PPARα signaling affects HCV replication

We next examined the potential role of PPARα signaling on HCV proliferation by monitoring HCV replication in 2D-HuS-E/2 cells that had been infected with HCV-RC5 and subsequently treated with the PPARα agonist fenofibrate [14] or the PPARα antagonist MK886 [14] (Fig. 3B). As outlined in Fig. 3A, a dose-dependent increase in HCV replication was observed in fenofibrate-treated cells. In contrast, a dose-dependent decrease in HCV proliferation was observed in the presence of MK886. Similarly, treatment with MK886 reduced HCV proliferation in 3D/TGP-HuS-E/2 cells (Fig. 3C). The response of HCV proliferation in response to fenofibrate and MK886 treatment was also analyzed in LucNeo#2 cells that contained HCV replicon RNA (LNMH14) derived from the HCV-1b genome (Fig. 4A). Luciferase expression in these cells represented replication of the HCV replicon [6] and, as shown in Fig. 4A, luciferase activity in the cells treated with fenofibrate or MK886 also showed either enhancement or suppression of replicon proliferation, respectively. In addition, the increased HCV replication following fenofibrate treatment was completely abolished when treated with MK886 simultaneously. As MK886 is known to induce apoptosis when administered in high doses [15], the cell viability was examined using the XTT assay. There were no significant effects on cell viability after treatment with fenofibrate. Although MK886 resulted in a minor reduction in XTT values when high doses (10–15 μM) were administered, this reduction was not statistically significant when compared to its effect on HCV replication (Fig. 4B). This result suggests that PPARα signaling is required for HCV replication and that suppression of PPARα signaling has an anti-HCV effect.

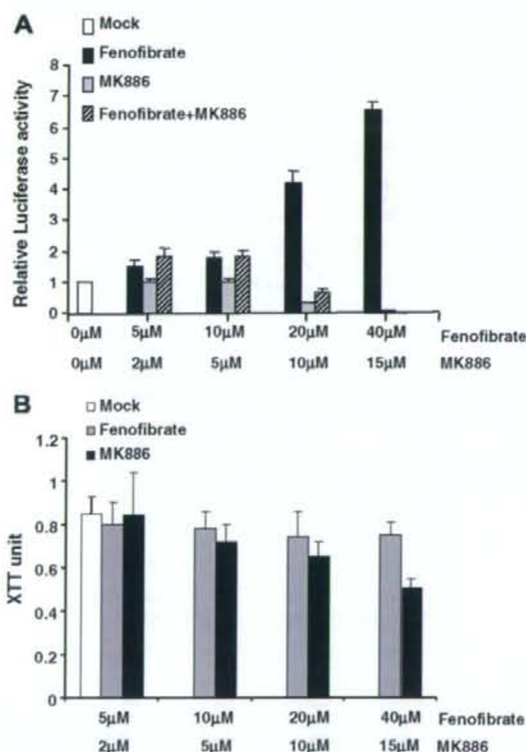

Fig. 4. The effects of PPARα agonists and antagonists on the replication of HCV subgenomic replicons. (A) LucNeo#2 cells containing a HCV subgenomic replicon termed LNMH14, were mock treated or treated with fenofibrate, MK886, or a combination of both fenofibrate and MK886 at the indicated concentrations for 2 days. Luciferase activity derived from the replicon was then measured as an indicator of HCV replication [7]. (B) Following treatment with fenofibrate and Mk886, LucNeo#2 cells were cultured for 2 days and cell viability measured using the XTT assay (Roche, Mannheim, Germany).

Discussion

In the current study, we demonstrated that immortalized hepatocyte HuS-E/2 cells cultured in 3D/TGP support the infection and replication of natural HCV derived from patient sera. Unlike recombinant HCVs, which have been required to adapt to sublines of HuH-7 cells [16], the population of the natural HCV is fairly polymorphic, demonstrating different responses to a variety of anti-viral agents [17,18]. The 3D/TGP-HuS-E/2 cells have the advantage of being a small-scale 3D cultured cells, which are cultured in 12-well plates at a density of 1×10^5/well, that allow the study of both viral and cellular events. In the current study, it demonstrated a 2 log increase in susceptibility to natural HCV infection and replication when compared to conventional 2D culture systems. Thus it offers an important advantage in the study of natural HCV infection and replication, and the response of natural HCV to anti-HCV drugs.

As the ability of HuS-E/2 cells to support infection and replication of natural HCV was greatly altered by the culture conditions, it is likely that the culture system described in our study will provide important information in regards to the cellular factors that support the HCV life cycle. The microarray study showed that the expression of some genes related to the PPARα signaling pathway were upregulated in the 3D cultured HuS-E/2 cells. Using both PPARα signaling agonists and antagonists, PPARα signaling was shown to affect infection and proliferation of natural HCV. PPARα is a ligand-activated transcription factor that is primarily expressed in tissues with high lipid metabolism including the liver, where it functions as one of three major nuclear receptors and is essential for its normal function [19]. Similar to a part of our data, a negative effect on HCV replication was previously observed in the replicon-bearing cells treated with siRNA for PPARα, with only 50% reduction of HCV-RNA [20]. In this study, even a large dose of PPARα agonist enhanced natural HCV replication in the 2D-HuS-E/2 cells for three times, despite the 2 logs enhancement of HCV proliferation in 3D/TGP culture. This implies that additional factors activated in 3D/TGP-HuS-E/2 cells may be required for the efficient HCV proliferation. Further analysis of the microarray data may provide us with further information on factors that may prove useful in the development of anti-HCV drugs.

In conclusion, the novel *in vitro* culture system combining TGP and immortalized hepatocytes described in this study demonstrated efficient support of natural HCV infection and replication. This system may be used in future virological studies to define new anti-HCV strategies. It may also prove useful for the specific design of effective individual therapy according to patient-specific strains.

Acknowledgments

This work was supported by Grants-in-Aid from the Ministry of Health, Labor and Welfare of Japan; and for scientific research from Ministry of Education, Sports, Culture, and Technology of Japan.

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Bioorganic & Medicinal Chemistry Letters 18 (2008) 4638–4641

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis and evaluation of 5′-modified 2′-deoxyadenosine analogues as anti-hepatitis C virus agents

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ARTICLE INFO

Article history:
Received 9 June 2008
Revised 3 July 2008
Accepted 5 July 2008
Available online 10 July 2008

Keywords:
Antiviral agent
Hepatitis C virus
HCV
Nucleoside

ABSTRACT

In order to study the effect of 5′-modification of 2′-deoxynucleoside on its anti-HCV activity, several analogues were synthesized and evaluated. Among the analogues, a 5′-deoxy-5′-phenacylated analogue exhibited a good anti-HCV activity with an EC_{50} of 15.1 μM. This compound is expected to operate via a type of mechanism that does not involve a generally known 5′-O-triphosphorylation process.

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Hepatitis C virus (HCV)[1] is a major causative agent of non-A and non-B hepatitis. It is estimated to have infected >170 million individuals, that is, 3.5% of the world's population. HCV infection is a leading cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Current therapy based on pegylated interferon and ribavirin is often poorly tolerated and is effective in only 50% of patients. Therefore, the development of further effective therapeutic agents against HCV is an urgent public health requirement.

In our previous study,[2] we revealed that several 5′-O-masked analogues of 6-chloropurine-2′-deoxyriboside, such as benzoate **1** and benzyl ether **2**, exhibit an effective anti-HCV activity in a subgenomic replicon cell line and are more potent than the corresponding unmasked analogue **3** (Fig. 1). Since it is generally accepted that most nucleoside antivirals exhibit their potency after being converted to the corresponding 5′-triphosphates,[3] the unmasked (or phosphorylated) 5′-hydroxyl group is indispensable for the antiviral activity. Accordingly, our result that the 5′-O-masking leads to an improvement in the anti-HCV activity appears to be inconsistent with the common understanding, interestingly.

We presume that the anti-HCV activity of certain 5′-O-masked analogues would arise from a new type of mechanism that does not involve the 5′-O-triphosphorylation process. However, there is still room for the discussion on the 5′-O-masking effect because certain carbon–oxygen bonds, for example, the carboxylic ester bond of compound **1** (i.e., the benzoate moiety in compound **1**), are often hydrolyzed in cultured cells; in other words, there is a possibility that compound **1** simply operates as a prodrug of **3**.[4,5] Therefore, in order to confirm the effectiveness of 5′-O-masking groups, particularly that of the benzoyl group of compound **1**, we planned the syntheses and anti-HCV evaluations of ketone analogues **4** and **5**, in which the 5′-oxgen atom was replaced with a methylene group to prevent the hydrolytic removal of the benzoyl group.

The synthesis of **4** began with readily available 3′-O-TBS-2′-deoxyadenosine (**6**)[6] (Scheme 1). First, we attempted to subject

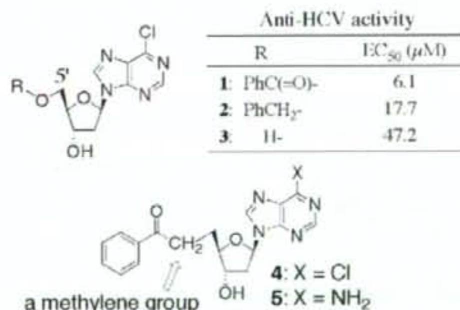

Figure 1. Structures of 5′-modified analogues.

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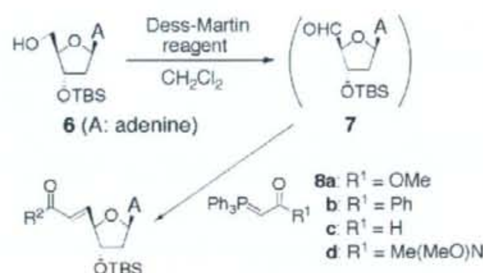
0960-894X/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2008.07.015



compd	R^2	yield (%)	E:Z
9	MeO–	90	E only
10	Ph–	60	13:1
11	H–	—	
12	Me(MeO)N–	—	

Scheme 1.

the isolated aldehyde **7** obtained via the oxidation of **6** to the following Wittig reaction; however, it was unsuccessful due to the instability of **7**. This issue was overcome by using a one-pot oxidation–Wittig reaction with Dess–Martin periodinane (DMP) and stabilized phosphorus ylide.[7] Among the four types of ylides examined (**8a–d**), two of them (**8a** and **8b**) successfully afforded the desired compounds **9** and **10** in 90% (E-isomer only) and 60% (E:Z = 13:1) yields, respectively, while the others (**8c** and **8d**) yielded complex mixtures. Since the Dess–Martin oxidation is not very suitable for the large-scale synthesis of **9** and **10** because of the explosive nature of DMP (and also its precursor, 2-iodoxybenzoic acid[8]), several other one-pot protocols such as Moffatt oxidation–Wittig,[9] PCC–Wittig,[10] TEMPO–BAIB–Wittig,[11] and TPAP–NMO–Wittig[12] were examined with **6** and **8a**. However, the TLC analyses of all the attempts revealed low yields and/or the formation of by-products.

With the thus-obtained products, the reduction of the C–C double bond was examined (Table 1). Compound **9** was converted to **13** under standard hydrogenation conditions (Pd/C, H_2, THF) with an excellent yield although the reaction required a long reaction time (~2 days) and comparatively large quantities of the catalyst (50 wt.%) (entry 1). In contrast, the conjugate reduction of **9** by sodium borohydride–transition metal salt (e.g., $NiCl_2$ and CuCl) systems[13] furnished **13** in a short time (1–3 h), but the yield was

Table 1
Chemoselective reduction of C–C double bond

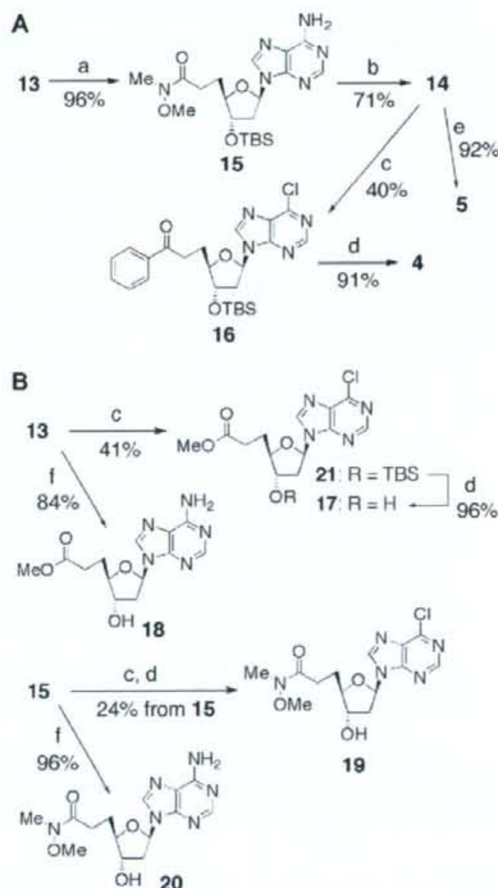
9 or 10 —entries 1–8→ **13**: R = MeO; **14**: R = Ph

Entry	Substrate	Conditions	Product	Yield (%)
1	**9**	Pd/C, H_2, THF, rt, 2 d	**13**	94
2	**9**	$NiCl_2$, $NaBH_4$, MeOH, 0 °C, 1 h	**13**	50
3	**9**	CuCl, $NaBH_4$, MeOH, 0 °C, 2.5 h	**13**	76
4	**9**	Mg, MeOH, reflux, 2 h	Complex mix.	
5	**10**	Pd/fibroin, H_2, MeOH, rt, 2 d	**14**	Trace[a]
6	**10**	Pd/C, Ph_2S, H_2, MeOH, rt, 2 d	**14**	31[b]
7	**10**	$PhSiH_3$, $In(OAc)_3$, EtOH, rt, over night	**14**	84
8	**10**	Bu_3SnH, $InCl_3$, i-PrOH −78 °C to rt, 2 h	**14**	93

[a] 93% of **10** was recovered.
[b] 56% of **10** was recovered.

moderate (entries 2 and 3). The use of elemental magnesium in methanol led to a complex mixture (entry 4). In the case of **10**, chemoselective hydrogenations by Sajiki's procedures (Pd/fibroin–H_2 or Pd/C–Ph_2S–H_2)[14] were ineffective, resulting in the recovery of a large amount of the starting material (entries 5 and 6), while the 1,4-reduction with indium hydride generated in situ by using $PhSiH_3$–$In(OAc)_3$ or Bu_3SnH–$InCl_3$[15] efficiently afforded the desired product **14** in good yields (entries 7 and 8). Consequently, the conditions in the case of entries 1 and 7 were employed for routine syntheses of **13** and **14**, respectively, in view of their simple experimental procedures as well as their good yields.

Compound **13** was readily converted to **14** with a two-step sequence, that is, via a Weinreb amide **15**, as illustrated in Scheme 2-A. Using a Grignard reagent (PhMgBr) led to a better yield (71%) than when phenyl lithium was used (58% yield). This two-step conversion will effectively serve for the synthesis of various analogues because the phenyl moiety of **14** can be easily replaced with other groups by changing the type of Grignard reagent. The amino group of **14** was substituted by a chloro group to afford **16** (40% yield),



Scheme 2. Reagents: (a) Me(MeO)NH·HCl, n-BuLi, THF; (b) PhMgBr, THF; (c) t-BuONO, Et_4NCl, CCl_4–CH_2Cl_2; (d) TBAF, AcOH, THF; (e) Et_3N·3HF, THF; (f) TAS-F, MeCN.

which was subsequently treated with a mixture of tetrabutylammonium fluoride (TBAF) and acetic acid, giving the desired product **4** in 91% yield. A moderate yield of **16** was mainly obtained due to the competitive elimination of its nucleobase moiety. The other desired compound **5** was prepared in 92% yield by exposing **14** to triethylamine trihydrofluoride.

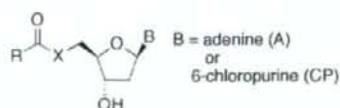
Since we are interested in the structure–activity relationship (SAR) of not only the benzoyl moiety but also the methyl ester and Weinreb amide moieties contained in the synthetic intermediates, we conducted syntheses of the corresponding analogues **17**–**20**, as shown in Scheme 2-B. 6-Chloropurine analogues **17** and **19** were prepared from **13** and **15**, respectively, under conditions almost identical to those used in the synthesis of **4** (i.e., *t*-BuONO–Et_4NCl and TBAF–AcOH). The conversion to **18** and **20** was effectively accomplished by the treatment of **13** and **15** with tris(dimethylamino)sulfonium difluorotrimethylsilicate (TAS-F),[16] while that with TBAF led to a mixture of the desired product and certain tertabutylammonium salts that were difficult to separate.

The synthesized nucleoside analogues mentioned above were assayed for their ability to inhibit HCV RNA replication in a subgenomic replicon Huh7 cell line (LucNeo#2),[17] and the result is presented in Table 2 and Figure 2. These cells contain an HCV subgenomic replicon RNA encoding a luciferase reporter gene as a marker. The antiviral potency of the analogues against the HCV replicon is expressed as EC_{50}, which was quantified by a luciferase assay after a two-day incubation period with the corresponding compound. In addition, the associated cytotoxicity (expressed as CC_{50} in Table 2) was evaluated in a tetrazolium (XTT)-based assay according to the manufacturer's protocol.

As shown in Table 2, the ketone analogue **4** exhibited an antiviral activity against the HCV replicon with an EC_{50} of 15.1 μM (entry 1), which is nearly comparable to that of benzoate analogue **1** (entry 7). The cytotoxicity of **4** was somewhat high (CC_{50}: 76.3 μM), but was not high enough to exert an influence on the EC_{50} value because the cytotoxicity at 15 μM was considerably low (ca. 0–2%) (Fig. 2A). Thus, the decrease in the luciferase activity with **4** results from its anti-HCV activity, not its cytotoxicity. Interestingly, compounds **17** and **19** also exhibited anti-HCV activities (entries 3 and 5, respectively). In contrast, the 6-amino analogues **5**, **18**, and **20** did not exhibit any significant anti-HCV activity (entries 2, 4, and 6).[18]

To confirm the anti-HCV potency of compound **4**, subgenomic replicon RNA levels were quantified by real-time RT-PCR analysis (Fig. 2B). Exposing the replicon cells to 12.5 and 25 μM of **4** reduced the replicon RNA amount up to approximately 60% and 25%, respectively. This result is almost consistent with that of the luciferase assay with **4**.

Table 2
Inhibitory potency (EC_{50}) and cytotoxicity (CC_{50}) of the synthesized analogues in HCV replicon assay



Entry	Compound	R	X	B	EC_{50}[a] (μM)	CC_{50}[a] (μM)
1	**4**	Ph	CH_2	CP	15.1 ± 0.4	76.3 ± 5.2
2	**5**	Ph	CH_2	A	>200	–
3	**17**	MeO	CH_2	CP	32.9 ± 1.6	>200
4	**18**	MeO	CH_2	A	>200	–
5	**19**	Me(MeO)N	CH_2	CP	40.4 ± 1.4	>200
6	**20**	Me(MeO)N	CH_2	A	>200	–
7	**1**	Ph	O	CP	6.1[b]	111[b]

[a] EC_{50}: 50% effective concentration; CC_{50}: 50% cytotoxic concentration.
[b] Extracts obtained from our previous study (Ref. 2).

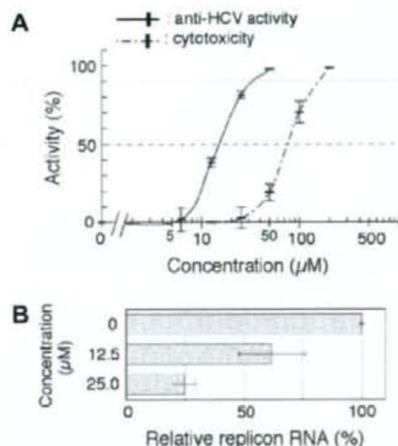


Figure 2. Anti-HCV activity and cytotoxicity of **4**: (A) result of luciferase assay and XTT assay; (B) result of real-time RT-PCR.

Taking these data into account, it appears that the phenacyl group ($BzCH_2$–) equipped at the C5′ position as well as the benzoyloxy group (BzO–) is effective functional group for anti-HCV activity; this should be noteworthy because the 5′-phenacyl group is expected to operate without being converted to the corresponding 5′-hydroxyl group (or 5′-triphosphate group). This result strongly supports our hypothesis that the 5′-*O*-masking group can contribute to the anti-HCV activity not only as a unit for the prodrug system but also as a part of the substrate. Although the detailed mechanism is unclear and the biological activity is still insufficient, the antiviral potency of such 5′-modified analogues is of great interest because they are likely to operate via a pathway that does not involve the 5′-*O*-phosphorylation process. We hope that the present study will contribute to developing a new class of HCV therapeutic agents.

Acknowledgments

This research was partly supported by a Grant-in-Aid for Young Scientists (B) (20790106) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by a Grant-in-Aid from Mitsubishi Chemical Corporation Fund.

Supplementary data

Supplementary data associated with this article (experimental details and spectroscopic data of new compounds **4–6**, **9**, **10**, **13–21**) can be found, in the online version, at doi:10.1016/j.bmcl.2008.07.015.

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Virology 375 (2008) 424–432

VIROLOGY

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

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Received 5 November 2007; returned to author for revision 27 November 2007; accepted 10 February 2008
Available online 18 March 2008

Abstract

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

Introduction

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

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

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

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

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doi:10.1016/j.virol.2008.02.009

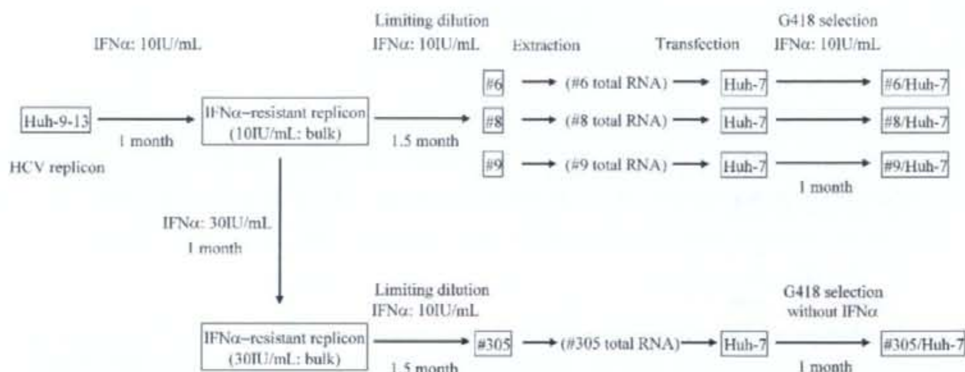
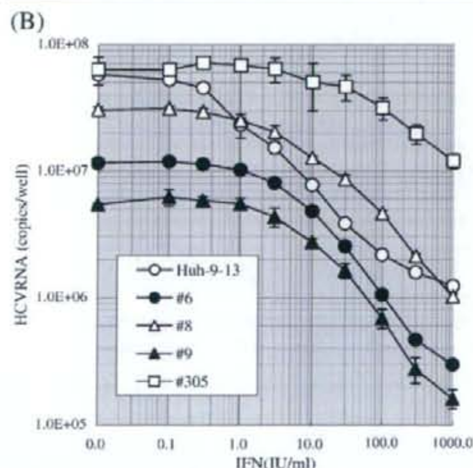


Fig. 1. An outline of the process used for isolation of replicon cells showing IFNα-resistance. Total RNA transfection derived from replicons to naive Huh-7 cells was performed using DMRIE-C reagent (Invitrogen).


(A)

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

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

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

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

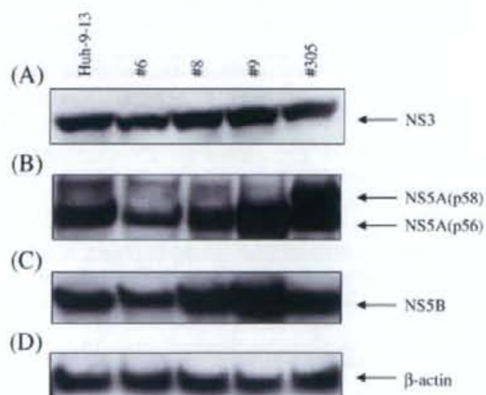


Fig. 3. Western blot analysis of the established IFNα-resistant replicon cells (#6, #8, #9, and #305) and original replicon cells (Huh-9-13). Expression of β-actin was used as an internal control of cellular protein in the replicon cells. Each cell line was inoculated on a 60-mm plate at 3×10^5 cells/well.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

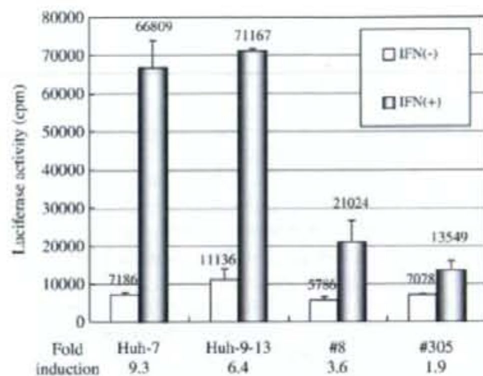


Fig. 4. Transactivation of ISRE in IFNα-resistant replicon cell lines (#8 and #305), original replicon (Huh-9-13), and parental Huh-7 cells by reporter gene (pISRE/Luc) analysis. The cells were stimulated with 1000 IU/mL of IFNα for 24 h after transfection of reporter plasmid DNA. White bars show control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

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

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

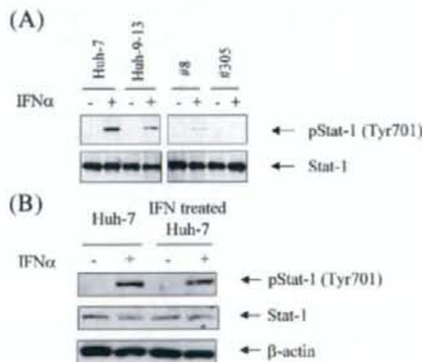


Fig. 5. (A) Change in phosphorylation of Stat-1 in IFNα-resistant replicon cell lines (#8 and #305), original replicon (Huh-9-13) and parental Huh-7 cell. Phosphorylation of Stat-1 was analyzed by western blot analysis using anti-phospho-Stat-1 (Tyr701) antibody. The cells were cultured in medium with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

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

Results

Establishment of IFNα-resistant replicon cell lines

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

(A)

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

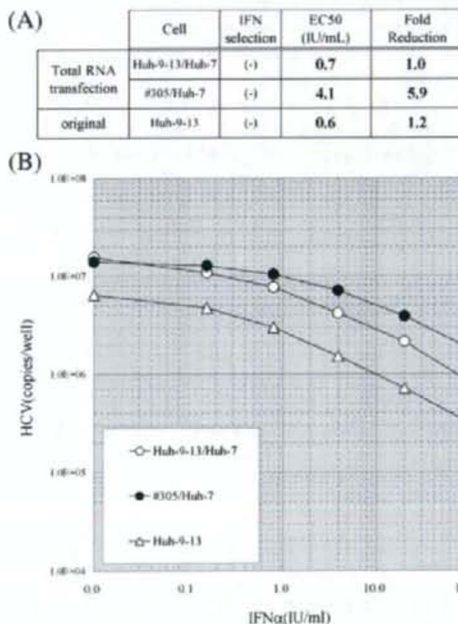


Fig. 6. Reactivity for IFNα in the Huh-7 cells, #305/Huh-7, transfected with total RNA of #305 replicon cells and in the Huh-7 cells, Huh-9-13/Huh-7, transfected with total RNA of original replicon cells (Huh-9-13). These transfected cells were selected with G418 in the absence of IFNα. The amount of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

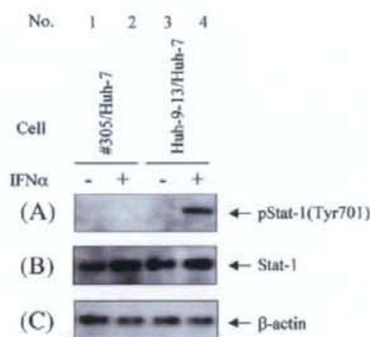


Fig. 7. Phosphorylation of Stat-1 in #305/Huh-7 and Huh-9-13/Huh-7 described in Fig. 6. The experiment was performed as described in Fig. 5. Each panel shows (A) phospho-Stat-1 (Tyr701), (B) Stat-1, and (C) β-actin. (Lanes 1 and 2) Huh-7 cells transfected with IFNα-resistant replicon (#305) total RNA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

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

Characterization of IFNα-resistant replicon cell lines

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

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

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

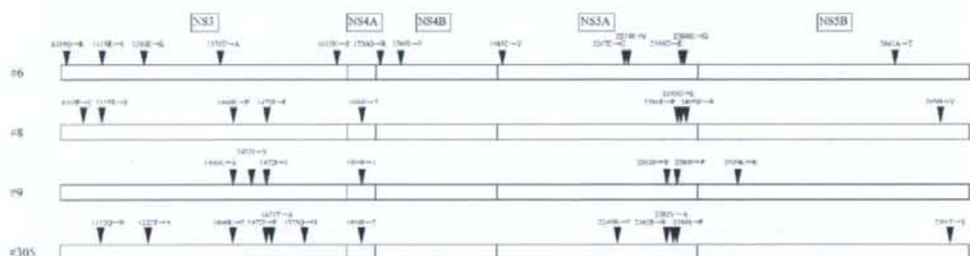


Fig. 8. The amino acid sequence deduced from nucleotide sequence in IFNα-resistant replicon cells. The nucleotide sequence was determined by an RT-PCR direct sequencing method. Arrows indicate the amino acid substitutions that were detected only in IFNα-resistant replicons compared with original replicon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

HCV replicon RNA confers IFNα-resistance

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

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

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

Direct sequencing analysis of IFNα-resistant replicons

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

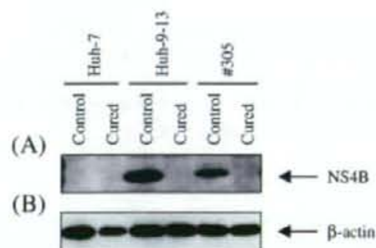


Fig. 9. Expression of NS protein (NS4B) (A) and β-actin (B) was confirmed in 'cured cells' by western blot analysis. Huh-7 cells with JTP-71892 as well as replicon cells (Huh-9-13 and #305) were analyzed likewise.

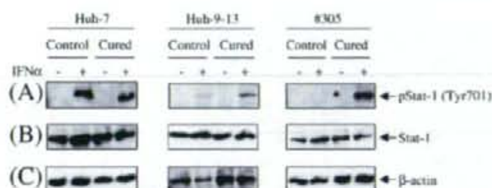


Fig. 10. Phosphorylation of Stat-1 (Tyr701) in 'cured cells'. Phosphorylation of Stat-1 (Tyr701) (A) by IFNα stimulation was investigated by western blot analysis. Stat-1 (B) and β-actin (C) were also analyzed. IFNα stimulation and western blot analysis were performed as described in Fig. 5.

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

Materials and methods

Cell culture

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

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

IFN treatment

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

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

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

Direct sequencing analysis of HCV replicon RNA

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

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

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

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

Reporter gene analysis

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

Western blot analysis

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

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

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

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

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

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

Acknowledgments

We thank M. Tomonaga, Y. Hori and K. Asahina for their technical assistance in this work. We also greatly acknowledge Dr. R. Bartenschlager for providing us the HCV subgenomic replicon (Huh-9-13) and naive Huh-7 cell lines for parental cell line of Huh-9-13.

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FEBS Letters 582 (2008) 3085–3089

Suppression of hepatitis C virus replication by baculovirus vector-mediated short-hairpin RNA expression

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Received 10 June 2008; revised 23 July 2008; accepted 30 July 2008

Available online 8 August 2008

Edited by Hans-Dieter Klenk

Abstract Short-hairpin RNAs (shRNAs) inhibit gene expression by RNA interference. Here, we report on the inhibition, by baculovirus-based vector-derived shRNAs, of core-protein expression in full-length hepatitis C virus (HCV) replicon cells. shRNAs were designed to target the highly conserved core region of the HCV genome. In particular, the core-shRNA452 containing nucleotides 452–472, as the target in the HCV core gene, dramatically inhibited the expression of the HCV core protein in replicon cells. Furthermore, HCV core-protein expression was inhibited more strongly by the vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped baculovirus vector than by the wild-type baculovirus vector.
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Keywords: Baculovirus vector; Hepatitis C virus; RNA interference; Short-hairpin RNA; Vesicular stomatitis virus glycoprotein

1. Introduction

The hepatitis C virus (HCV) is a major cause of liver-related morbidity and mortality [1]. The virus establishes a persistent liver infection, leading to the development of chronic hepatitis, liver cirrhosis, and hepatocellular carcinomas [1,2]. However, a highly effective anti-HCV drug has yet to be developed, partly due to the lack of detailed information about the life cycle of the virus. In an effort to develop an alternative to combined interferon–ribavirin treatment [3,4], we used RNA interference (RNAi) based on short-hairpin RNA (shRNA), which is a powerful tool for suppressing gene function [5]. RNAi is triggered by small-interfering RNAs (siRNAs) that are processed from long double-stranded or hairpin precursors, and become part of the ribonucleoprotein complex, the RNA-induced silencing complex (RISC) [6,7]. siRNAs expressed from DNA templates through the action of the Dicer enzyme silence gene expression as effectively as exogenously introduced synthetic siRNAs [5,8,9]. The use of RNAi has recently been extended to differentiated cultured mammalian cells [10]. It has also been used to inhibit viral replication in the HCV subgenomic replicon [11–13].

The baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) has long been used as a biopesticide, and as a tool for efficient recombinant protein production in insect cells [14]. Its host specificity was originally thought to be restricted to cells derived from arthropods; however, with an appropriate eukaryotic promoter, it can express foreign genes in several types of mammalian cells [15–17] and animal models [18,19]. Its advantages for use in gene-therapy applications are its inherent inability to replicate, its lack of cytopathic effect (CPE) in mammalian cells even at a high multiplicity of infection (MOI), and the absence of preexisting antibodies against baculoviruses in animals.

Here we designed baculovirus vector-mediated shRNAs against the highly conserved core-protein region of the HCV [20,21]. The shRNA452 construct mediated more effective inhibition of HCV replication than the other core-shRNAs (Ac-shRNA479 and Ac-shRNA523). We also found that HCV core-protein expression was more significantly inhibited by the vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped baculovirus vector than by the wild-type baculovirus vector.

2. Materials and methods

2.1. Baculovirus transfer vector constructs

We designed baculovirus transfer vectors expressing shRNAs against the following regions of the HCV core-protein sequence: nucleotides 452–472, containing the nuclear localization signal site (pU6-core-shRNA452); nucleotides 479–499 (pU6-core-shRNA479); and nucleotides 523–543 (pU6-core-shRNA523) [22]. Sense and antisense strands of shRNA oligonucleotides were synthesized, annealed at 95 °C for 3 min, and then slowly cooled in phosphate-buffered saline (PBS; pH 7.4, containing 50 mM NaCl). The oligonucleotides contained the loop CCACACC sequence, and Kpn I and BamHI ends, which were inserted into a pU6 vector, based on pSV2-neo. A Pol III-type U6 promoter allowed the constant expression of shRNAs. The following three sites in the core region of the common sequences of the HCV strain M1LE (GenBank accession number AB080299) were chosen as the targets for shRNAs: 5′-GCCGCGCAGGGGCCCCAGGUU-3′ (shRNA452); 5′-GCGCGCGACUAGGAAGACUUC-3′ (shRNA479); and 5′-GCGACAACCUAUCCCCAAGGC-3′ (shRNA523). Fragments of U6-core-shRNAs, ranging from the EcoRI site upstream of the U6 promoter to the BamHI site downstream of the terminating sequences, were sequenced and then inserted into the cloning site of the baculovirus transfer vector, pVL1393 (BD Biosciences, San Jose, CA, USA) in an opposite orientation to the PH promoter (Fig. 1C), in order to create pVL1393-core-shRNA452, shRNA479, shRNA523, and pVL1393-U6-terminator. A spacer was inserted between the inverted sequences to form a hairpin structure,

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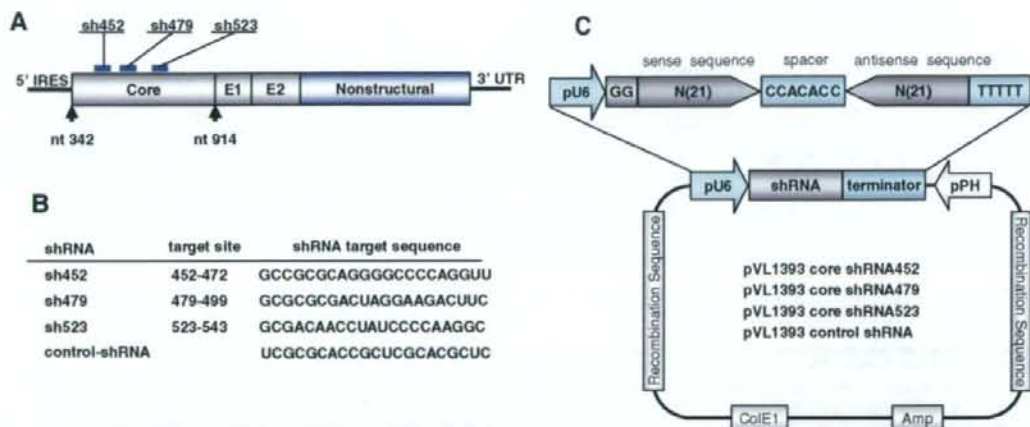
doi:10.1016/j.febslet.2008.07.056



shRNA	target site	shRNA target sequence
sh452	452-472	GCCGCGCAGGGGCCCCAGGUU
sh479	479-499	GCGCGCGACUAGGAAGACUUC
sh523	523-543	GCGACAACCUAUCCCCAAGGC
control-shRNA		UCGCGCACCGCUCGCACGCUC

Fig. 1. (A) Genomic profile of HCV showing both coding and non-coding genes. (B) HCV core-region target sites and sequences used for the design of shRNAs. (C) Construction and schematic representation of baculovirus transfer vector expressing HCV core-shRNA.

and to enhance its stability (Fig. 1C). Scrambled shRNA (control-shRNA) cloned into the same vector was used as a negative control (pVL1393-control-shRNA) in all experiments. The VSV-G-pseudotyped baculovirus vector-transduced shRNA452 was constructed following previously published procedures [23].

2.2. Preparation of baculoviruses

Recombinant baculovirus containing the shRNA genome (Ac-shRNA) was generated by homologous recombination of the transfer vector and linearized baculovirus DNAs (BD Biosciences) following previously published procedures [23].

2.3. Cell culture

NNC#2 (NN/1b/FL) cells [24] carrying a full genome replicon were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), non-essential amino-acids, L-glutamine, and 1 mg/ml G418 (Invitrogen, Carlsbad, CA, USA).

2.4. Measurement of HCV core protein

AcU6-HCV-core-shRNAs or AcU6-VSV-HCV-core-shRNAs were used to infect HCV replicon cells. After 48 h, intracellular HCV core-protein levels were measured using a fully automated HCV core-protein antigen chemiluminescent enzyme immunoassay (CLEIA) according to the manufacturer's instructions [25]. The relative chemiluminescence unit was measured, and was used to determine the concentration of HCV core antigen according to a standard curve generated using recombinant HCV core antigen. The concentration was expressed in units of femto-mol/L (fmol/L). Each CLEIA assay was performed in triplicate.

3. Results

3.1. Construction of baculovirus transfer vectors carrying shRNA-synthesizing cassettes

The core-protein forms the nucleocapsid, and modulates gene transcription, cell proliferation, and apoptosis [21]. HCV functions as a messenger RNA (mRNA) with a single-stranded RNA genome; thus, we hypothesized that cleavage of the core-protein mRNA would inhibit nuclear transport and virus duplication.

To characterize the efficiency of baculoviruses as vehicles for gene therapy, we selected the HCV core region as a target site (Fig. 1A and B), and constructed a recombinant baculovirus vector expressing the shRNA. Fig. 1C shows the baculovirus-transfer vectors used in this study. The baculovirus-transfer vector pVL1393-core-shRNA carries core-shRNA under the control of the PolIII, U6 promoter. Recombinant baculovirus containing the shRNA genome (Ac-shRNA) was generated by homologous recombination of the transfer vector and linearized baculovirus DNAs (BD Biosciences) in Sf9 cells. Viruses were produced at high titers, ranging from 1.5×10^8 to 1.2×10^9 pfu/ml. shRNA452 expression was confirmed by Northern blot analysis in Ac-shRNA452-infected Huh-7 cells (Supplementary Fig. S1 and Supplementary methods).

3.2. Inhibition of HCV RNA replication of baculovirus-mediated shRNA-expression vectors in the HCV replicon

We investigated whether intracellular expression of shRNA inhibited viral replication and affected HCV RNA levels in NNC#2 cells. The baculovirus-infection efficiency of NNC#2 cells ranged from 80 to 90% (Fig. 2A and Supplementary methods). The real-time reverse-transcription (RT) polymerase chain reaction (PCR) was used to examine the presence of HCV RNA, and the ability to induce RNA silencing in NNC#2 cells 48 h post-infection. When NNC#2 cells were infected with Ac-shRNAs at a MOI of 100, a significant reduction in HCV RNA levels was observed compared with a non-related shRNA control (Fig. 2B and Supplementary methods). Although inhibition of HCV RNA levels occurred with all three constructs, the greatest effect occurred with sh452 (68%), while sh479 decreased the levels by approximately 55% and sh523 by 25%. By contrast, the control baculovirus vectors (Ac-U6-terminator and Ac-control-shRNA [random sequence]) had no inhibitory effect on HCV replication (Fig. 2B). These results point to a sequence-specific inhibitory effect of shRNA on HCV replication.

3.3. Silencing of HCV core-protein expression through baculovirus-mediated shRNA in the HCV replicon

We confirmed the inhibitory effect of shRNAs using fluorescence microscopy to assess the localization and expression of the HCV core protein 48 h post-infection. The core proteins

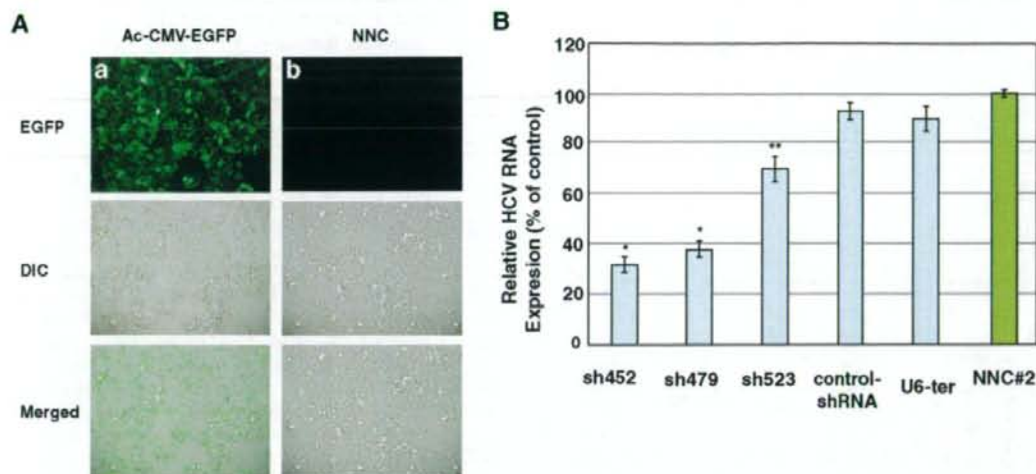


Fig. 2. Inhibition of HCV RNA by baculovirus-mediated shRNA expression vector. (A) Fluorescence-microscopic analyses of HCV full replicon cells (NNC#2, 4×10^4 cells/well) infected with enhanced green fluorescent protein (EGFP) baculovirus (Ac-CMV-EGFP) at a MOI of 100. Images were taken at a ×10 magnification 2 d post-infection: (a) Ac-CMV-EGFP; (b) NNC cells alone. (B) Real-time PCR analysis of HCV RNA expression after transduction of HCV full replicon cells (NNC#2, 4×10^4 cells/well) with a MOI of 100 for baculovirus-mediated shRNA. Relative HCV RNA values are shown. Error bars represent S.E. of the mean from three different experiments. $^*P < 0.01$; $^{**}P < 0.05$.

were localized in the cytoplasm of core protein-shRNA (sh452, sh479, and sh523)-expressing NNC#2 cells (Fig. 3A and Supplementary methods), as well as control cells (control-shRNA). Inhibition of the HCV core protein was observed following infection by all three constructs, although the greatest effect occurred with sh452.

We next examined whether shRNA against the HCV core protein inhibited viral replication, using a CLEIA assay. When NNC#2 cells were infected with Ac-shRNAs at MOIs of 100 and 200, a significant reduction in core-protein expression was observed compared with a non-related shRNA control (Fig. 3B). The sh452 construct mediated more effective inhibition of HCV replication than the other core-shRNAs (Ac-shRNA479 and Ac-shRNA523). The control-Ac-U6-terminator and Ac-control-shRNA baculovirus vectors had no inhibitory effect on HCV replication.

Furthermore, enhanced gene-transfer efficiency was observed in a range of cell lines with recombinant baculovirus vectors expressing surface glycoprotein G of VSV-G (Fig. 4A). VSV-G enhances the escape of baculovirus vectors from intracellular endosomes, thereby increasing the transduction efficiency of the virus [17]. The VSV-G-modified baculovirus

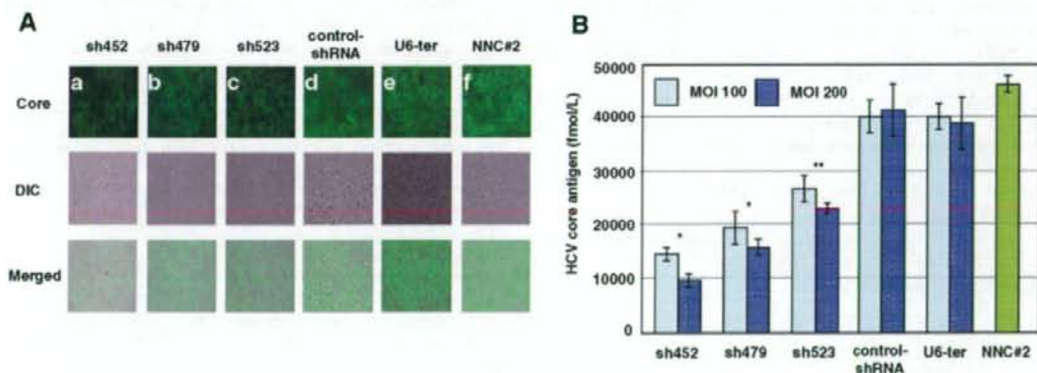


Fig. 3. Suppression of HCV core protein by baculovirus-mediated core-shRNA expression vectors. (A) Immunofluorescence microscopy of anti-HCV core-protein antibody (green) following infection of NCC#2 cells with the following: (a) sh452 vector; (b) sh479 vector; (c) sh523 vector; (d) control-shRNA vector; (e) U6-terminator vector; (f) NNC cells alone. (B) Ac-shRNAs were used to infect NCC#2 cells and intracellular HCV core-protein levels were measured after 48 h by an HCV protein antigen CLEIA assay. Error bars represent S.E. of the mean from three different experiments. $^*P < 0.01$; $^{**}P < 0.05$.

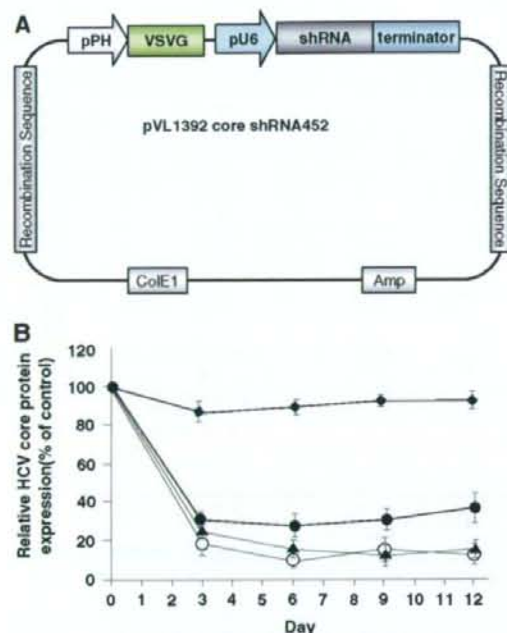


Fig. 4. Inhibition of HCV core protein by VSV-G-pseudotyped and wild-type baculovirus-mediated shRNAs. (A) Construction and schematic representation of VSV-G-pseudotyped baculovirus transfer vector expressing HCV core-shRNA. (B) CLEIA analysis of HCV core-protein expression after transduction of HCV full replicon cells (NNC#2, 4×10^4 cells/well) with Ac-shRNA452 (MOI = 100 [solid circle] or 200 [solid triangle]), Ac-VSV-G-shRNA452 (MOI = 100 [open circle]) or control-shRNA expression vector (MOI = 100 [solid diamond]).

(Ac-VSV-G-shRNA452) was propagated following previously published procedures [23]. Based on the shRNA452-expression data, we evaluated the effectiveness of shRNA452-mediated inhibition of HCV core expression by VSV-G-pseudotyped or wild-type baculovirus vector-transduced shRNA in NNC#2 cells. When Ac-VSV-G-shRNA452 (MOI = 100) or Ac-shRNA452 (MOI = 00 or 200) was added to the cells at 3 d intervals for 12 d, a significant reduction in HCV RNA (70–90%) was sustained from day 3 to day 12. The results indicated that HCV core protein expression was more strongly inhibited by the VSV-G-pseudotyped baculovirus vector than by the wild-type baculovirus vector (Fig. 4B). The ability of Ac-VSV-G-shRNA452 to reduce HCV core-protein levels was not due to a cytotoxic effect (Supplementary Fig. S2 and Supplementary methods).

4. Discussion

There is a need for a highly effective anti-HCV drug to be developed. The current study aimed to establish a universal baculovirus vector for intracellular shRNA generation and inhibition of HCV gene expression in NNC#2 cells. shRNAs that were designed to target core regions of HCV replicon RNA varied in their ability to inhibit HCV replication. It is important to note that the core protein is synthesized as the initial polypeptide from the HCV polyprotein precursor. However, the inhibition of the HCV core protein upon the introduction of siRNAs was not determined in these studies [11,12]. Our current work revealed that baculovirus-mediated shRNA452 expression significantly inhibits HCV replication.

The delivery of siRNA to appropriate cells or tissues is a major challenge. Several approaches have been described for generating loss-of function phenotypes in mammalian systems using siRNA; however, these techniques are limited and are not suitable for generating a long-term silencing effect in vivo [26,27]. Efficient and safe delivery systems have not yet been established for the suppression of HCV replication. Baculoviruses appear to be useful viral vectors, not only for the abundant expression of foreign genes in insect cells, but also for efficient gene delivery to the hepatoma lines HepG2 and Huh7 [28], and the osteogenic sarcoma line Saos2 [29].

In this study, the introduction of three different baculovirus-mediated shRNAs into target cells containing HCV replicon RNA caused a dose-dependent reduction in the level of viral proteins and RNA. In particular, protein-expression analyses revealed that shRNA452 at a MOI of 200 suppressed HCV core-protein levels by 75%. By contrast, the control baculovirus vectors (Ac-U6-terminator and Ac-control-shRNA) had no inhibitory effect on HCV replication. We also examined the suppression of HCV core protein by VSV-G-pseudotyped baculovirus-mediated shRNA452. HCV core-protein expression was more strongly inhibited by the VSV-G-pseudotyped baculovirus vector than by the wild-type baculovirus vector (Fig. 4B). Baculovirus vectors carrying shRNA thus appear to be able to inhibit HCV replication more effectively than simple siRNA.

In conclusion, we have demonstrated efficient inhibition of intracellular HCV replication by baculovirus-based shRNA-expressing vectors. The reduction in the amount of HCV mRNA is consistent with an shRNA-mediated degradation effect at the post-transcriptional level; this suggests that our RNAi approach might provide effective gene therapy for HCV infection, especially at the nuclear localization signal site (the shRNA452 target site) within the conserved core region. Furthermore, the VSV-G-modified baculovirus (Ac/VSV-G) transduced genes into NNC#2 cells more efficiently than wild-type baculovirus.

Acknowledgements: This work was supported, in part, by Grants-in-aid for research on hepatitis from the Ministry of Health, Labor, and Welfare of Japan, by Grant-in-Aid for High Technology Research No. 09309011 from the Ministry of Education, Science, Sports, and Culture, Japan, and by a Grant from the Research and Development Program for New Bio-industry Initiatives.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.07.056.

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