). Following receptor ligation, the activation of receptor-regulated Smad (R-Smad, Smad2, and Smad3) enhances complex formation with the common-mediator Smad (Co-Smad, Smad4). These complexes translocate to the nucleus, where they directly regulate the transcription of various target genes. TGF-β receptor ligation also activates members of the mitogen-activated protein kinase (MAPK) family, including p38 MAPK, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK).

In this study, we demonstrate that TGF-β inhibits HCV RNA replication and viral protein expression using a HCV subgenomic replicon system. The anti-viral effect of TGF-β was associated with growth arrest of cells and the activation of Smad, not MAPK, signaling. Our results provide insight into the mechanisms of liver disease pathogenesis caused by HCV.

Results

Construction of a highly efficient and sensitive replicon system

Although we had previously developed subgenomic HCV replicon cell lines (Kishine et al., 2002), we desired a highly efficient replicon system to study the molecular mechanisms of HCV replication. Among the G418-resistant subgenomic replicon cell lines, we identified a replicon cell clone (MH14) in which the viral RNA levels were higher than those in other replicon cells (Miyanari et al., 2003). The amount of replicon RNA present in MH14 cells was approximately five times greater than that present in typical MH5 replicon cells (Fig. 1B). The production of NS5A protein in MH14 cells was also greater (Fig. 1C), suggesting efficient replication of the viral RNA. Sequence analysis of replicon RNA in the MH14 cells revealed two point mutations; S2204R, the replacement of the Ser residue at position 2204 with Arg, and a silent mutation L1882L in the NS4B coding region, which did not encode an amino acid substitution. The S2204R mutation corresponded to a previously reported adaptive mutation in NS5A (Lohmann et al., 2003). At least two forms of NS5A, p56 (basally phosphorylated form), and p58 (hyper-phosphorylated form), have been reported. Residue Ser-2204 is important for hyper-phosphorylation of the protein (Tanji et al., 1995). As expected, only the basally phosphorylated p56 form was detected and hyper-phosphorylated p58 was missing in the MH14 cells, while MH5 cells, which do not carry a mutation at the sequences liable for the hyper-phosphorylation, produce both the p56 and p58 forms of NS5A (Fig. 1C).

To test permissiveness of MH14 cells for HCV replication, cells were cured of the HCV replicon RNA by prolonged treatment with IFN-α,resulting in the curedMH14 line (Figs. 1B, C). MH5 replicon cells were treated with IFN-α in parallel, for use as controls. To examine permissiveness, cured cells were transfected with replicon RNAs in which the firefly luciferase gene was inserted (Fig. 1A). We, here, used luciferase gene as a marker since it is more convenient and has the sensitivity for better quantitation. Cells were harvested at various time points after transfection and cellular luciferase activities were measured subsequently (Figs. 1D–F). Luciferase activity in transfected cells reflects the replication of the replicon RNA. Polymerase-defective RNA replicon constructs, in which the catalytic GDD motif of the NS5B polymerase was substituted to the inactive GHD motif, were used as negative controls. When cells were transfected with the prototype NN replicon RNA, luciferase activity decreased rapidly 3 to 5 days after transfection (Figs. 1D–F, NN). Use of the MH14 RNA, which is identical to the prototype NN RNA with the exception of the L1882L and S2204R mutations, resulted in higher luciferase activities (Figs. 1D–F, MH14) than those observed in cells transfected with the NN RNA. For the curedMH14 cells, luciferase activity did not decrease (Fig. 1F, MH14), but increased, peaking 3 to 5 days after transfection, suggesting highly efficient replication.

We also tested the effect of the mutations and cured cell lines on G418-resistance transduction efficiencies (not shown) and confirmed that the numbers of G418-resistant colonies exhibited a similar trend as seen for the luciferase activities described above.

These results suggest that the curedMH14 cells were highly permissive for replication of RNA containing the adaptive mutations.

Furthermore, when curedMH14 cells were transfected with the highest efficiency replicon RNA, high luciferase activity persisted for greater than 1 month (data not shown) in the absence of selection.

Suppression of HCV replicon with luciferase by TGF-β

As we have constructed a highly efficient and sensitive replicon system using a luciferase reporter and curedMH14 cells, we used this system to screen anti-HCV compounds. Treatment for 3 days with IFN-α, IL-1β, or cyclosporin A reduced the observed luciferase activities to 3.8%, 9.5%, or 3.4% of control levels, respectively (Fig. 2A). As all three treatments have been reported to repress HCV replicon (Blight et al., 2000; Watashi et al., 2003; Zhu and Liu, 2003), the system is an effective method to screen for potential anti-HCV drugs. We also observed the suppressive effect of TGF-β on luciferase activity (Fig. 2A). While treatment with 2 ng/ml TGF-β (Fig. 2B, open circle) for 36 h had little effect on luciferase activity, enzymatic activity decreased to 11%, 12%, 10% that of the mock-treated cells (black circle) at 48, 60, and 72 h, respectively. To examine

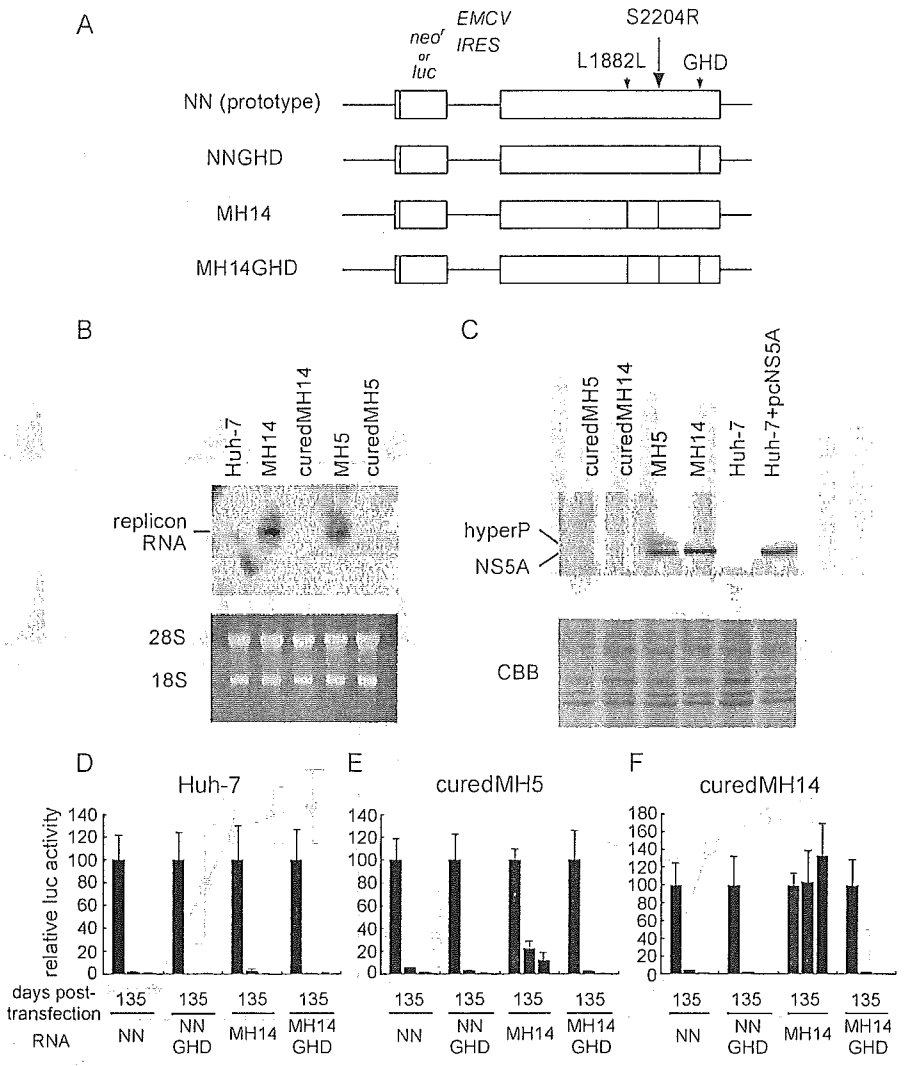


Fig. 1. Constructions and cells used in this study. (A) Schematic representation of the subgenomic replicon RNA constructs. The prototype NN RNA was used for transfection to obtain subgenomic replicon cell lines, including MH5 and MH14. NNGHD denotes polymerase-defective mutant, in which the catalytic GDD motif of the NS5B polymerase was substituted to the inactive GHD motif, and was used as a negative control. MH14 RNA carries two mutations, which were found in the replicon RNA in MH14 subgenomic replicon cells. MH14GHD is a negative control for the MH14 RNA. The ORFs are depicted as open boxes. The locations of the mutations introduced into the viral proteins are indicated by vertical lines. (B) Northern blot analysis of total RNA extracted from replicon cells. RNA from Huh-7, MH14, curedMH14, MH5, or curedMH5 cells was electrophoresed on denaturing agarose gels, blotted, and probed with an HCV RNA (upper panel). As an internal control, the ethidium bromide-staining pattern of 28S and 18S ribosomal RNA is shown (lower panel). (C) Western blot analysis of NS5A protein expressed in Huh-7, MH14, curedMH14, MH5, or curedMH5 cells (upper panel). As a control, wild-type NS5A protein was exogenously produced in Huh-7 cells from an expression plasmid (Huh-7 + pcNS5A). The position of the hyper-phosphorylated form of the protein is designated HyperP. As an internal control, the Coomassie brilliant blue (CBB) staining pattern of the same blot is shown (lower panel). (D–F) Replication of replicon RNAs with mutations in Huh-7 cells or its derivatives. Huh-7 (D), curedMH5 (E), or curedMH14 (F) cells were transfected with replicon RNA constructs containing the luciferase gene (depicted in A). Luciferase activity was measured at 1, 3, and 5 days after transfection. Each bar represents the mean and SD of three independent transfections.

the effect of TGF-β on luciferase expression and activity, the pCMV-Luc, in which the firefly luciferase gene is driven under the control of the CMV promoter, was used in Fig. 2C. While TGF-β treatment repressed the luciferase expression from replicon (Fig. 2B), we found that the cytokine enhanced the luciferase expression from the CMV promoter (Fig. 2C), provably because of the activation of transcriptional factors (Derynck and Zhang, 2003; Miyazono et al., 2000).

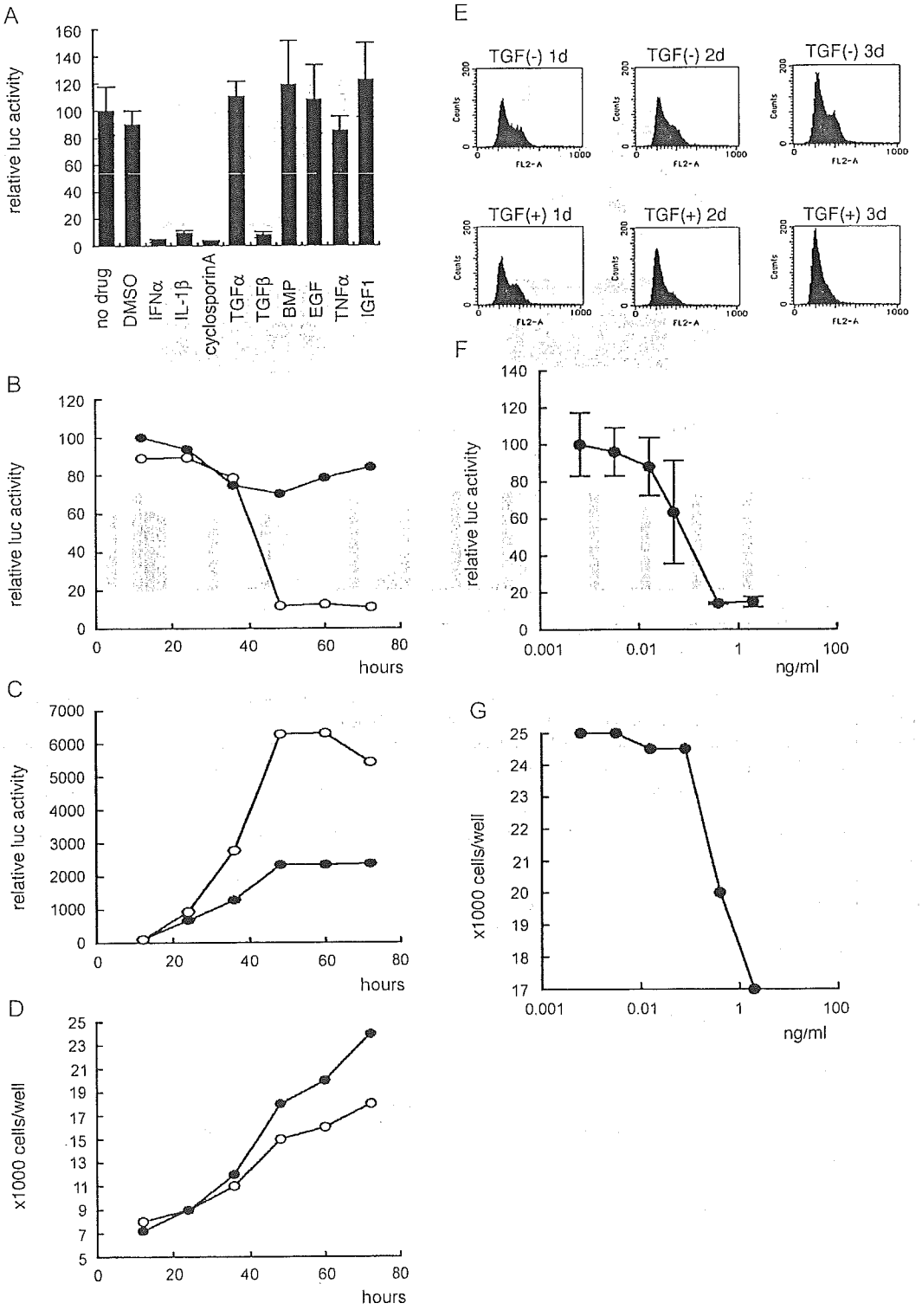
TGF-β is a multifunctional cytokine that exerts a range of biological activities, including cell growth inhibition (Derynck and Zhang, 2003; Miyazono et al., 2000). When exposed to TGF-β, cells generally arrest in the G(1)/S phase of the cell cycle. We, therefore, examined cell growth of curedMH14 cells (Fig. 2D). As expected, the growth of curedMH14 cells was partially inhibited by cytokine treatment (Fig. 2D, open circle). As assessed by FACS, administration of TGF-β for 2–3 days resulted in G(1)/S-arrest, while a 1-day treatment had no effect on cell cycle (Fig. 2E).

Fifty percent suppression of luciferase activity, after 3 days of treatment, was observed in the presence of

approximately 0.1 ng/ml TGF-β (Fig. 2F). Cell growth started to be suppressed at similar concentrations (Fig. 2G).

Smad-dependent suppression of HCV replicon

The cellular effects of TGF-β are mediated by both type I and type II serine/threonine kinase receptors. Receptor ligation is followed by the activation of both the canonical Smad and the MAPK signaling pathways, which include p38 MAPK, JNK, and ERK (Derynck and Zhang, 2003; Miyazono et al., 2000). To clarify the signaling pathways crucial for the suppression of HCV replication, we utilized two constitutive active receptors. One constitutively active TGF-β type-I receptor, TβR-I(T/D), evokes both Smad and



MAPK pathways, even in the absence of the TGF-β ligand (Imamura et al., 1997). The other constitutively active type-I receptor, TβR-ImL45(T/D), only activates the MAPK pathways, lacking the ability to excite Smad signaling (Yu et al., 2002). Three days after transfection of curedMH14 cells with the combination of the luciferase-replicon (LMH14) RNA and the TβR-I(T/D) expression plasmid, luciferase activity was reduced to 37% of the control levels, even in the absence of TGF-β (Fig. 3A). In contrast, co-transfection with TβR-ImL45(T/D) produces 71% of the control levels of luciferase activity, suggesting that the second constitutively active receptor had little effect on HCV replication (Fig. 3A). As summarized in Figs. 4B and C, the TβR-I(T/D) receptor likely activated transcription from promoters containing either AP-1-or Smad-responsive elements, while the TβR-ImL45(T/D) evoked transcription from the AP-1-dependent promoter alone. These data serve as evidence that the antiviral activity of TGF-β is dependent on Smad signaling.

Upon TGF-β stimulation, R-Smads specific for this cytokine, Smad2 and Smad3, are activated, forming complexes with the Co-Smad, Smad4, to activate transcription from the corresponding promoters. As co-expression of Smad2 with Smad4 or Smad3 with Smad4 mimics the effects of TGF-β stimulation (Yingling et al., 1997), we tested if such the co-expression of these molecules would also suppress HCV replicon production. Transfection of either Smad2/4 or Smad3/4 reduced the luciferase activity to 16% or 13%, respectively, even in the absence of TGF-β (Fig. 3A). Expression of an inhibitory-Smad (I-Smad), Smad7, which inhibits TGF-β mediated Smad signaling (Derynck and Zhang, 2003), only modestly reduced (81% of the control) the luciferase activity (Fig. 3A). The enhancement of transcription from AP-1-responsive (Fig. 3C), as well as TGF-β-responsive (Fig. 3B), promoters by co-transfection of either Smad2/4 or Smad3/4 was expected, as Smad4 itself has been reported to elicit transcription from AP-1 binding site-containing promoters (Liberati et al., 1999; Yingling et al., 1997).

To verify whether the Smad but not MAPK signaling is crucial for the suppression of HCV replicon by TGF-β,we used several specific MAPK inhibitors to examine if the antiviral effect of TGF-β is associated with the activation of specific MAPKs. The addition of inhibitors of ERK, p38, or JNK, U0126, SB20350, or dexamethasone, respectively, did not cancel TGF-β suppression of luciferase activity (Fig. 4A), despite effective inhibition of kinase activity by the inhibitors (Figs. 4B–D). The phosphorylation of ERK by TGF-β was not observed under these conditions (Fig. 4B), as ERK was already activated by growth factors contained within the bovine serum supplementing the culture medium.

Suppression of G418-resistant replicon by TGF-β

We then investigated the effect of TGF-β on G418-resistant replicon RNA or protein levels. MH14, G418-resistant subgenomic replicon cells, were treated with IFN-α, TGF-β, or BMP-4. BMP-4 was used here because it is a member of TGF-β superfamily cytokines and it does not induce inhibition of cell proliferation at least in Huh-7 cells. Total RNA and protein were collected at various time points. RNAs were subjected to Real-Time RT-PCR (Fig. 5A) or Northern blotting (Fig. 5B), while proteins were examined by Western blotting (Fig. 5C). Replicon RNA levels gradually decreased following treatment with 2 ng/ml TGF-β to 0.6% of the levels observed in mock-treated samples on the 7th day. This inhibition was similar to that seen following treatment with 100 IU/ml IFN-α. The NS5A protein was virtually undetectable by the 5th day after transfection (Fig. 5C). The suppressive effect of TGF-β on viral protein production was also observed by indirect immunofluorescence (not shown).

Because the replicon RNA of MH14 cells has the EMCV IRES to produce NS proteins, one could not deny the possibility that the EMCV IRES might cause the inhibition by TGF-β. Therefore, we next used full-genome replicon cell line SNC#2 (Fig. 6), which has the HCV IRES instead of the EMCV IRES, and tested if TGF-β would affect the replication. As shown in Fig. 6B, replicon RNA in SNC#2 cells treated with TGF-β decreased clearly while BMP-4 did not suppress the RNA levels.

Simultaneous suppression of viral RNA replication and protein synthesis by TGF-β

The suppression of HCV replication by TGF-β treatment was associated with the inhibition of cell growth. We examined the kinetics of the suppression of HCV replication

Fig. 2. Suppression of luciferase-replicon by TGF-β. (A) curedMH14 cells transfected with the luciferase-replicon RNA construct (LMH14), were administered with DMSO (0.1%), IFN-α (100 IU/ml), IL-1β (10 ng/ml), cyclosporin A (1 μg/ml), TGF-α (1 ng/ml), TGF-β (2 ng/ml), BMP-4 (10 ng/ml), mEGF (100 ng/ml), TNF-α (10 ng/ml), or IGF-1 (1 ng/ml). Three days later, cellular luciferase activity was measured. Bars represent the mean and SD of three independent experiments. (B) curedMH14 cells transfected with LMH14 luciferase-replicon RNA construct were mock-treated (black circle) or treated with TGF-β (2 ng/ml, white circle). At the indicated times, cells were harvested for determination of luciferase activity. The activity was normalized to cell number. (C) In parallel with the experiments in Fig. 3B, cells transfected with pCMV-Luc were mock-treated (black circle) or treated with TGF-β (2 ng/ml, white circle). At the indicated times, cells were harvested for determination of luciferase activity. The activity was normalized to cell number. (D) In parallel with the experiments in Fig. 3B, cells were mock-treated (black circle) or treated with TGF-β (2 ng/ml, white circle). Cell numbers were counted at the indicated time points. (E) Flow cytometric analysis of cell cycle progressing in curedMH14 cells transfected with the S2204R luciferase-replicon RNA construct. Cells were incubated in the presence or absence of TGF-β (2 ng/ml) for 1, 2, or 3 days. The DNA content of these cells was analyzed as described in Materials and methods. Dose-dependence of luciferase-replicon (F) and cell growth inhibition (G). curedMH14 cells transfected with the LMH14 luciferase-replicon RNA construct were treated with varying concentrations of TGF-β for 3 days. Luciferase activity (F) and cell number (G) were subsequently determined. The luciferase activity was normalized to cell number and shown with the SD value of three experiments in F.

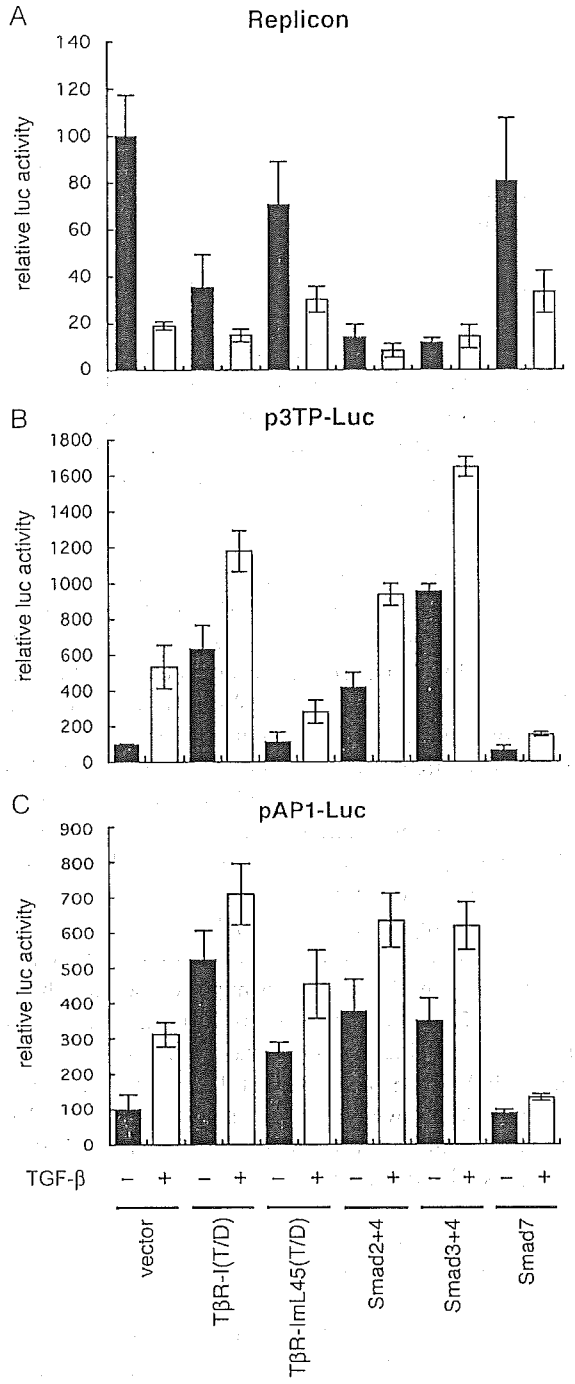


Fig. 3. Expression of TGF-β signaling-related proteins affected HCV replicon. curedMH14 cells were transfected with the expression plasmid designated below panel C together with (A) the luciferase-replicon construct with the mutations (LMH14), (B) p3TP-Luc, to monitor Smad-dependent transcription, or (C) pAP1-Luc, used to monitor AP-1-dependent transcription. Four hours after transfection, TGF-β (2 ng/ml) was added. Cells were incubated with or without the cytokine for 3 days (A) or 12 h (B, C) and then cellular luciferase activity was measured. The luciferase activity in A was normalized to cell number and then, the mean and SD value of three transfections are shown.

by examining viral RNA and protein synthesis rates at various times after the inoculation of replicon cells with the cytokine (Fig. 7). Aphidicolin and IFN-α were used as controls. The inhibition of viral protein synthesis by 100 IU/ml IFN-α began 12 h after treatment, while viral RNA synthesis was not affected until 24 h after cytokine addition. These results suggest that IFN-α first represses protein synthesis, thereby blocking RNA replication. In contrast, both aphidicolin and TGF-β inhibited protein synthesis and RNA replication concurrently, by 48 h after treatment. As both aphidicolin and TGF-β have growth inhibitory effects on cells, it is likely that both prevent HCV replicon in similar manners.

Anti-HCV activity of TGF-β was not mediated by IFN-induced signaling pathway

Although the antiviral activity of TGF-β was dependent on Smad signaling, the possibility remains that TGF-β may exert an antiviral activity via the same mechanisms as IFN-α and -γ. The binding of IFN-α or -γ to cellular receptors activates the JAK tyrosine kinase, which in turn phosphorylates effector Stat proteins. These proteins stimulate transcription from promoters with the specific sequences, ISRE or GAS, respectively. We prepared reporter plasmids that produce firefly luciferase following IFN-α or -γ stimulation by placing the gene under the control of a promoter containing either ISRE or GAS sequence. While 100 IU/ml IFN-α stimulation enhanced transcription from the ISRE-promoter 3.5-fold, enhancement of promoter activity was not observed following TGF-β treatment (Fig. 8A). The addition of 1000 IU/ml IFN-γ activated the GAS-dependent promoter by 5.8-fold, while TGF-β had little effect (Fig. 8B). The results suggest that TGF-β exerts its antiviral activity in a manner independent of IFN signaling.

Discussion

First, we have developed an efficient HCV subgenomic replicon system in this study. When maintained in cells, HCV replicon RNA often acquires cell culture-adaptive mutations. We found that the replicon RNA in MH14 cells carries two mutations, L1882L and S2204R. Among them, the S2204R, but not the L1882L, mutation was necessary and sufficient for the high efficiency (not shown). Although the mechanism by which the adaptive mutation produce high replication efficiency is not known, the interaction of the NS5A protein with a co-factor, such as hVAP-A (Gao et al., 2004; Tu et al., 1999), might explain the phenomenon.

As cytokines can play major roles in pathogenetic process during the courses of viral diseases, the relationships between viruses and cytokines, such as TGF-β, are of great importance. In this study, we found that TGF-β inhibits viral RNA replication and protein expression in the HCV replicon system.

The mechanism by which IFN-α suppresses HCV replicon is not well understood. Here, we showed that

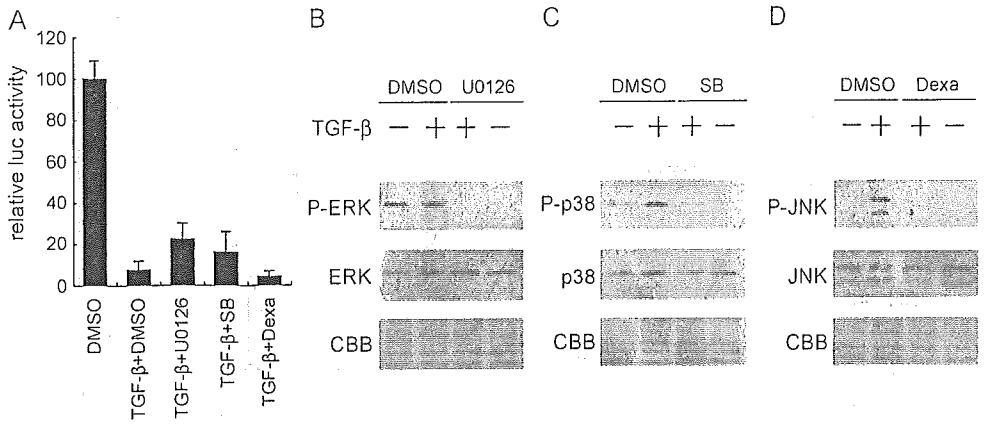


Fig. 4. Effect of MAPKs on the HCV replicon. (A) curedMH14 cells transfected with the luciferase-replicon construct (LMH14) were treated with DMSO, TGF-β (2 ng/ml) plus DMSO, or TGF-β (2 ng/ml) with either U0126 (3 μM), SB203580 (30 μM), or dexamethasone (1 μM). Inhibitors were added 1 h prior to the addition of TGF-β . After a 3-day treatment, cellular luciferase activity was measured. The mean and SD of three independent transfections are depicted after normalization to cell number. (B–D) The effect of inhibitors on the phosphorylation of ERK (B), p38MAPK (C), and JNK (D). Western blotting examined the phosphorylation of these molecules in cells treated with or without the designated reagents. The upper panels depict phosphorylated MAPKs, while the middle panels display the total amount of the MAPKs. CBB staining pattern of the same blot is used as a loading control (lower panel).

IFN-α repressed protein synthesis first, then suppressed RNA replication of the HCV replicon (Fig. 7). These data seem to support the previous report (Guo et al., 2004) and may aid our understanding of the suppression mechanism by IFN-α. IFN-α might suppress HCV translation trough the La (Shimazaki et al., 2002)-, ISG56 (Sumpter et al., 2004) or PKR (Wang et al., 2003)-dependent manner.

In contrast, either aphidicolin or TGF-β simultaneously inhibited both protein synthesis. and RNA replication from the replicon de novo (Fig. 7). While the mechanism of simultaneous suppression by aphidicolin or TGF-β remains unknown, both reagents arrest cell cycle progression at G(1)/S, suggesting a common target in the repression of HCV replicon expression. Recently, Scholle et al. (2004) reported that the replication of the HCV replicon RNA depends on host cell growth. Our results clearly correspond with that report, in which viral RNA levels remained unchanged during a 24-h period of cell cycle arrest followed by drops by 48 h.

It has been demonstrated that cell cycle arrest by TGF-β is mainly caused in the Smad pathway-dependent manner and the MAPK signaling serves as an accessory modifier of the arrest (Ten Dijke et al., 2002). Therefore, our result is convincing in that Smad, but not the MAPK pathway, played an essential role in the suppression of HCV replication.

Broadly speaking, serological investigations of HCV in chronically infected patients imply an inverse relationship between viral RNA load and TGF-β levels. Increased TGF-β expression significantly correlates with the degree of hepatic fibrosis (Calabrese et al., 2003; Nelson et al., 1997). A 3-year follow-up study demonstrated that TGF-β levels were elevated in patients with fibrosis that was

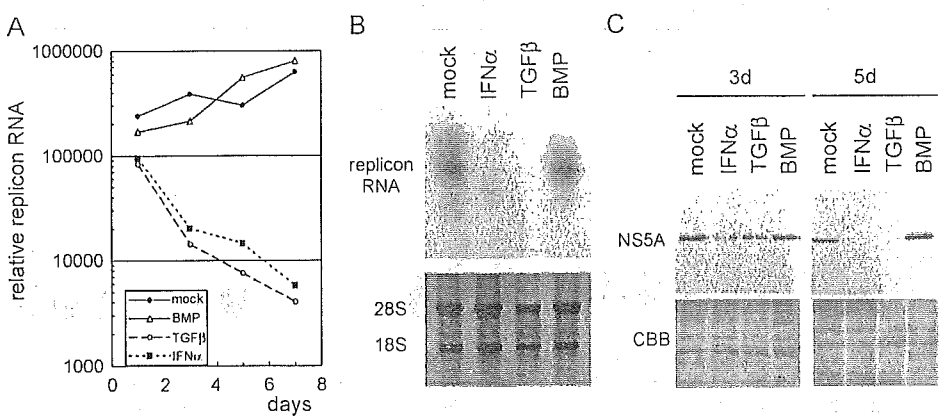


Fig. 5. Suppression of G418-resistant subgenomic replicon by TGF-β. (A) MH14, a G418-resistant subgenomic replicon cell line, was mock-treated or treated with BMP-4 (10 ng/ml), TGF-β (2 ng/ml), or IFN-α (100 IU/ml) for 1, 3, 5, or 7 days. Following the extraction of total RNA, the quantity of HCV replicon RNA was determined by real-time RT-PCR analysis. (B) Total RNA was also subjected to Northern blot analysis (upper panel). The ethidium bromide-staining pattern of ribosomal RNA is shown as an internal control (lower panel). (C) Total protein from cells prepared as above was harvested after either 3 or 5 days of cytokine treatment. Western blot analysis was performed using an antibody against NS5A. CBB staining pattern of the same blot is shown as a loading control (lower panel).

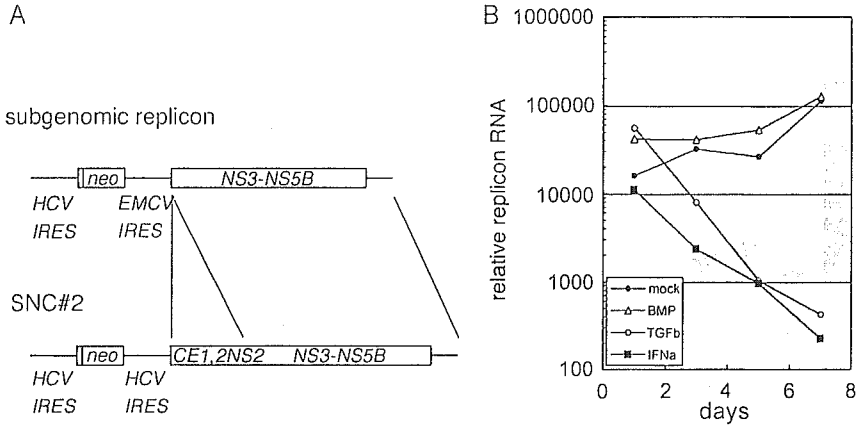


Fig. 6. Suppression of G418-resistant replicon without EMCV IRES by TGF-β. (A) Schematic representation of an RNA construct in typical subgenomic replicon cells (upper) and that in SNC#2 replicon cells (lower). RNA in SNC#2 carries the sequence for the whole HCV ORFs, driven by the HCV IRES instead of the EMCV IRES. The ORFs are depicted as open boxes. (B) SNC#2 cells were mock-treated or treated with BMP-4 (10 ng/ml), TGF-β (2 ng/ml), or IFN-α (100 IU/ml) for 1, 3, 5, or 7 days. Following the extraction of total RNA, the quantity of HCV replicon RNA was determined by real-time RT-PCR analysis.

increasing in severity (Neuman et al., 2002), which correlated with lower levels of viremia in patients than those with less progressed fibrosis (Adinolfi et al., 2001). These reports suggest that the presence of TGF-β, which may be induced by HCV core protein (Taniguchi et al., 2004), has a suppressive influence on viral RNA load. Gewaltig et al. (2002) demonstrated that polymorphisms in the TGF-β gene were associated with progression of HCV-induced liver fibrosis, suggesting again that the cytokine and the cytokine signaling have a certain influence on the virus.

The precise molecular mechanism of the anti-HCV activity of TGF-β remains to be clarified. Additional studies, including clinical studies, may reveal a novel mechanism of HCV replication regulation, potentially providing a target for novel anti-HCV therapies in the future.

Materials and methods

Cell culture, antibodies, and reagents

Huh-7, curedMH5, and curedMH14 cells were maintained in Dulbecco's modified Eagle medium (Gibco BRL) supplemented with 10% fetal bovine serum, 100 units/ml nonessential amino acids (Invitrogen), and 100 μg/ml of both penicillin and streptomycin sulfate (Invitrogen). MH5, MH14, and SNC#2 replicon cells were cultured in the above medium supplemented with 300 μg/ml G418 (Geneticin, Invitrogen). Cured cells were prepared by treating cells with 5000 IU/ml of IFN-α for 2 weeks. Absence of replicon RNA and viral proteins was checked by Northern blotting, Western blotting, and RT-PCR.

Rabbit antisera raised against p38, JNK, ERK, and phospho-ERK were purchased from Cell Signaling Tech-

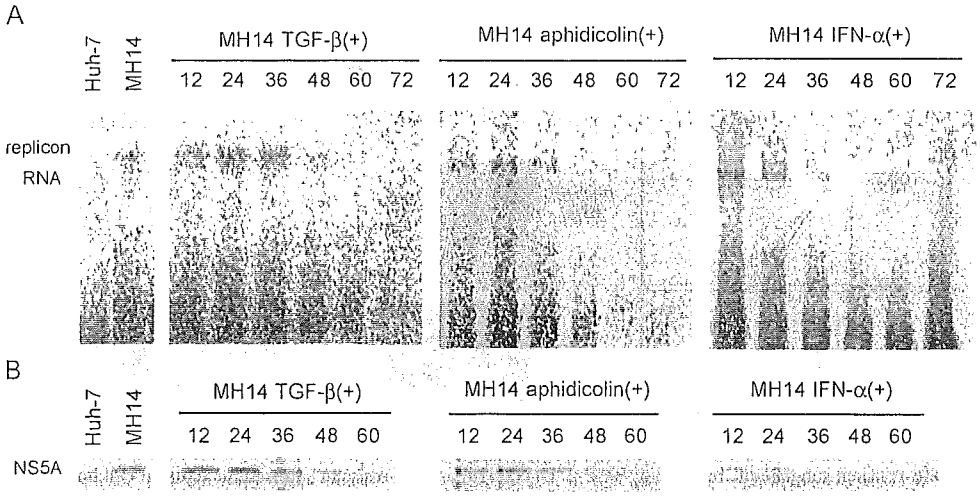


Fig. 7. Simultaneous suppression of viral RNA replication and protein synthesis by TGF-β. (A, B) MH14, a G418-resistant subgenomic replicon cell line, was mock-treated or treated with TGF-β (2 ng/ml), aphidicolin (5 μg/ml), or IFN-α (100 IU/ml) for 12, 24, 36, 48, 60, or 72 h. Cells were then subjected to semi-intact replication assay (A) or [^{35}S]-methionine metabolic labeling and immunoprecipitation using an anti-NS5A antibody (B) as described in Materials and methods.

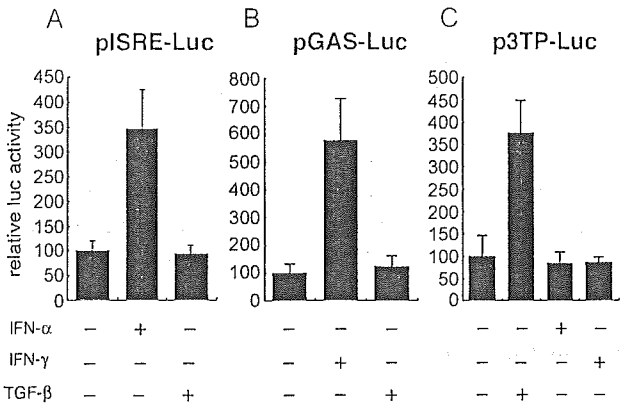


Fig. 8. TGF-β exerts an anti-HCV activity independent of the signal transduction pathway activated by IFN-α or -γ. curedMH14 cells were transfected with either pISRE-Luc (A), pGAS-Luc (B) or p3TP-Luc (C) and treated with TGF-β (2 ng/ml), IFN- (100 IU/ml), or IFN-γ (1000 IU/ml). Luciferase activities were measured 12 h after transfection. Data represent the means and SD values of relative luciferase activities in three independent experiments.

nology. Mouse antibodies specific for phospho-p38 and phospho-JNK were acquired from BD Biosciences and SIGMA, respectively. Horseradish peroxidase-conjugated goat antibodies to mouse and rabbit IgG were procured from Amersham Biosciences. TGF-β, BMP-4, and SB203580 were obtained commercially from Calbiochem. U0126 and Dexamethasone were purchased from SIGMA.

Northern and Western blot analysis

RNA was extracted from cells using Sepasol RNAI super reagent (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol. Northern blot analysis was performed as described (Kishine et al., 2002). The 1.5-kb *Eco*RI fragment of pNNRZ2 was used, which corresponds to the C-terminal half of the NS5A gene and the N-terminal half of the NS5B gene as a probe.

Plasmid construction

pNNRZ2 was used to prepare G418-resistant prototype NN replicon RNA (Kishine et al., 2002). To generate pMH14, L1882L and S2204R mutations in NS4B and NS5A were inserted into pNNRRZ2 by PCR-based site-directed mutagenesis. The following primers were used for mutagenesis: 5′-CTGGTCAATCTACTTCCTGCC-3′ and 5′-GGCAGGAAGTAGATTGACCAG-3′ (Bold letters in the primers denote the substituted nucleotides for L1882L). 5′-CTTCAGCTAGACAGTTGTCTGC-3′ and 5′-GCAGACAACTGTCTAGCTGAAG-3′ (same for S2204R). In addition, 5′-CACCCAAATGTACACC-AATG-3′ and 5′-CGATCCTCATGGAACCGTTC-3′, 5′-GAACGGTTCCATGAGGATCG-3′, and 5′-TGATGGG-CAGCTTGCTTTCC-3′ were used for amplification of appropriate fragments. The *neo* genes in pNNRZ2 and pMH14 were replaced with the luciferase gene from the pGL3 vector (Promega, Tokyo, Japan) to create pLNNRZ2 and pLMH14. To prepare the NS5B (RNA polymerase)-defective luciferase-replicon constructs, we inserted a GHD motif into either pLNNRZ2 or pLMH14 by replacing the corresponding sequence with pNNRZ2GHD (Kishine et al., 2002) to create pLNNRZ2GHD or pLMH14GHD, respectively. pSNC was generated to prepare G418-resistant full-genome replicon cell line, SNC#2. To prepare the plasmid, the sequence from NS3 to the end of the NS5B was cloned from I377NS3-3′ (Lohmann et al., 1999) with the S2204I adaptive mutation and other parts were from pM1LE (Kishine et al., 2002).

The coding region for NS5A in the pNNRZ2 plasmid was cloned into the *Sma*I site of pCALNL5/pBR (kindly provided by Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science). The neomycin-resistance gene was removed by *Xho*I digestion to prepare the expression vector pcNS5A.

pcDNA3 (Invitrogen)-based plasmids expressing FLAG-tagged human Smad2 or Smad4, HA-tagged constitutively active TGF-β type I receptor (TβR-I[T/D]), and p3TP-Luc have been described previously (Ohshima and Shimotohno, 2003; Imamura et al., 1997). The combined mutant type I receptor, R-ImL45(T/D), which possesses a constitutively active kinase domain, but lacks the ability to phosphorylate Smad, was generated by PCR-based mutagenesis as described (Ohshima and Shimotohno, 2003; Yu et al., 2002). The pAP1-Luc reporter plasmid was obtained commercially (PathDetect Reporter System; Stratagene, LaJolla, CA). Two additional reporter plasmids, pISRE (IFN-α-stimulated response element)-Luc and pGAS (gamma activation site)-Luc, were based on pGL3-Promoter Vector (Promega, Tokyo, Japan) that contains the SV40 basal promoter sequence without an enhancer sequence. To create pISRE-Luc, the ISRE consensus sequence (ACTTT-CAGTTTCAT) was repeated five times in tandem and inserted between the *Mlu*I and *Xho*I cloning sites of the pGL3-Promoter vector. For pGAS-Luc, three tandem repeats of the GAS sequence (TTTCCCCGAAA) were cloned into the pGL3-Promoter Vector at the *Kpn*I–*Bgl*II cleavage site.

RNA synthesis

HCV subgenomic RNA was transcribed in vitro using a MEGAscript T7 kit (Ambion) according to the manufacturer's instructions. Following DNase treatment, RNA was purified by lithium chloride precipitation.

Transfection and luciferase assay

For the luciferase assay to monitor luciferase-replicon, curedMH14 or other cells seeded on 48-well plate (5×10^3 cells/well) were transfected with 0.25 μg of the luciferase-replicon RNA using DMRIE-C reagent (Invitrogen) according to the manufacturer's instructions. Proteins in cells were

extracted in a lysis buffer supplied in a Dual-Luciferase Reporter Assay System (Promega) kit and the luciferase activity was measured using the kit. Assays were performed in triplicate; standard deviations are denoted by bars in the figures. Plasmid DNA was transfected into cells using FuGENE6 reagent (Roche).

Real time RT-PCR analysis

To monitor the effect of cytokines on neo-resistant replicon RNA, TGF-β, IFN-α, or BMP-4 was added in the media of replicon cells seeded on 6-well plate (4 × 10 4 cells/well). At various times, total cellular RNA was collected and subjected to Real time RT-PCR analysis. The 5′-UTR of HCV genomic RNA was quantified using the ABI PRISM 7700 sequence detector (Applied Biosystems) as described (Watashi et al., 2003) using the 5′-CGGGAGAGCCATAGTGG-3′ (forward) and 5′-AGTACCACAAGGCCTTTCG-3′ (reverse) primers and the fluorogenic probe 5′-CTGCGGAACCGGTGAGTACAC-3′. As an internal control, ribosomal RNA was quantified using TaqMan ribosomal RNA control reagents (Applied Biosystems).

Flow cytometry analysis Cells were trypsinized, fixed in formaldehyde, washed with PBS, and treated with staining solution containing 50 μg/ml propidium iodide (PI) (Sigma), 50 μg/ml of RNaseA (Wako), and 0.1% Triton X-100 (nacalai tesque) in PBS for 15 min. PI fluorescence was analyzed using a FACScalibur flow cytometer (Becton Dickinson). Twenty thousand events were collected and analyzed using CellQuest software (Becton Dickinson).

Semi-intact cell replication assay

A semi-intact cell replication assay was performed as described (Miyanari et al., 2003). In brief, cells were permeabilized by incubation in reaction buffer containing digitonin. Following two washes, samples were incubated for 4 h in the labeling reaction mixture containing 10 μCi of [^{32}P]UTP (Amersham Biosciences) in reaction buffer at 27 °C. Total cellular RNA was collected and fractionated by denaturing agarose gel electrophoresis. Radioactivity incorporated into newly synthesized replicon RNA was visualized using a Fujix Bio-Imaging Analyzer BAS2000 System (Fuji Photo Film, Japan).

Metabolic labeling and immunoprecipitation of NS5A protein

Cells were washed in PBS and incubated in methionine-free DMEM (ICN biomedicals) containing 10% dialyzed FBS and 100 μC i/ml of [^{35}S]-methionine (Tran 35S label, ICN biomedicals) for 4 h. After washing in PBS, cells were lysed in RIPA buffer (10 mM Tris–HCl pH 7.4, 1% NP40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, and 1 mM EDTA) for 1 h. Cellular debris was removed by centrifugation at 15,000 rpm for 10 min. Following preclearing, lysates were incubated with a monoclonal antibody against NS5A in the presence of protein-G sepharose. After extensive washing, immune complexes were recovered by low-speed centrifugation and subjected to SDS-PAGE. Radioactivity incorporated into newly synthesized NS5A protein was visualized using a Fujix Bio-Imaging Analyzer BAS2000 System (Fuji Photo Film, Japan).

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Short Communication

Interferon resistance of hepatitis C virus replicon-harbouring cells is caused by functional disruption of type I interferon receptors

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Hepatitis C virus (HCV) replicon-harbouring cell lines possessing interferon (IFN)-resistant phenotypes have recently been established. These were divided into two classes; partially IFN resistant and highly IFN resistant. Here, the viral and cellular factors contributing to the IFN resistance of HCV replicon-harbouring cells were evaluated. The results revealed that cellular factors rather than viral factors contributed to a highly IFN-resistant phenotype. The possibility of genetic abnormality of the factors involved in IFN signalling was investigated. As a result, nonsense mutations and deletions in type I IFN receptor genes (IFNAR1 and IFNAR2c) were found in replicon-harbouring cells showing a highly IFN-resistant phenotype, but rarely appeared in cells showing a partially IFN-resistant phenotype. Furthermore, similar genetic alterations were also found in IFN-resistant phenotype, replicon-harbouring cell lines obtained additionally by IFN-β treatment. Moreover, it was shown that ectopic expression of wild-type IFNAR1 in IFN-resistant phenotype, replicon-harbouring cells possessing the IFNAR1 mutant restored type I IFN signalling.

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Persistent infection by hepatitis C virus (HCV) is a major cause of chronic hepatitis (Choo *et al.*, 1989; Kuo *et al.*, 1989), which can progress to liver cirrhosis and hepatocellular carcinoma (Saito *et al.*, 1990). Since at least 170 million people are currently infected with HCV worldwide, this infection constitutes a global health problem (Thomas, 2000). HCV is an enveloped RNA virus belonging to the family *Flaviviridae*, the genome of which consists of a positive-stranded RNA encoding an approximately 3000 aa polyprotein precursor (Kato *et al.*, 1990). This precursor protein is processed by the host and viral proteases to generate at least 10 proteins in the following order: core, envelope 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B (Kato, 2001).

Since 1998, combined treatment with interferon (IFN)-α and ribavirin has been standard clinical therapy for patients with chronic hepatitis C; however, the effectiveness of IFN is limited to approximately 50% (Hadziyannis *et al.*, 2004). This clinical result suggests that HCV directly or indirectly attenuates the antiviral actions of IFN (Pawlotsky, 2000).

Although an HCV replicon system carrying autonomously replicating HCV subgenomic RNA containing the NS3–NS5B regions (Lohmann *et al.*, 1999) was considered to be useful in studies on the mechanism(s) of IFN resistance of HCV, all HCV replicons established to date have been highly sensitive to IFN-α, -β and -γ (Frese *et al.*, 2001, 2002; Kato *et al.*, 2003). This seems to contradict the fact that half of the patients with chronic hepatitis C are resistant to current IFN therapy. Therefore, we assumed that prolonged IFN treatment might change HCV replicons from an IFN-sensitive phenotype to an IFN-resistant phenotype.

Based on this assumption, we recently established nine HCV replicon cell lines possessing two IFN-resistant phenotypes: a partially resistant phenotype (αR series: 1αR, 3αR, 4αR, 5αR and αRmix) and a highly resistant phenotype (βR series 1βR, 3βR, 4βR and 5βR) obtained by IFN-α and -β treatment, respectively (Namba *et al.*, 2004). Genetic analysis of these replicons found one common amino acid substitution (Q1737H) in the NS4B region and several additional amino acid substitutions (such as M2174V and T2242N) in the NS5A region of the βR series (Namba *et al.*, 2004). To examine which viral and cellular factors contribute to the IFN resistance of HCV replicons, we evaluated the IFN sensitivity of replicon-harbouring cells (6M.m/6Mc, 1βR.m/6Mc and 4βR.m/6Mc) established by transfection of total RNAs isolated from an IFN-sensitive clone (6M) and from highly IFN-resistant clones (1βR and 4βR) into cured 6Mc cells, from which 50-1 replicons (Kishine *et al.*, 2002) had been eliminated by IFN-γ treatment (500 IU ml^{-1} for

Supplementary material is available in JGV Online.

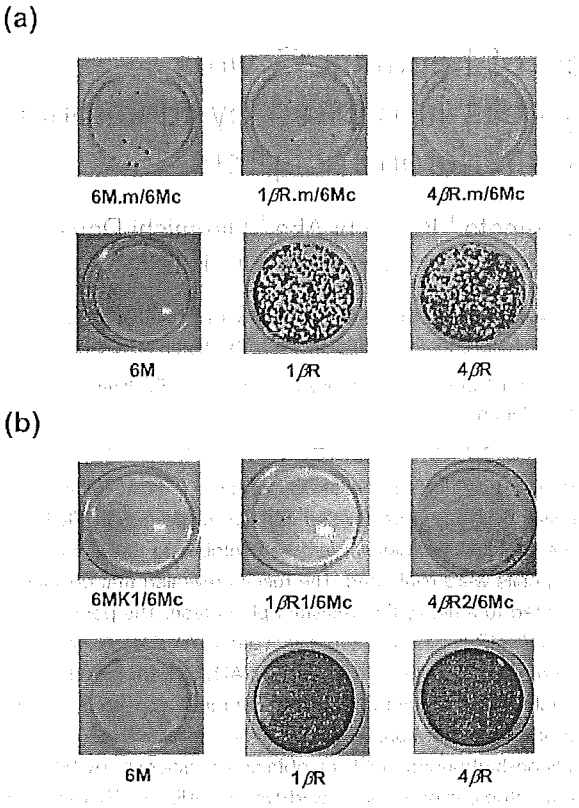

Fig. 1. IFN sensitivities of various HCV replicon-harbouring cells. (a) 6M.m/6Mc, 1βR.m/6Mc and 4βR.m/6Mc cells obtained as G418-resistant mixed colonies were treated with IFN-α (400 IU ml^{-1}) for 3 weeks in the presence of G418 (300 μg ml^{-1}). 6M, 1βR and 4βR cells were also used for control experiments. G418-resistant colonies were stained with Coomassie brilliant blue as described previously (Naganuma *et al.*, 2004). (b) IFN sensitivities of 6MK1/6Mc, 1βR1/6Mc and 4βR2/6Mc cells were examined as described in (a) except that they were treated with IFN-β (200 IU ml^{-1}).

3 weeks). Although many colonies were found to have survived in 1βR and 4βR cells after IFN-α treatment, only a few colonies survived in 1βR.m/6Mc and 4βR.m/6Mc cells or in 6M and 6M.m/6Mc cells (Fig. 1a). Similar results were obtained when these replicon-harbouring cells were treated with IFN-β (400 IU ml^{-1}) (data not shown), although two colonies (named 6βR and 7βR, described below) derived from 6M.m/6Mc and 4βR.m/6Mc cells, respectively, proliferated as highly IFN-resistant clones.

We further examined the IFN sensitivity of replicon-harbouring cells (6MK1/6Mc, 1βR1/6Mc and 4βR2/6Mc) established by the transfection of *in vitro*-synthesized replicon RNAs (6MK1, 1βR1 and 4βR2 obtained from 6M, 1βR and 4βR cells, respectively) (Namba *et al.*, 2004; Kato *et al.*, 2005) into 6Mc cells. The results revealed that few or no colonies survived in 1βR1/6Mc and 4βR2/6Mc cells, as in 6M and 6MK1/6Mc cells, whereas many colonies survived in 1βR and 4βR cells (Fig. 1b). These results suggested that cellular factors rather than viral factors contributed to the highly IFN-resistant phenotype of HCV replicon-harbouring cells. However, the present results obtained under a high concentration of IFN do not necessarily rule out a possible role for HCV mutations in conferring low degrees of IFN resistance, because effects of HCV mutations on IFN activity are presumably weaker than those of the cellular factors.

To obtain evidence in favour of the idea that alterations in cellular factor(s) are involved in the emergence of an IFN-resistant phenotype, we attempted to prepare cured cells from the replicon-harbouring cells possessing a highly IFN-resistant phenotype. Since phosphorylation of signal transducer and activator of transcription 1 (STAT1) occurred in the 1βR and 4βR cells treated with IFN-γ (Fig. 2a), these replicon-harbouring cells were treated with IFN-γ (500 IU ml^{-1}) for 3 weeks, and cured 1βRc and 4βRc cells were obtained. Western blot (Fig. 2b) and RT-PCR (data not shown) analyses showed that no replicons were detected in either type of cured cells. Analysis of a luciferase reporter assay indicated that the complete defect of the IFN-α/β signalling was not restored in the cured 1βRc and 4βRc cells (Fig. 2c).

To clarify whether or not the signalling defect in these replicon-harbouring cells was restricted to type I IFN, we examined the phosphorylation status of STAT3 in 6M, 1βR and 4βR cells treated with interleukin-6 (IL6). Since it has been reported that STAT3 is also activated by IFN-α treatment (Pfeffer *et al.*, 1997), the phosphorylation status of STAT3 in these replicon-harbouring cells after IFN-α treatment was also examined. Our results revealed that STAT3 was not phosphorylated in 1βR and 4βR cells treated with IFN-α, while phosphorylation of STAT3 was observed in 6M, 1βR and 4βR cells treated with IL6 and in 6M cells treated with IFN-α (Fig. 2d), indicating that only type I IFN signalling was defective in 1βR and 4βR cells. These results suggested that the initial reaction following the addition of IFN-α/β was defective in replicon-harbouring cells possessing a highly IFN-resistant phenotype.

Following up this suggestion, we examined the genetic status of tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1). Sequence analysis of TYK2 and JAK1 cDNAs obtained from 1βR and 4βR cells was performed after cloning into the pCXbsr vector (Akagi *et al.*, 2000), as described previously (Nozaki *et al.*, 2003). However, the results showed no mutations in these cDNAs (data not shown). We next focused on type I IFN receptors (IFNAR1 and IFNAR2c). Our results showed that the mRNA levels of the two receptors were almost equal among all examined replicon-harbouring cells including 6Mc cells (see Supplementary Fig. S1a, available in JGV Online). More than three independent clones of each cDNA (1708 bp for IFNAR1 and 1582 bp for IFNAR2c) were sequenced as described above. Table 1 shows a summary of sequence analysis of IFNAR1

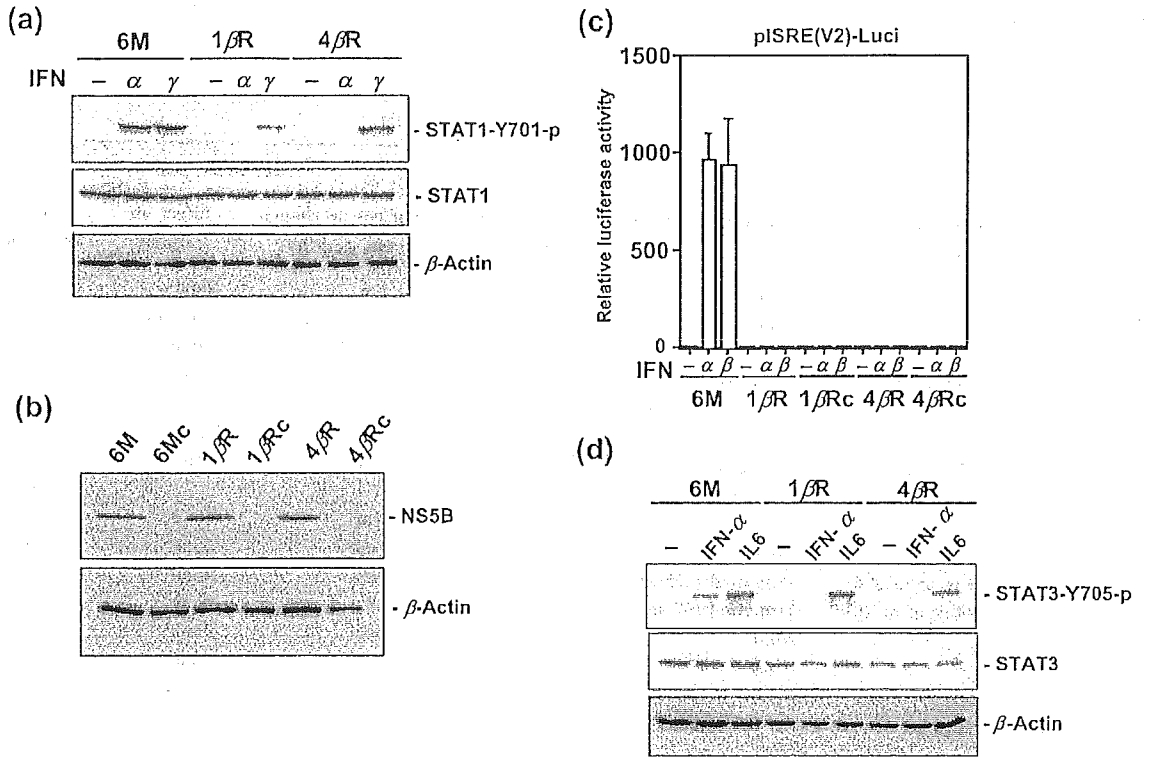

Fig. 2. Cellular factors rather than HCV replicons contribute to defects in type I IFN signalling. (a) Western blot analysis of STAT1 in 6M, 1βR and 4βR cells treated with IFN-α or -γ. The replicon-harbouring cells were treated with or without IFN-α or -γ (500 IU ml^{-1} each) for 30 min, and Western blot analysis for STAT1 and its phosphorylation status (Tyr-701) was then performed as previously described (Hijikata *et al.*, 1993). β-Actin was used as a control for the amount of protein loaded per lane. (b) Western blot analysis of NS5B. Anti-NS5B antibody was used for immunoblotting. β-Actin was used as described in (a). (c) Analysis of IFN signal transduction. Dual luciferase assays using pISRE(V2)-Luci (Dansako *et al.*, 2003) were performed as previously described (Naganuma *et al.*, 2000). Cells were treated with IFN-α or -β (500 IU ml^{-1} each) for 6 h. (d) The defect in the signalling pathway in 1βR and 4βR cells is restricted to type I IFN. Replicon-harbouring cells were left untreated or treated with IFN-α (500 IU ml^{-1}) or IL6 (100 ng ml^{-1}) for 30 min. Western blot analysis for STAT3 and its phosphorylation status (Tyr-705) was performed as described in (a).

and IFNAR2c mRNAs. Surprisingly, we found that nt 475 of IFNAR2c mRNA from 1βR cells had a U substituted for G in 13/13 clones, resulting in a nonsense mutation at codon 159 from glutamic acid (GAG) to the termination codon UAG (see Supplementary Fig. S1b). Furthermore, nt 319 of IFNAR1 mRNA from 4βR cells was also found to have a U substituted for G in 12/12 clones, resulting in a nonsense mutation at codon 107 from glutamic acid (GAA) to the termination codon UAA (see Supplementary Fig. S1c). However, interestingly, IFNAR1 and IFNAR2c mRNAs obtained from 1αR and 4αR cells, which were derived from clone 1 and clone 4 and were counterparts of 1βR and 4βR cells, respectively (see Supplementary Fig. S2, available in JGV Online), did not possess the nonsense mutations found in the mRNAs from 1βR and 4βR cells. In addition, several kinds of deletion and another nonsense mutation (lysine to a termination codon at codon 458) were found in approximately half of IFNAR1 cDNA clones obtained from 3βR and 5βR cells. In contrast to the finding of frequent mutations and deletions in IFNAR mRNAs from the βR series, such genetic abnormalities in IFN receptors were quite rare in the αR series (Table 1).

To evaluate the possibility that genetic mutants might pre-exist in the cloned replicon-harbouring cells (clones 1, 3, 4 and 5 shown in supplementary Fig. S2) or that genetic mutants had appeared during the IFN-β treatment of the cloned replicon-harbouring cells, we repeated IFN-β treatment of cloned 1, 3, 4 and 5 cells following the method described previously (Namba *et al.*, 2004). The result was almost identical to that obtained previously (Namba *et al.*, 2004), indicating the good reproducibility of the experiment with IFN-β treatment (data not shown). In the present study, each of the three colonies showing resistance to IFN-β was isolated and proliferated (see Supplementary Fig. S2) and we then performed sequence analysis of IFNAR mRNAs

Table 1. Genetic alterations of type I IFN receptors in HCV replicon-harbouring cell lines possessing IFN-resistant phenotypes

The determined nucleotide sequences were compared with those of 6M and 6Mc cells, which confirmed that the deduced amino acid sequences were identical to the human IFNAR1 (GenBank accession no. NM_000629) and IFNAR2c (GenBank accession no. L41942) sequences. NM, Not mutated.

Cell line	IFNAR1 mRNA			IFNAR2c mRNA		
	Nucleotide change and position	Effect on protein	Mutation frequency*	Nucleotide change and position	Effect on protein	Mutation frequency*
6M	NM	None	0/3	NM	None	0/3
1βR	NM	None	0/3	G to U at nt 475	E to stop at codon 159	13/13
3βR	Deletion of 5 nt at nt 376	Truncated (135 aa)	2/11	NM	None	0/3
	A to U at nt 1372	K to stop at codon 458	3/11			
4βR	G to U at nt 319	E to stop at codon 107	12/12	NM	None	0/3
5βR	Deletion of 176 nt at nt 201	Truncated (78 aa)	4/10	NM	None	0/3
	Deletion of 79 nt at nt 201	Truncated (67 aa)	2/10			
1αR	NM	None	0/3	NM	None	0/3
3αR	NM	None	0/3	NM	None	0/3
4αR	Deletion of 5 nt at nt 376	Truncated (135 aa)	1/3	NM	None	0/3
5αR	Deletion of 79 nt at nt 201	Truncated (67 aa)	1/3	NM	None	0/3
αRmix	NM	None	0/3	NM	None	0/3
6Mc	NM	None	0/3	NM	None	0/3

*Number of mutated or truncated clones/number of examined clones.

as described above. The results revealed that the nonsense mutations or deletions identified at this time (see Supplementary Table S1, available in JGV Online) were quite different from those obtained from the βR series (Table 1). Therefore, it is unlikely that the identified IFNAR mutants pre-existed in cloned 1, 3, 4 and 5 cells when these cells were obtained as colonies surviving IFN-α treatment.

To examine whether or not additional HCV replicon cell lines possessing the IFN-resistant phenotype could be obtained from HCV replicon-harbouring cells other than the parental replicon-harbouring cells used for the isolation of the αR and βR series, 6M.m/6Mc, 1βR.m/6Mc, 4βR.m/6Mc and 50-1 replicon-harbouring cells were treated with IFN-β (see Supplementary Fig. S2). Finally, we obtained four replicon-harbouring cell lines (6βR obtained from the 4βR.m/6Mc cells, 7βR obtained from the 6M.m/6Mc cells, and 8βR and 9βR obtained from the 50-1 cells) showing resistance to IFN-β. These results indicated that HCV replicon-harbouring cells showing the IFN-resistant phenotype were obtained from HCV replicon-harbouring cells established immediately. By sequence analysis of IFNAR1 and IFNAR2c cDNAs as described above, the E107stop nonsense mutation in IFNAR1 cDNA, which was the same mutation found in the 4βR cells, was found again in the 8βR and 9βR cells, while no IFNAR mutations were detected in the 6βR and 7βR cells (see Supplementary Table S1). The observation that IFNAR mutations occurred preferentially after IFN-β treatment is interesting. Since a variety of mutations and deletions in the IFN receptors were obtained from the cloned replicon-harbouring cells surviving after IFN-β treatment, such genetic alterations might occur accidentally in order to impair the antiviral states caused after IFN-β treatment. Thereafter, only replicon-harbouring cells possessing the IFNAR mutants might be able to proliferate in the presence of G418, resulting in the βR series.

To clarify whether or not the IFNAR mutations found in the βR series were determinants for the IFN sensitivity of HCV replicons, we prepared 4βR cells (possessing the IFNAR1 mutant) stably expressing wild-type IFNAR1 and examined its IFN sensitivity. Analysis of a luciferase reporter assay (see Supplementary Fig. S3a, available in JGV Online) clearly showed that IFN signalling in 4βR cells was restored by the expression of wild-type IFNAR1 in comparison with those of 4βR cells expressing the IFNAR1 mutant (see Supplementary Fig. S3b). The quantitative RT-PCR analysis of replicon RNA in the cells treated with IFN-β clearly showed that the level of 4βR replicon in cells expressing wild-type IFNAR1 was drastically decreased after IFN-β treatment, as was the level of 6M replicon in cells expressing wild-type IFNAR1 (see Supplementary Fig. S3c). In summary, we demonstrated that the IFNAR mutation found in 4βR cells was a major determinant for a strongly IFN-resistant phenotype of 4βR cells, suggesting that IFNAR mutations, which lead to the impairment of IFN signalling, convert HCV replicon-harbouring cells from an IFN-sensitive phenotype to a highly IFN-resistant phenotype.

IFNAR1 and IFNAR2c belonging to the class II cytokine receptor superfamily are structurally conserved transmembrane receptors located on the cell surface (see Supplementary Fig. S4a, available in JGV Online). However, since both the IFNAR1 E107stop mutant and the IFNAR2c E159stop mutant found in 4βR and 1βR cells, respectively, were N-terminally truncated and probably soluble forms, these truncated proteins may not be functional as IFN receptors or may act as dominant-negative inhibitors, and will lead to the interception of IFN signalling (see Supplementary Fig. S4b). Thus, the cause of the IFN-resistant phenotype of 1βR or 4βR cells appeared to be the functional disruption of IFNAR. The present results suggest that the downstream JAK/STAT pathway is intact, at least in 4βR cells.

Although for the most part we could clarify the mechanism underlying a highly IFN-resistant phenotype of HCV replicon-harbouring cells, at least in the case of 4βR cells, the mechanism underlying a partially IFN-resistant phenotype remains unclear, because IFNAR mutations were rare in the αR series. Since the expression levels of IFNAR, TYK2 and JAK1 were not decreased in the αR series, a functional deficiency of other cellular factor(s) involved in the IFN signalling may contribute to the acquisition of IFN resistance. Alternatively, certain HCV mutation(s) may account for the partially IFN-resistant phenotype of the αR series.

Since Machida *et al.* (2004a, b) recently reported that the frequency of genetic mutation was enhanced by HCV replication in *in vitro*-infected B cells and that the HCV core and NS3 were involved in the induction of a mutator phenotype mediated through the activation of inducible nitric oxide synthase, we cannot exclude the possibility that persistent HCV replication induces some irreversible genetic mutations. To clarify whether or not HCV acts as a mutagen for cellular factors, further study using an HCV RNA replication system (Ikeda *et al.*, 2002, 2005; Naka *et al.*, 2005) will also be necessary.

The HCV replicon-harbouring cells including 1βR and 4βR, in which IFN signalling is impaired, used or obtained in the present study may be useful for future studies, not only of the mechanism(s) underlying the IFN resistance of the replicons but also of the functional characterization of IFN receptors. Furthermore, these replicon cells may also be useful for screening novel anti-HCV reagents that act by mechanisms unrelated to IFN signalling.

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Enhancement of internal ribosome entry site-mediated translation and replication of hepatitis C virus by PD98059

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Abstract

Translation initiation of hepatitis C virus (HCV) occurs in an internal ribosome entry site (IRES)-dependent manner. We found that HCV IRES-dependent protein synthesis is enhanced by PD98059, an inhibitor of the extracellular signal-regulated kinase (ERK) signaling pathway, while cellular cap-dependent translation was relatively unaffected by the compound. Treatment of cells with PD98059 allowed for robust HCV replication following cellular incubation with HCV-positive serum. Though the molecular mechanism underlying IRES enhancement remains elusive, PD98059 is a potent accelerator of HCV RNA replication.

Keywords: HCV; IRES; ERK; Replication

Introduction

Hepatitis C virus (HCV), a member of the family Flaviviridae, is an enveloped virus with a positive-, single-stranded, 9.6-kb RNA genome (Murphy et al., 1995). The virus is the major causative agent of non-A, non-B hepatitis (Choo et al., 1989) and an estimated 170 million people throughout the world are persistently infected. Although acute phase HCV infection, in most cases, is asymptomatic, the virus frequently establishes a persistent infection, which is associated with serious clinical diseases such as chronic hepatitis followed by liver cirrhosis and hepatocellular carcinoma (Goodman and Ishak, 1995).

Like other positive-stranded RNA viruses, the 5′-untranslated region (UTR) of HCV RNA genome functions as an internal ribosomal entry site (IRES) and mediates translation initiation in a cap-independent manner (Tsukiyama-Kohara et al., 1992). Nearly the entire 5′-UTR (340 nt) and a short sequence of the coding region downstream of the initiator AUG codon of the HCV genome serve as an IRES (Honda et al., 1996). Unlike encephalomyocarditis virus (EMCV) or poliovirus, the 5′ end of the HCV genome is modified by neither cap structure nor VpG but bears a phosphate residue (Takahashi et al., 2005).

Molecular biological investigations of HCV have been hampered for a long time because of the lack of cell culture system that efficiently supports HCV replication. However, establishment of an HCV subgenomic replicon cell culture system in 1999 (Lohmann et al., 1999) allowed for such studies to be undertaken. The subgenomic replicon RNA is composed of, in this order, the HCV 5′-UTR containing an IRES, neomycin phosphate transferase or luciferase gene, HCV nonstructural (NS) proteins 3 through 5B directed by an EMCV IRES and the HCV 3′-UTR. As the replicon RNA replicates autonomously in cultured cells, the system provides a unique tool to analyze the molecular mechanisms governing viral genome replication and protein synthesis. Additionally, this system facilitates the screening of anti-HCV compounds.

PD98059 was identified as a potent inhibitor of mitogenic-extracellular signal-regulated kinase (MEK)–extracellular signal-regulated kinase (ERK) signaling pathway and has been widely used as a specific inhibitor of the pathway. The MEK–ERK pathway is elicited by broad

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range of growth factors or hormones and plays a crucial role in various events including cell growth promotion, differentiation, cell death and morphogenesis in eukaryotic cells (reviewed in Robinson and Cobb, 1997).

In this study, we demonstrate that PD98059 enhances HCV IRES-dependent translation. Because several lines of evidence suggest that IRES-mediated translation regulates replication in cultured cells (Lerat et al., 2000; He et al., 2003) and in vivo (Lott et al., 2001; Laporte et al., 2003; Forton et al., 2004), we examined the effect of PD98059 on viral replication. Although the RNA levels in replicon cells were relatively unaffected, PD98059 increased viral RNA levels in cultured cells infected with HCV-positive serum. Our results provide insight into the mechanisms of HCV IRES-dependent translation initiation and, in addition, suggest a simple infection system in cultured cells that supports HCV replication very efficiently.

Results

Enhancement of luciferase-replicon or HCV IRES by PD98059

We previously developed a highly efficient subgenomic HCV replicon system (Murata et al., 2005). Briefly, we used cured cells (curedMH14) as a host cell line, and the adaptive mutations were introduced into the subgenomic replicon construct for efficient replication. The luciferase gene was then placed under the control of the HCV IRES for rapid, quantitative and sensitive detection (Fig. 1, LMH14RNA). We have used this system to screen for compounds that inhibit HCV IRES-mediated translation. Treatment with IFN-α, IL-1β, cyclosporin A (CsA) or TGF-β, all factors known to repress HCV replication (Blight et al., 2000; Zhu and Liu, 2003; Watashi et al., 2003; Murata et al., 2005), reduced the observed luciferase activity (Fig. 2A), demonstrating the effectiveness of this system. Conversely, the compound PD98059 increased the luciferase activity by 348% compared to vehicle (DMSO)-treated control (Fig. 2A). The increase in luciferase activity induced by PD98059 was not apparent at 6 h after compound addition, but the activity was significantly elevated by 12 h and remained high for at least 3 days (Fig. 2B). Since PD98059 is an inhibitor of the MEK–ERK pathway, we examined its effects on ERK phosphorylation (Fig. 2C). PD98059 treatment blocked ERK phosphorylation, but a clear band of phospho-ERK was seen in DMSO-treated cells due to growth factors present in the growth medium. Luciferase activity increased in a dose-dependent manner following PD98059 treatment (Fig. 2D). Treatment with the inhibitor at 30 μM slowed cell growth (Fig. 6B) but did not put cells to death, while >30 μM of the chemical resulted in a high degree of toxicity (not shown). We next wished to examine whether PD98059 specifically affected HCV IRES-mediated translation. Using a plasmid based di-cistronic vector

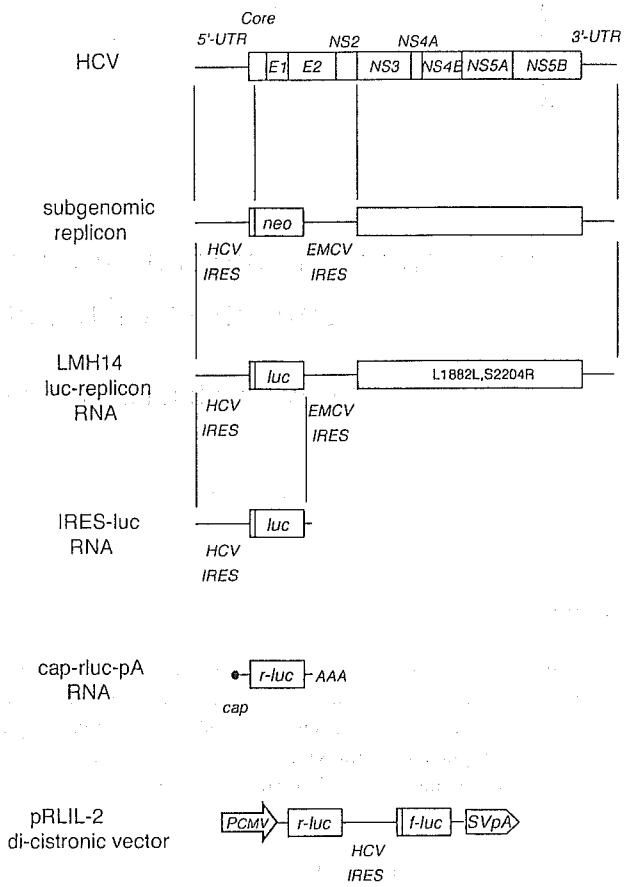

Fig. 1. Constructs used in this study. Schematic representation of the HCV RNA genome (HCV), G418-resistant subgenomic replicon (subgenomic replicon), subgenomic luciferase-replicon (LMH14), mono-cistronic luciferase expression vector with HCV IRES (IRES-luc), mono-cistronic renilla luciferase expression vector with cap and polyA (cap-rluc-pA) or di-cistronic plasmid-based vector construct. The ORFs are depicted as open boxes.

(Fig. 1 pRLIL-2), we found that PD98059 increased the ratio of IRES-dependent translation to cap-dependent translation (Fig. 3B, 247 and 278% at 30 and 10 μM, respectively). Translation downstream of a mono-cistronic mRNA was also enhanced, while cap-dependent translation was not affected (Figs. 3C,D). These results suggest that the positive response of the luciferase-replicon is primarily explained by increased IRES activity. Similar results were obtained when another inhibitor of the MEK–ERK signaling pathway, U0126, was used (Figs. 3E–H).

Effect of CGP57380 on HCV IRES

It has been known that either mitogen-activated protein kinase (MAPK)-interacting protein kinase (MNK) or eukaryotic initiation factor 4E (eIF4E)-binding protein (4EBP) regulates translation initiation downstream of the MEK–ERK pathway (Raught and Gingras, 1999). We first examined the involvement of MNK in the IRES activation using CGP57380, a specific inhibitor of MNK (Knauf et al., 2001). ERK interacts with and phosphorylates MNK in