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Development of hepatitis C virus production reporter-assay systems using two different hepatoma cell lines

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A hepatitis C virus (HCV) infection system was developed previously using the HCV JFH-1 strain (genotype 2a) and HuH-7 cells, and this cell culture is so far the only robust production system for HCV. In patients with chronic hepatitis C, the virological effects of pegylated interferon and ribavirin therapy differ depending on the HCV strain and the genetic background of the host. Recently, we reported the hepatoma-derived Li23 cell line, in which the JFH-1 life cycle is reproduced at a level almost equal to that in HuH-7-derived RSc cells. To monitor the HCV life cycle more easily, we here developed JFH-1 reporter-assay systems using both HuH-7- and Li23-derived cell lines. To identify any genetic mutations by long-term cell culture, HCV RNAs in HuH-7 cells were amplified 130 days after infection and subjected to sequence analysis to find adaptive mutation(s) for robust virus replication. We identified two mutations, H2505Q and V2995L, in the NS5B region. V2995L but not H2505Q enhanced JFH-1 RNA replication. However, we found that H2505Q but not V2995L enhanced HCV RNA replication of strain O (genotype 1b). We also selected highly permissive D7 cells by serial subcloning of Li23 cells. The expression levels of claudin-1 and Niemann–Pick C1-like 1 in D7 cells are higher than those in parental Li23 cells. In this study, we developed HCV JFH-1 reporter-assay systems using two distinct hepatoma cell lines, HuH-7 and Li23. The mutations in NS5B resulted in different effects on strains O and JFH-1 HCV RNA replication.

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INTRODUCTION

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis and leads to liver cirrhosis and hepatocellular carcinoma. Elimination of HCV by antiviral reagents seems to be the most efficient therapy to prevent fatality.

HCV belongs to the family *Flaviviridae* and contains a positive ssRNA genome of 9.6 kb. The HCV genome encodes a single polyprotein precursor of approximately 3000 aa, which is cleaved by host and viral proteases into at least 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B (Kato, 2001; Kato *et al.*, 1990; Tanaka *et al.*, 1996).

Evaluation of anti-HCV reagents was difficult before the development of the HCV replicon system (Lohmann *et al.*, 1999). The HCV replicon system enabled investigation of anti-HCV reagents and the cellular factors involved in HCV RNA replication. Following introduction of the replicon system, genome-length HCV RNA-replication systems and reporter-assay systems were also developed (Ikeda *et al.*, 2002, 2005; Lohmann *et al.*, 2001; Pietschmann *et al.*, 2002). In 2005, an HCV infection system was developed using the genotype 2a JFH-1 strain (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005). The JFH-1 infection system has been used to study not only viral RNA replication, but also virus infection and release. This HCV cell-culture system was developed using the human hepatoma cell line HuH-7 and, thus far, HuH-7 is the only cell line to exhibit robust HCV production. Therefore, we intended to test the susceptibility of various other cell lines to HCV RNA replication. We reported previously that the hepatoma cell line Li23 supports robust HCV RNA replication and is also susceptible to authentic JFH-1 infection (Kato *et al.*, 2009). Microarray analysis

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Three supplementary figures are available with the online version of this paper.

revealed that HuH-7 and Li23 cells exhibited distinct gene-expression profiles (Mori *et al.*, 2010). For example, we identified three genes (New York oesophageal squamous cell carcinoma 1, β-defensin-1 and galectin-3) showing Li23-specific expression. Using HuH-7 and Li23 cells in combination with HCV strain O (genotype 1b), we developed drug-assay systems (OR6 and ORL8, respectively) by introducing the *Renilla* luciferase (RL) gene (Ikeda *et al.*, 2005; Kato *et al.*, 2009). We found and reported that the sensitivities to anti-HCV reagents were different between the HuH-7 and Li23 assay systems; for example, the Li23 assay system was 10 times more sensitive to ribavirin than the HuH-7 assay system (Mori *et al.*, 2011). Methotrexate showed very strong anti-HCV activity in the Li23 assay system, although it showed very weak anti-HCV activity in the HuH-7 assay system (Ueda *et al.*, 2011). These results encouraged us to develop a JFH-1 reporter-assay system using HuH-7 and Li23 cells. This JFH-1 reporter-assay system not only facilitated monitoring of virus infection and release steps, but also provided us with new information that could be missed in these steps when using only a HuH-7 assay system. However, increasing the size of the viral genome by introducing exogenous genes [RL and the encephalomyocarditis virus internal ribosomal site (EMCV-IRES)] reduced the efficiency of HCV RNA replication. To overcome this issue, we tried to improve the efficiency of HCV RNA replication by introducing adaptive mutations and by subcloning the parental cells.

Here, we developed JFH-1 HCV production reporter-assay systems in HuH-7- and Li23-derived cells using adaptive mutations and subcloned cells, which monitor the life cycle of HCV with luciferase activity. We also tested the effect of the mutations in NS5B from the JFH-1 strain on RNA replication of the specific genotype 1b O strain.

RESULTS

HCV mutations caused by long-term cell culture

The efficiency of HCV RNA replication depends on viral genetic mutations, host cells and viral genome size. For development of the HCV reporter-assay system, use of a longer viral genome reduced the efficiency of virus replication. To compensate for this issue, we tried to introduce adaptive mutations into the JFH-1 genome. We examined the viral sequences of JFH-1 130 days after infection of HuH-7-derived RSc cells. We performed RT-PCR for three parts of the viral genome: Core to NS2, NS3 to NS5A, and NS5B to 3′X. These three parts were separated by the *AgeI*, *SpeI*, *Bsr*GI and *XbaI* sites on the viral genome. We introduced PCR products into the cloning vector and three independent clones were subjected to sequencing analysis.

In the Core to NS2 region between the *AgeI* and *SpeI* sites (designated AS), there were eight common mutations with amino acid substitutions: lysine to glutamate at aa 78 (K78E) in Core, P251L and A351D in E1, V402A, I414T and K715N in E2, Y771C in p7, and D962G in NS2 (Fig. 1a). In the NS3 to NS5A region between *SpeI* and *Bsr*GI sites (designated SB), there were eight common mutations with amino acid substitutions: V1460I and M1611T in NS3, and I2270T, Q2307R, S2363L, M2392T, S2426A and C2441S in NS5A (Fig. 1b). In the NS5B to 3′X region between the *Bsr*GI and *XbaI* sites (designated BX), there was only one common mutation with an amino acid substitution, V2995L in NS5B (Fig. 1c). The determined sequences were studied further to enhance HCV RNA replication in the JFH-1 reporter assay.

Effect of genetic mutations on HCV RNA replication

To monitor the virus life cycle more easily, we constructed dicistronic JFH-1 with a reporter gene, pJR/C-5B. The first cistron contained the RL gene and was translated by the HCV-IRES. The second cistron contained the JFH-1 ORF and was translated by the EMCV-IRES. This construct facilitated monitoring of all steps of the virus life cycle by quantification of RL activity. However, the use of a longer viral genome resulted in lower replication efficiency. We tested the effect on HCV RNA replication of amino acid substitution caused during long-term cell culture.

The amino acid substitution clusters from three independent clones in Core to NS2 (AS-1, AS-2, AS-3) were introduced into pJR/C-5B. *In vitro*-transcribed HCV RNA was introduced into HuH-7-derived RSc cells, and RL activities were monitored 24, 48 and 72 h after electroporation (Fig. 2a). AS-3 exhibited higher replication efficiency than the wild type (WT). However, the replication efficiency of AS-2 was almost equal to that of the WT, and AS-1 exhibited lower replication efficiency than the WT. AS-3 possessed the highest replication efficiency among the tested JFH-1 mutants: at 72 h, the luciferase value of this clone was approximately 100 times that at 24 h.

The three pJR/C-5B constructs with mutations in NS3 to NS5A (SB-2, SB-3 and SB-4) were transcribed and introduced into RSc cells to compare the efficiency of HCV RNA replication (Fig. 2b). Unexpectedly, RL activity was not increased over 72 h after electroporation and exhibited a pattern similar to that of JFH-1 without the GDD motif. This result indicates that the mutation in NS3 to NS5A exhibited a negative effect on HCV RNA replication.

Finally, we tested the effect of the mutations in the NS5B region on HCV RNA replication. BX-2 contains two mutations with amino acid substitution (H2505Q and V2995L) and BX-7 contains only V2995L (Fig. 2c). JFH-1 with mutation(s) of BX-2 or BX-7 exhibited strong enhancement of HCV RNA replication. These results indicate that V2995L works as a strong replication-enhancing mutation (REM) in JFH-1 HCV RNA replication.

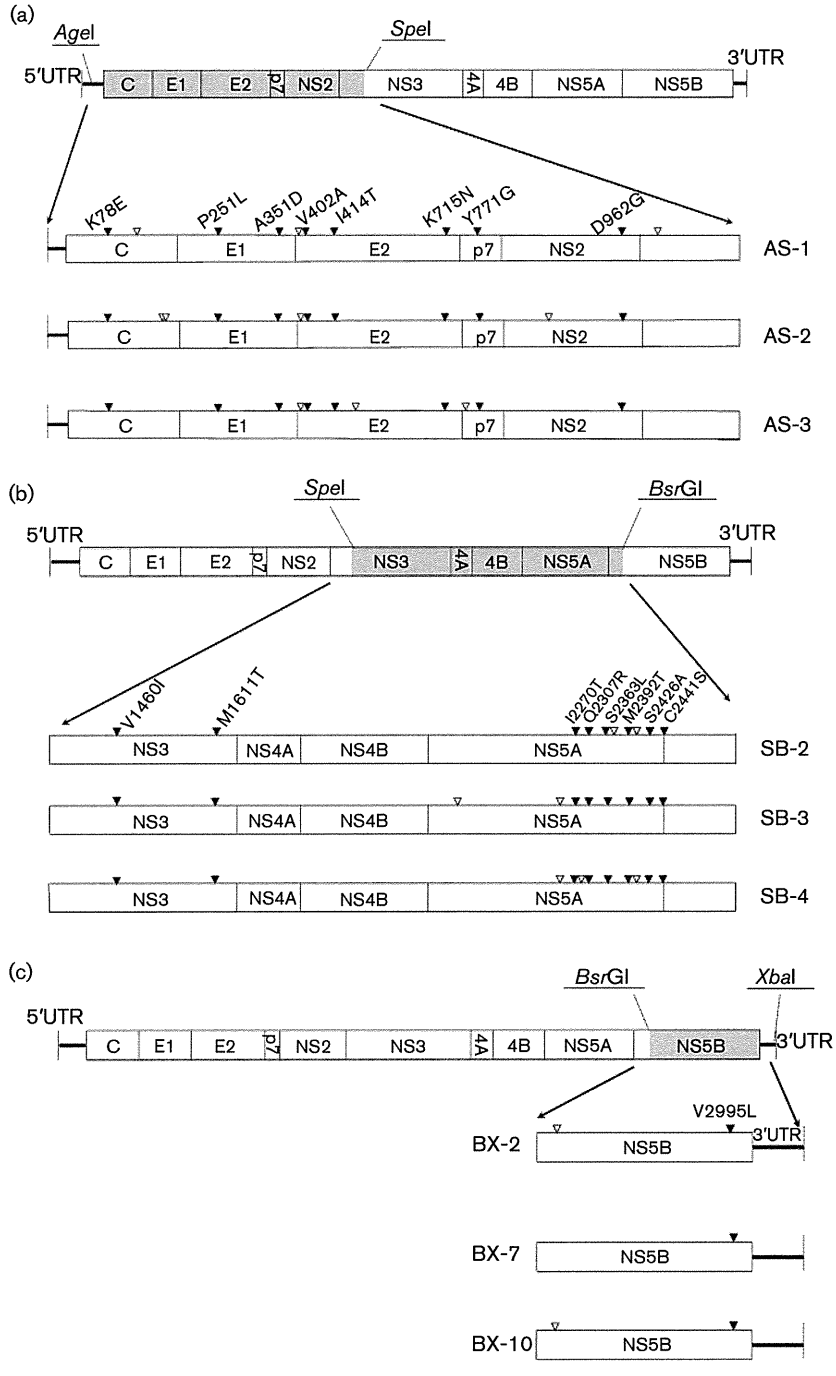

Fig. 1. Genetic mutations causing amino acid substitutions during long-term HCV infection. RT-PCR was performed for HCV RNAs from HuH-7 cells 130 days after JFH-1 infection. PCR products were subcloned into the pBluescript II plasmid. Three clones of (a) the Core to NS2 region between the *Age*I and *Spe*I sites (AS), (b) the NS3 to NS5A region between the *Spe*I and *Bsr*GI sites (SB) and (c) the NS5B to 3′X region between the *Bsr*GI and *Xba*I sites (BX) were subjected to sequencing analysis. ▼ and ▽ represent conservative and non-conservative amino acid substitutions, respectively.

Mutations in NS5B enhanced HCV RNA replication differently in genotypes 1b and 2a

V2995L in NS5B is a common substitution, occurring in three clones, and H2505Q is conserved in two clones (BX-2 and BX-10). We examined the corresponding amino acids at positions 2995 and 2505 in genotype 1b replication-competent HCV strains O, 1B-4 and KAH5 (Fig. 3a) (Nishimura *et al.*, 2009). The histidine at aa 2505 in JFH-1 is conserved in O, 1B-4 and KAH5 at the corresponding position, aa 2482. The valine at aa 2995 in JFH-1 is an alanine in O, 1B-4 and KAH5 at the corresponding position, aa 2972 (Fig. 3a). It is not clear whether the adaptive mutation found in genotype 2a is effective in genotype 1b HCV. Therefore, we investigated the effect of V2995L and/or H2505Q substitution on genotype 1b HCV RNA replication. We introduced substitutions V2995L and/or H2505Q into the subgenomic replicon, pOR/3-5B (HCV-O). In contrast to the case of JFH-1, H2505Q but not V2995L enhanced HCV-O RNA replication (Fig. 3b). These results indicate that the mutations in NS5B derived from JFH-1 functioned differently in genotype 1b HCV-O RNA replication.

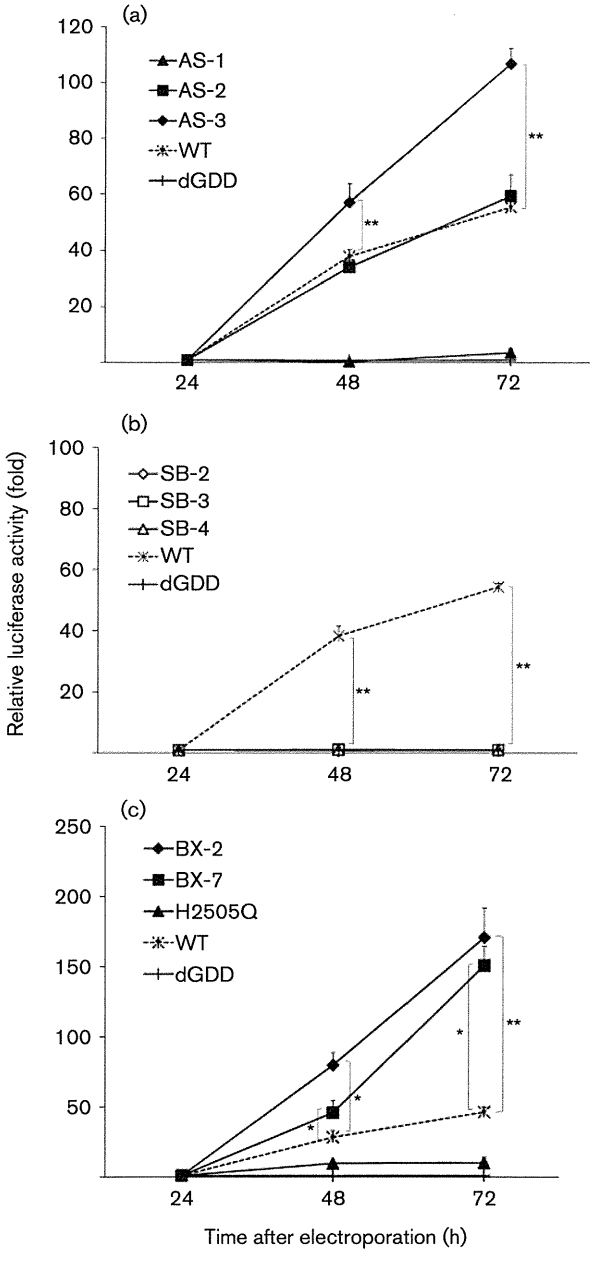

Fig. 2. Effect of amino acid substitutions on HCV RNA replication. (a) The Core to NS2 region; (b) the NS3 to NS5A region; (c) the NS5B to 3′X region. Amino acid substitutions were introduced into pJR/C5B and *in vitro*-synthesized RNAs were electroporated into HuH-7-derived RSc cells. RL activity was determined 24, 48 and 72 h after electroporation. dGDD, Negative control without the GDD motif; WT, wild type. $*P<0.05$; $**P<0.01$.

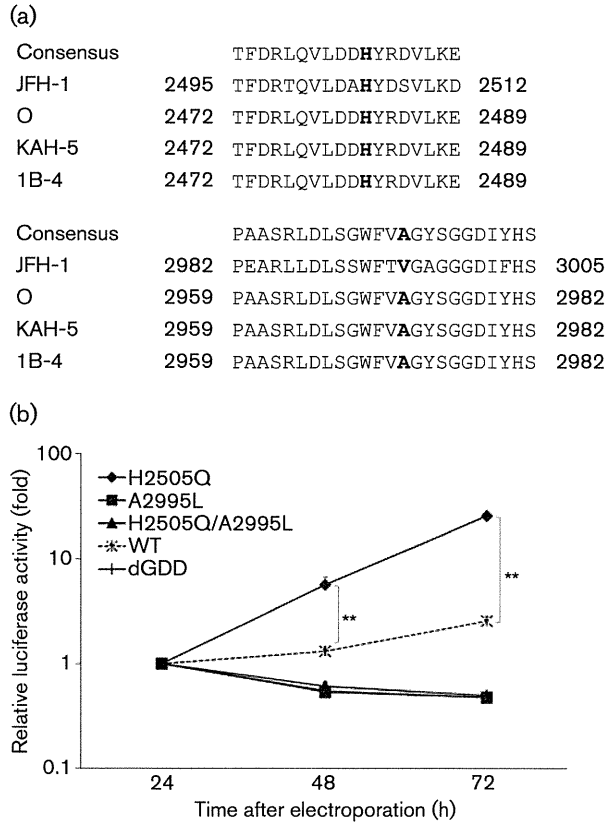

Fig. 3. Effect of amino acid substitutions in NS5B on genotype 1b and 2a HCV RNA replication. (a) Alignment of amino acids at positions 2505 (JFH-1) and 2482 (genotype 1b) and around the adjacent region (upper panel). Alignment of amino acids at positions 2995 (JFH-1) and 2972 (genotype 1b) and around the adjacent region (lower panel). The HCV strains O, KAH5 and 1B-4 belong to genotype 1b. (b) H2505Q and/or V2995L were introduced into the HCV-O subgenomic replicon (pOR/3-5B), and transcribed RNAs were electroporated into RSc cells. RL activities were tested 24, 48 and 72 h after infection. dGDD, Negative control without the GDD motif; WT, wild type. $**P<0.01$.

HCV infection in HuH-7- and Li23-derived cell lines

As well as viral genetic mutations, the choice of host cells is important for the efficiency of HCV RNA replication. Cured cells in which HCV RNAs were eliminated by IFN-α, such as HuH-7.5, HuH-7.5.1 and our RSc cells, exhibit higher replication efficiency than their parental HuH-7 cells (Ariumi *et al.*, 2007; Blight *et al.*, 2002; Zhong *et al.*, 2005). Therefore, we examined whether subcloned Li23 cells might enhance HCV RNA replication. We performed serial subcloning of Li23 cells from Li23-derived ORL8c cells by the limiting-dilution method (Fig. 4a). ORL8c cells are a cured cell line in which genome-length HCV RNAs were eliminated by interferon (IFN) treatment (Kato *et al.*, 2009). The subclonal Li23-derived cell lines were selected from among 50–100 independent single cells in 96-well plates by three-round limiting dilution from ORL8c cells (Fig. S1a, available in JGV Online). First, L8c15 cells were selected from their parental ORL8c cells by limiting dilution. Then, C22 cells were selected from their parental L8c15 cells by limiting dilution. Finally, D7 cells were selected from C22 cells by limiting dilution (Fig. S1b). Together, these steps resulted in the

selection of three subclonal cell lines that respectively exhibited the strongest replication efficiency in each round of selection. The lineages of the selected cell lines after three rounds of subcloning were designated L8c15, C22 and D7 cells, respectively.

We tested the subcloned cells for their HCV infectivities in comparison with those of HuH-7 and HuH-7-derived RSc cells. We reported previously that RSc cells could strongly support HCV replication and production (Kato *et al.*, 2009). Li23 and its derived ORL8c, L8c15, C22 and D7 cell lines were infected using the supernatant from RSc cells replicating JR/C-5B with BX-2 mutations at an m.o.i. of 0.2 (Fig. 4b, c). RL activities were determined 24, 48, 72 and 96 h after infection and f.f.u. ml^{-1} were determined 48 h after infection. The efficiency of HCV infectivity was highest in D7 cells, followed in order by C22, L8c15 and Li23 cells. HCV RNA replication in D7 cells was almost equal to that in RSc cells. These results suggest that the subcloned cell lines exhibit higher susceptibility to HCV infection than their parental cells.

Next, we further characterized the susceptibility of D7 cells to HCV infection in comparison with RSc cells, because D7 cells exhibited the highest susceptibility to HCV infection among the Li23-derived cell lines. D7 cells also exhibited the highest production and release of Core into the supernatant among the parental C22-derived subclonal

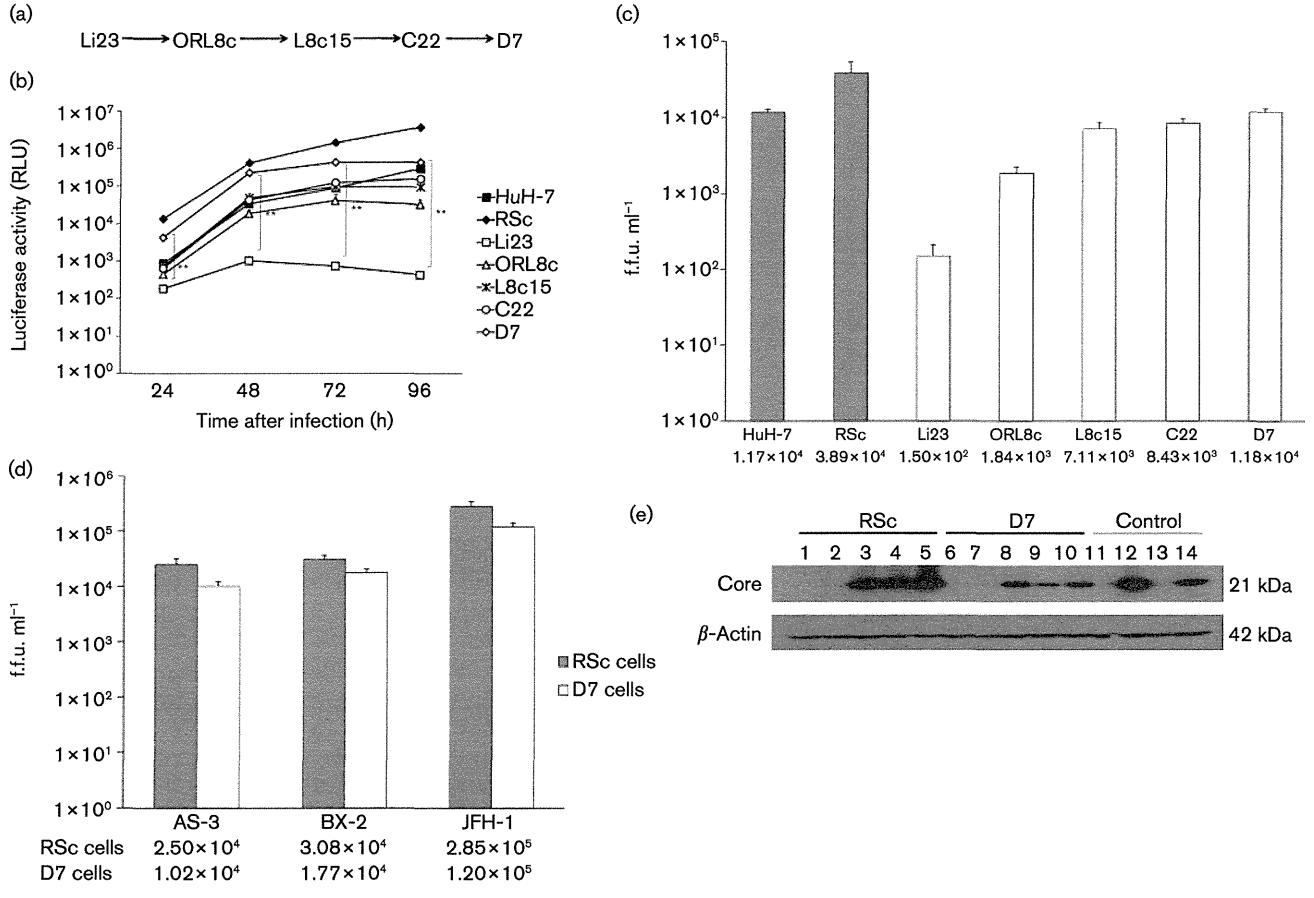

Fig. 4. HCV infection in HuH-7- and Li23-derived cell lines. (a) History of the selection of subclonal Li23-derived cell lines. (b) HuH-7, HuH-7-derived RSc, and Li23-derived ORL8c, L8c15, C22 and D7 cells were inoculated with supernatant from RSc cells replicating JR/C5B/BX-2. $^{**}P<0.01$. (c) f.f.u. ml^{-1} values were determined 48 h after infection of HuH-7- and Li23-derived cells with HCV using the supernatant from RSc cells replicating JR/C5B/BX-2 . (d) f.f.u. ml^{-1} values were determined 48 h after infection of RSc or D7 cells with HCV using the supernatant from RSc cells replicating JR/C5B/AS-3 or JR/C5B/BX-2. Supernatant from authentic JFH-1-replicating RSc cells was used as a positive control. (e) Core expression levels in RSc or D7 cells were determined 1, 2, 3 and 4 days after infection with JFH-1 with BX-2 mutations. Lanes: 1 and 6, mock-infected cells; 2 and 7, cells 1 day after infection; 3 and 8, cells 2 days after infection; 4 and 9, cells 3 days after infection; 5 and 10, cells 4 days after infection; 11 and 12, OR6c and OR6 cells, respectively; 13 and 14, ORL8c and ORL8 cells, respectively. OR6 and ORL8 were used as positive controls; OR6c and OR8c were used as negative controls. β-Actin was used as a control for the amount of protein loaded per lane.

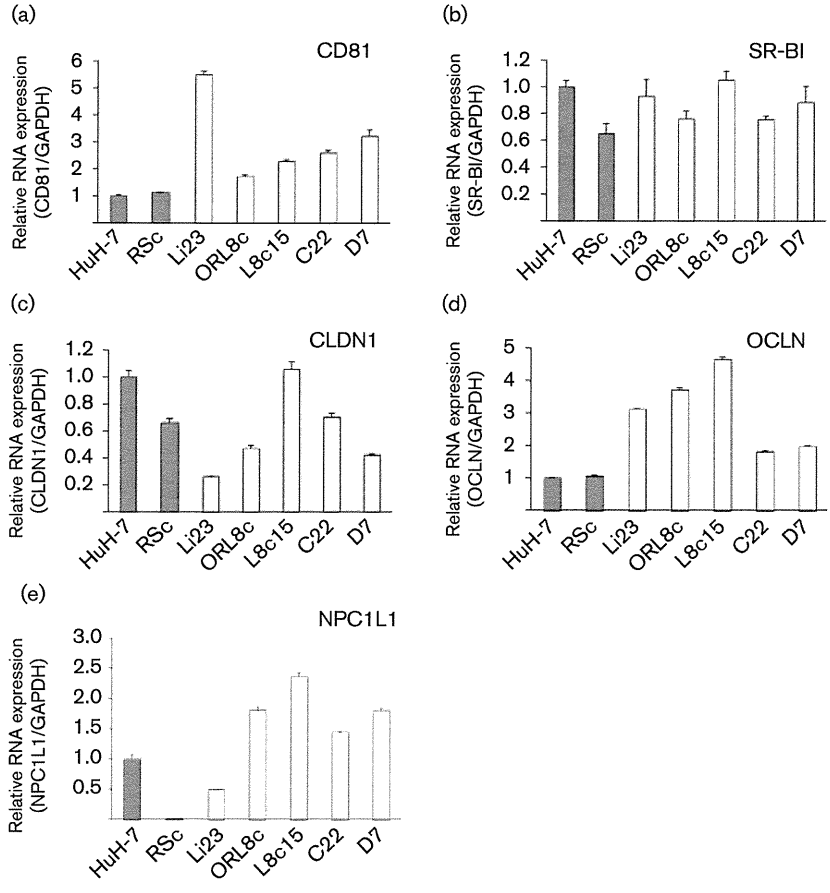

Fig. 5. Expression levels of HCV receptors in HuH-7- and Li23-derived cells. Quantitative RT-PCR was performed for CD81, SR-BI, CLDN1, OCLN and NPC1L1 as described in Methods. Relative expression levels of mRNA are shown, when the expression level of each receptor in HuH-7 was assigned to be 1. GAPDH was used as an internal control. Experiments were done in triplicate.

cells (Fig. S1b). The susceptibility of the HCV reporter-assay system to HCV infection was examined using HuH-7- and Li23-derived cells. Supernatants from RSc cells replicating JR/C-5B with AS-3 or BX-2 mutations were used as inocula. The supernatant from authentic JFH-1-replicating RSc cells was used as a positive control. RSc and D7 cells were inoculated with each HCV-containing supernatant and f.f.u. ml^{-1} were determined 48 h after infection. As shown in Fig. 4(d), the values of f.f.u. ml^{-1} for AS-3 were 2.5×10^4 and 1.0×10^4 in RSc and D7 cells, respectively; those for BX-2 were 3.1×10^4 and 1.8×10^4 in RSc and D7 cells, respectively; and those for authentic JFH-1 were 2.9×10^5 and 1.2×10^5 in RSc and D7 cells, respectively. These results indicate that the infectivities of these three inocula were almost equal in RSc and D7 cells.

Next we examined Core expression after infection of RSc and D7 cells with HCV, as D7 cells exhibited the highest infectivity among the Li23-derived cell lines (Fig. 4e). Core was detected 2, 3 and 4 days after infection of the supernatant from RSc cells infected by JR/C-5B with BX-2. Although Core expression in D7 cells was slightly weaker than that in RSc cells, the signal of Core in HCV-infected D7 cells was equal to that in stable ORL8 cells. These results suggest that the JFH-1 reporter-assay system in Li23 cells is useful not only for the RL assay, but also for Core expression.

Expression of HCV receptors in parental and subclonal hepatoma cell lines

We tested expression of the HCV receptors CD81, scavenger receptor class B member I (SR-BI), claudin-1 (CLDN1) and occludin (OCLN). We also examined the expression of the recently reported HCV entry factor Niemann–Pick C1-like 1 (NPC1L1) (Sainz *et al.*, 2012). Expression levels of CD81 in Li23 and its subclonal cells were higher than those in HuH-7 and RSc cells (Fig. 5a). Although expression of CD81 in Li23-derived cell lines was lower than that in parental Li23 cells, interestingly the expression levels of CD81 increased during the rounds of selection. There is no difference in the expression of SR-BI among the cell lines tested (Fig. 5b). The expression of CLDN1 in Li23-derived cells was higher than that in parental Li23 cells (Fig. 5c). Expression levels of OCLN in Li23 and its subclonal cells were higher than those in HuH-7 and RSc cells (Fig. 5d). Finally, the expression of NPC1L1 in Li23-derived cell lines was higher than that in parental Li23 cells (Fig. 5e). It is noteworthy that the expression level of NPC1L1 in RSc cells was approximately 2 $\log_{10}$ lower than that in parental HuH-7 cells. Taken together, these results indicate that the expression levels of CLDN1 and NPC1L1 in Li23-derived cells were higher than those in parental Li23 cells.

Life cycle of the HCV reporter-assay system in Li23-derived cells

We investigated whether D7 cells produce infectious HCV. First, D7 cells were inoculated using the supernatant from RSc cells replicating JR/C5B with BX-2, and the supernatant was stored at 17 days after infection. Then, the supernatant derived from the D7 cells was used as an inoculum for reinfection of naïve D7 cells. RL activities were determined 2, 6, 10 and 14 days after reinfection (Fig. 6). RL activity was increased after reinfection in D7 cells and reached a plateau 10 days after reinfection. These data indicate that the JFH-1 reporter-assay system is also useful for monitoring the HCV life cycle in Li23-derived cell lines.

DISCUSSION

In this study, we developed an HCV production reporter-assay system using two distinct hepatoma cell lines, HuH-7 and Li23. Robust HCV RNA replication and virus production were achieved by the introduction of REMs into the structural region or the NS5B region. These REMs were obtained from JFH-1-infected long-term-cultured cells. The two REMs in NS5B (H2505Q and V2995L substitutions) derived from JFH-1 had different effects on replication of genotype 1b HCV-O RNA and genotype 2a JFH-1 RNA. Furthermore, the subcloned Li23-derived D7 cells produced by serial limiting dilution supported this HCV production reporter-assay system.

Several groups have reported JFH-1 reporter virus systems (Koutsoudakis *et al.*, 2006; Marcello *et al.*, 2006; Pietschmann *et al.*, 2002; Wakita *et al.*, 2005). However, robust reporter virus production was limited within the study using HuH-7-derived cells. Therefore, we attempted to developed a JFH-1 reporter virus assay system using our previously reported line of Li23 cells (Kato *et al.*, 2009).

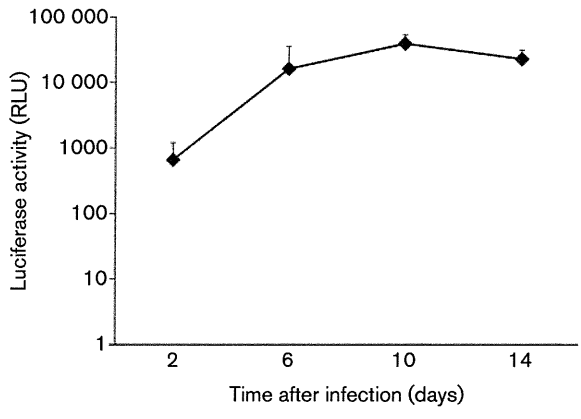

Fig. 6. HCV life cycle in Li23-derived D7 cells. D7 cells were inoculated with the supernatant from D7 cells after infection with JFH-1 with BX-2 mutants. RL activities were tested 2, 6, 10 and 14 days after infection.

The introduction of RL and EMCV-IRES genes into the HCV gene lengthened the genome of HCV by approximately 1.9 kb and led to a reduction in the efficiency of HCV RNA replication. To overcome this disadvantage, we adopted the following strategies: (i) introduce the REMs; (ii) select cloned Li23-derived cells with a highly permissive host condition by the serial limiting-dilution method. For the first purpose, we performed sequence analyses for HCV RNA from JFH-1-infected RSc cells. Mutations in the region from Core to NS2 or NS5B enhanced HCV RNA replication. However, combination of mutations from two different regions reduced HCV RNA replication (Fig. S2). The reason for this may be that these two mutation clusters were obtained from distinct RT-PCR-amplified clones and they were not necessarily located on the same viral genome. It has been reported that the combination of REMs exhibited an antagonistic effect on HCV RNA replication (Lohmann *et al.*, 2001). For the second purpose, we selected highly permissive Li23-derived clonal cells by the limiting-dilution method. We obtained three Li23-derived subclonal cell lines, L8c15, C22 and D7, in order from parental Li23-derived ORL8c cells. The efficiency of infectivity was highest in D7 cells, followed in order by C22, L8c15 and Li23 cells. D7 cells were highly permissive for infection of HCV with NS5B mutations.

As shown in Fig. 3(a), the histidine at aa 2505 in JFH-1 was conserved in the replication-competent O, 1B-4 and KAH5 strains at the corresponding position, aa 2482. The valine at aa 2995 in JFH-1 was alanine in strains O, 1B-4 and KAH5 at the corresponding position, aa 2972. The REMs in genotype 1b HCV were usually obtained by selection with neomycin after HCV RNA electroporation. Pietschmann *et al.* (2009) reported that REMs impaired infectious virus production. Most REMs are located in the NS3 and NS5A regions (Abe *et al.*, 2007; Blight *et al.*, 2002; Lohmann *et al.*, 2001; Pietschmann *et al.*, 2002). NS5A is a key molecule for virus production, and REMs affect the phosphorylation status of NS5A and the interaction with Core (Kato *et al.*, 2008; Masaki *et al.*, 2008; Tellinghuisen *et al.*, 2008). In contrast, our REMs in NS5B were obtained in JFH-1-infected long-term cell culture without drug selection. Taking this information into account, we considered that H2505Q in NS5B might not interfere with genotype 1b virus production. We attempted to apply this REM from genotype 2a to genotype 1b and found that H2505Q enhanced replication of the genotype 1b HCV-O replicon. We are currently investigating whether our NS5B REM could enhance genotype 1b HCV production. As for the substitution at aa 2995 in JFH-1 (aa 2972 in genotype 1b), we should be careful in interpretation, because the backgrounds at this position are different between genotypes 2 and 1. Analysis of an HCV database (http://s2as02.genes.nig.ac.jp/) revealed that the consensus amino acids at position 2995 in genotype 2 and at 2972 in genotype 1 were valine and alanine, respectively. Furthermore, alanine and valine are not found at position 2995 in genotype 2 or at 2972 in genotype 1, respectively. These observations

indicate that amino acid substitution between alanine and valine at these positions may be lethal for HCV of both genotypes. The amino acid at position 2995 in genotype 2 (2972 in genotype 1) is just upstream of a *cis*-acting replication element in NS5B. Therefore, the nucleotide at this position may affect the HCV RNA replication. To clarify this issue, further study will be needed.

A comparative study using HuH-7- and Li23-based JFH-1 reporter-assay systems would be expected to reveal new information on virus entry and release steps, because the backgrounds of these cells are different. Our recent study of these cells revealed the difference in sensitivities to anti-HCV reagents including ribavirin and methotrexate (Mori *et al.*, 2011; Ueda *et al.*, 2011). Furthermore, the IL28B genotype was different between HuH-7 and Li23 cells. The IL28B genotype (rs8099917) of HuH-7 cells renders them resistant to pegylated IFN and ribavirin, and Li23 cells are sensitive to pegylated IFN and ribavirin (M. Ikeda and N. Kato, unpublished data).

Recently, it was reported that stable expression of miR122 enhanced JFH-1 HCV production in Hep3B and HepG2 (Kambara et al., 2012; Narbus et al., 2011). It is noteworthy that the expression of miR122 in Li23-derived cells was almost the same as that in HuH-7 cells (Fig. S3). High-level expression of miR122 in Li23 cells may be one of the reasons that Li23 cells can support HCV production as robust as that in HuH-7 cells among the hepatocyte-derived cell lines. Interestingly, the expression levels of miR122 are higher in ORL8c, L8c15 and D7 cells, but not in C22 cells, than those in parental Li23 cells (Fig. S3). This result suggests that the expression level of miR122 may partly contribute to the fitness of HCV replication and production.

So far, we have only little information regarding the mechanism by which subclonal cells support HCV replication and production more efficiently than the parental cells. In this study, we found that the expression levels of CLDN1 and NPC1L1 in Li23-derived subclonal cells were higher than those in the parental Li23 cells. These results suggest that a high expression level of these entry factors in Li23-derived subclonal cells may contribute to enhanced virus entry. In the course of the experiment to determine the expression levels of NPC1L1 in HuH-7- and Li23-derived cell lines, we found that RSc cells expressed a very low level of NPC1L1 compared with the parental HuH-7 cells. Possible mechanisms for this are: (i) very low-level expression of NPC1L1 is sufficient for HCV entry; (ii) an unknown entry factor compensates for NPC1L1 in the entry step in RSc cells. Further study will be needed to clarify this issue.

In summary, we have developed JHF-1 reporter-assay systems using HuH-7-derived RSc and Li23-derived D7 cells. Expression levels of CLDN1 and NP1C1L were higher than those in the parental Li23 cells. We found different effects of REMs (V2995L and H2505Q) in NS5B on virus RNA replication in genotype 2a and 1b HCV strains. These findings will become useful tools for the study of the life cycle of HCV.

METHODS

Cell cultures. RSc and ORL8c cells were derived from the cell lines HuH-7 and Li23, respectively, as described previously (Kato *et al.*, 2009). L8c15, D7 and C22 cells were selected from ORL8c, L8c15 and C22 cells, respectively, by limiting dilution. HuH-7 and RSc cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% FBS (Life Technologies). Li23-derived cell lines were maintained in F12 medium (Life Technologies) and DMEM (1 : 1 in volume) supplemented with 1% FBS and epidermal growth factor (50 ng ml^{-1}; PeproTech, Inc.) as described previously (Kato *et al.*, 2009).

RT-PCR and sequencing analysis. RSc cells were infected with cell-culture-grown HCV (HCVcc) and cultured for 130 days. Total RNAs from these cells were prepared using an RNeasy extraction kit (Qiagen). These RNA samples were used for RT-PCR in order to amplify the Core to NS2 (4.0 kb), NS3 to NS5A (3.6 kb) and NS5B to 3′X (1.9 kb) regions. Reverse transcription was performed with an oligo$(dA)_{23}$ primer. The following primer pairs were employed: to amplify the Core to NS2 region, JFH-1/*Age*I (5′-CCCAAGCTTAC-CGGTGAGTACACCGGAATTGC-3′) and JFH-1/*Spe*IR (5′-TGCCA-TGTGCCTTGGATAGGTACG-3′); for the NS3 to NS5A region, JFH-1/*Spe*I (5′-CCCAGGGGTACAAAGTACTAGTGC-3′) and JFH-1/*Bsr*GIR (5′-CCCAAGCTTTACCTTTTTAGCCCTCTGTGAGGC-3′); for the NS5B to 3′X region, JFH-1/*Bsr*GI (5′-CCGCTCGAGACCC-TTTGAGTAACTCGCTGTTGC-3′) and JFH-1/*Xba*IR (5′-GCTCTA-GACATGATCTGCAGAGAGACCAGTTAC-3′). SuperScript III reverse transcriptase (Invitrogen) and KOD-plus DNA polymerase (TOYOBO) were used for reverse transcription and PCR, respectively. PCR products were ligated into pBluescript II (Fermentas) and three independent clones were subjected to sequencing analysis.

Plasmid construction. pJR/C-5B plasmid is a dicistronic HCV JFH-1 construct. The RL gene and HCV ORF were introduced into the first and second cistrons, respectively. To construct this plasmid, we fused the JFH-1 5′UTR with the RL gene by overlap PCR, and the PCR products were ligated into pFGR-JFH-1 (GenBank accession no. AB237837) at the *Age*I and *Pme*I sites. For the first PCR, the primer pair 5′-GCGCCTAGCCATGGCGTTAGTATG-3′ (J5dC) and 5′-AAGCCATGGCCGGCCCTGGGCGACGGTTGGTGTTTCTTTTGG-3′ (J5dCR) was employed to amplify the 5′UTR, and the primer pair 5′-AACCGTCGCCCAGGGCCGGCCATGGCTTCCAAGGTGTACG-ACCCC-3′ (JRL) and 5′-TCGAAATCTCGTGATGGCAGGTTGG-3′ (JRLR) was employed to amplify the RL region. These first PCR products were used in the second PCR as the templates. For the second PCR, the primer pair J5dC and JRLR was employed to amplify the 5′UTR and RL. KOD-plus DNA polymerase was used for PCR.

The H2505H and/or A2995L mutations were introduced into the HCV-O replicon by QuikChange mutagenesis (Stratagene) as described previously (Ikeda *et al.*, 2002).

Luciferase reporter assay. For the luciferase assay, approximately $1.0–1.5 \times 10^4$ HCV-harbouring cells were plated onto 24-well plates in triplicate and were cultured for 24–96 h after electroporation or infection, as described previously (Ikeda *et al.*, 2005). The cells were harvested with *Renilla* lysis reagent (Promega) and subjected to RL assay according to the manufacturer's protocol.

Western blot analysis. Preparation of cell lysates, SDS-PAGE and immunoblotting were performed as described previously (Kato *et al.*, 2003). The antibodies used in this study were Core (CP11; Institute of Immunology, Tokyo, Japan) and β-actin (AC-15; Sigma) antibodies. Immunocomplexes were detected with a Renaissance enhanced chemiluminescence assay (PerkinElmer Life Science).

HCV infection and determination of f.f.u. To determine f.f.u. ml^{-1}, 6×10^3 cells were plated onto a 96-well plate 24 h before infection. The supernatant of HCV RNA-replicating cells was diluted serially and was used as an inoculum. Forty-eight hours after infection, the cells were fixed and Core was stained with anti-Core antibody and HRP-conjugated mouse anti-IgG antibody. Then, the expression of Core was visualized with a DAB substrate kit (DAKO). Culture supernatants and cells were collected for quantification of Core by ELISA (Mitsubishi Kagaku Bio-Clinical Laboratories).

Quantitative RT-PCR analysis. Quantitative RT-PCR analysis for HCV receptors was performed using real-time LightCycle PCR (Roche Diagnostics) as described previously (Ikeda *et al.*, 2005). The primer pairs for CD81, SR-BI, CLDN1 and OCLN were reported previously (Nakamuta *et al.*, 2011). The primer pair NPC1L1 (5′-AGATCTTCTTCTTCCGCCTCCA-3′) and NPC1L1R (5′-TGCCAG-AGCCGGGTTAAC-3′) was used for NPC1L1.

Statistical analysis. Luciferase activities were compared statistically between the various treatment groups using Student's *t*-test. *P*-values of <0.05 were considered statistically significant. The mean $\pm$ SD was determined from at least three independent experiments.

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Raloxifene inhibits hepatitis C virus infection and replication

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ABSTRACT

Postmenopausal women with chronic hepatitis C exhibited a poor response to interferon (IFN) therapy compared to premenopausal women. Osteoporosis is the typical complication that occurs in postmenopausal women. Recently, it was reported that an osteoporotic reagent, vitamin D3, exhibited anti-hepatitis C virus (HCV) activity. Therefore, we investigated whether or not another osteoporotic reagent, raloxifene, would exhibit anti-HCV activity in cell culture systems. Here, we demonstrated that raloxifene inhibited HCV RNA replication in genotype 1b and infection in genotype 2a. Raloxifene enhanced the anti-HCV activity of IFN-α. These results suggest a link between the molecular biology of osteoporosis and the HCV life cycle.

1. Introduction

Hepatitis C virus (HCV) belongs to the *Flaviviridae* family and contains a positive single-stranded RNA genome of 9.6 kb. The HCV genome encodes a single polyprotein precursor of approximately 3000 amino acid residues, which is cleaved by the host and viral proteases into at least 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, nonstructural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [1–3].

The virological study and screening of antiviral reagents for HCV was difficult until the replicon system was developed [4–7]. In 2005, an infectious HCV production system was developed using genotype 2a HCV JFH-1 and hepatoma-derived HuH-7 cells, and the HCV life cycle was reproduced in a cell culture system [8]. We previously developed genome-length HCV reporter assay systems using HuH-7-derived OR6 cells [4]. In OR6 cells, the genotype 1b HCV-O with renilla luciferase (*RL*) replicates robustly. We also developed an HCV JFH-1 reporter infection assay system [9].

HCV infection frequently causes chronic hepatitis (CH) and leads to serious liver cirrhosis and hepatocellular carcinoma. Therefore, HCV infection is a major health problem worldwide. The elimination of HCV by antiviral reagents seems to be the most efficient therapy for preventing the fatal state of the disease. Pegylated-interferon (PEG-IFN) with ribavirin (RBV) is the current standard therapy for CH–C, but its sustained virological response (SVR) rate has remained 40–50%. Recently, a protease inhibitor, telaprevir, improved the SVR rate by up to 60–70% in combination with PEG-IFN/RBV [10]. The response to PEG-IFN/RBV therapy depends on host factors as well as viral factors. Among the host factors, age and gender are known to be associated with the outcome of IFN/RBV therapy [11,12]. Postmenopausal women with CH–C exhibited a poor response to IFN therapy compared to premenopausal women [11]. The decrease in estrogen may affect the response to IFN therapy. Dyslipidemia and osteoporosis are the typical complications in postmenopausal women. We and other groups reported that statins, which are dyslipidemia reagents, inhibited HCV proliferation in vitro and in vivo [13–17]. Recently it was reported that vitamin D3, an osteoporotic reagent, exhibited anti-HCV activity in vitro and in vivo [18–21]. It was also reported that 17β-estradiol inhibited the production of infectious HCV [22]. Taken together, these reports suggest an association between hepatitis C and complications due to the decrease of estrogen.

Raloxifene and tamoxifen are synthetic selective estrogen receptor modulators (SERMs) and are used for breast cancer and osteoporosis, respectively, in clinical settings. The responses of SERMs are mediated by estrogen receptors (ERs), either ERα or ERβ. SERMs exhibit agonistic actions in some tissues but antagonistic actions in others. Both raloxifene and tamoxifen are antagonists in breast and agonists in bone. However, only tamoxifen, and not raloxifene, exhibited agonistic activity in the uterus. It was reported that tamoxifen inhibited HCV RNA replication [23]. However, tamoxifen's agonist action leads to uterine cancer. Raloxifene belongs to an antiosteoporotic reagent and offers the advantage of safety without uterine cancer. Therefore, we decided to investigate whether or not raloxifene would exhibit anti-HCV activity in our developed cell culture systems.

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2. Materials and methods

2.1. Reagents and antibodies

Raloxifene was purchased from LKT Laboratories, Inc. (St. Paul, MN). IFN-α and tamoxifen were purchased from Sigma–Aldrich (St. Louis, MO). Pitavastatin (PTV) was purchased from Kowa Company (Nagoya, Japan). The antibodies used in this study were those specific to HCV Core (CP11, Institute of Immunology, Tokyo, Japan), NS3 (Novocastra Laboratories, Newcastle, UK), and β-actin (Sigma).

2.2. Cell culture and HCV RNAs

HuH-7 cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% fetal bovine serum, penicillin, and streptomycin. HuH-7-derived OR6 and sOR cells were genome-length and subgenome HCV (O strain of genotype 1b) RNA harboring cells, respectively and cultured in the above medium supplemented with G418 (0.3 mg/ml; Geneticin, Invitrogen) [4]. HCVs replicating in OR6 and sOR cells contain *RL* and neomycin phosphotransferase (*NPT*) genes after 5′-untranslated region (UTR). HuH-7-derived RSc cells are cured cells, in which HCV RNA was eliminated by IFN-α; they are used for HCV JFH-1 infection [9]. RSc cells are also used for subgenomic JFH-1 RNA (JRN/35B) replication. JRN/35B contains *RL* and *NPT* genes after 5′-UTR.

2.3. RL assay

For the RL assay, 1.5×10^4 OR6 were plated onto 24-well plates in triplicate and cultured for 24 h. The cells were treated with each reagent for 72 h. Then the cells were harvested with *Renilla* lysis reagent (Promega, Madison, WI) and subjected to RL assay according to the manufacturer's protocol.

2.4. WST-1 cell proliferation assay

The cells (2×10^3 cells) were plated onto a 96-well plate in triplicate at 24 h before treatment with each reagent. At 72 h after treatment, the cells were subjected to a WST-1 cell proliferation assay (Takara Bio, Otsu, Japan) according to the manufacturer's protocol.

2.5. Western blot analysis

For Western blot analysis, 4×10^4 cells were plated onto 6-well plates, cultured for 24 h, and then treated with reagent(s) for 72 h and 120 h. Preparation of the cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting were then performed as previously described [24]. Immunocomplexes on the membranes were detected by enhanced chemiluminescence assay (Renaissance; Perkin Elmer Life Science, Wellesley, MA).

2.6. HCV infection

RSc cells (1.5×10^4 cells) were plated onto a 24-well plate 24 h before infection. To evaluate the effect of the treatment prior to infection, the cells were first treated with raloxifene for 24 h, then inoculated with reporter JFH-1 (JR/C5B/BX-2) supernatant at a multiplicity of infection (MOI) of 0.2, cultured for 48 h, and subjected to RL assay as described previously [9]. The JR/C5B/BX-2 contains the *RL* gene in the first cistron following the encephalomyocarditis virus-internal ribosomal entry site (*EMCV-IRES*) gene and the open reading frame (ORF) of JFH-1 in the second cistron. To evaluate the effect of the treatment after infection, the cells were inoculated with reporter JFH-1 supernatant at MOI of 0.2, cultured for 72 h, and subjected to RL assay.

3. Results

3.1. Raloxifene inhibited HCV RNA replication

The HCV RNA that replicated in HuH-7-derived OR6 cells was a genome-length HCV with *RL*, *NPT*, and *EMCV-IRES* in the first cistron and the ORF of HCV (O strain of genotype 1b) in the second cistron [4]. OR6 cells could not produce infectious HCV. Therefore, we can monitor the replication step in the HCV life cycle using OR6 cells. Raloxifene inhibited HCV RNA replication in a dose-dependent manner, and its 50% effective concentration (EC_{50}) was 1 μM (Fig. 1A). Raloxifene did not exhibit cytotoxicity to OR6 cells until 2.5 μM (Fig. 1B). Raloxifene also inhibited intracellular Core and NS3 production in a dose- and time-dependent manner (Fig. 1C). The intensities of Core and NS3 in OR6 cells treated with 2.5 μM of raloxifene decreased to almost the level of cells treated with 10 IU/ml of IFN-α at 120 h after treatment. We also examined anti-HCV activity of raloxifene using subgenomic HCV replicon harboring sOR cells. Raloxifene exhibited weak anti HCV activity to sOR cells as compared with OR6 cells (Supplementary Figs. 1A and 1B). These results suggest that raloxifene exhibits anti-HCV activity and decreased the expression levels of HCV proteins more slowly compared to IFN-α.

3.2. Raloxifene enhanced anti-HCV activity of IFN-α

We investigated the anti-HCV activity of raloxifene in combination with a representative anti-HCV reagent, IFN-α. HCV RNA replication decreased in a dose-dependent manner after co-treatment with IFN-α and raloxifene (Fig. 2A). The results were almost similar to the expected effect of raloxifene in combination with IFN-α calculated from the anti-HCV activity of each reagent (Fig. 2B). These results indicate that the anti-HCV activity of raloxifene and IFN-α exhibited additive effect. We also examined the anti-HCV activity of previously reported SERM, tamoxifen. Tamoxifen also exhibited additive anti-HCV activity on HCV RNA replication in combination with IFN-α (Supplementary Figs. 2A–C). These results indicate that raloxifene as well as tamoxifen enhanced the anti-HCV activity of IFN-α. As both raloxifene and IFN-α are clinically used reagents, raloxifene seemed to be a candidate reagent as an add-on treatment to IFN-α in patients with CH–C.

3.3. Raloxifene antagonized anti-HCV activity of statin

We previously reported that statins exhibited anti-HCV activity using the OR6 assay system [14]. Statin is the first-choice reagent for dyslipidemia. As dyslipidemia and osteoporosis are major complications in postmenopausal women, we invested the effect of raloxifene on the anti-HCV activity of PTV. Raloxifene did not enhance the anti-HCV activity of PTV (Fig. 3A). Fig. 3B exhibits the expected anti-HCV activity of co-treatment with raloxifene and PTV calculated from the anti-HCV effect of either raloxifen or PTV alone. Raloxifene exhibited an antagonistic effect on PTV's anti-HCV activity. Raloxifene's antagonistic effect on PTV increased dose-dependently. The co-treatment with raloxifene (2.5 μM) and PTV (0.25, 0.5, and 1 μM) resulted in lower anti-HCV activity than did treatment with raloxifene alone (2.5 μM). These results suggest that we should be careful in the administration of statins with raloxifene to postmenopausal woman with CH–C.

3.4. Raloxifene inhibited infection of genotype 2a HCV

To further investigate the anti-HCV activity of raloxifene, we examined whether or not raloxifene could inhibit HCV infection. For this purpose, we used our recently developed JFH-1 reporter infection assay system [9]. HuH-7-derived RSc's are highly HCV-permissive cell lines. Raloxifene was pretreated at 24 h before HCV infection. The cells were inoculated with HCV JFH-1 virion with *RL* (JR/C5B/BX-2), and

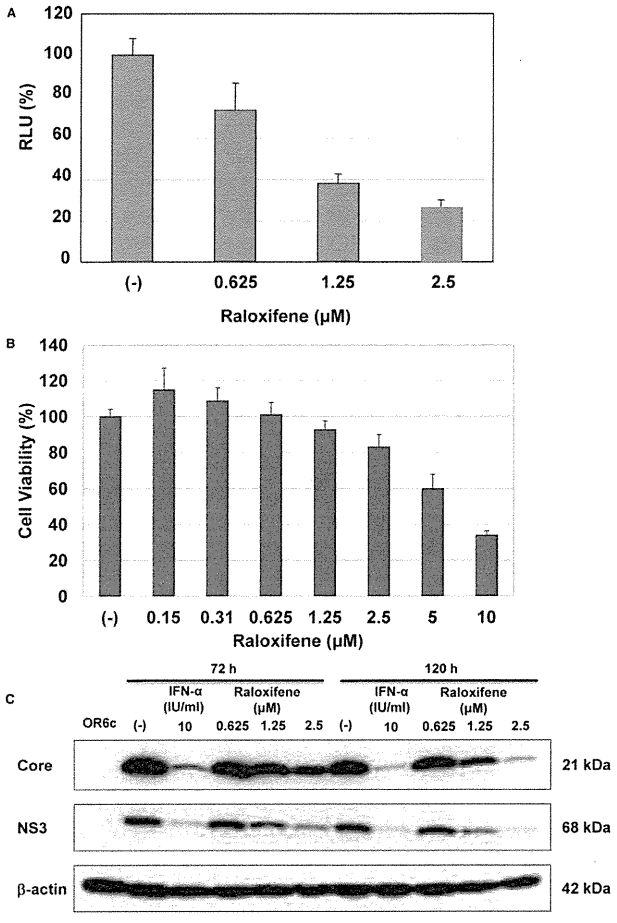

Fig. 1. Raloxifene inhibited HCV RNA replication. (A) Anti-HCV activity of raloxifene in OR6 cells. OR6 cells were treated with raloxifene (0, 0.625, 1.25, and 2.5 μM) for 72 h. Relative RL activity (relative light unit: RLU) for HCV RNA replication is expressed as a percentage of control. Each bar represents the average with standard deviations of triplicate data points. (B) Effect of raloxifene on OR6 cell viability. Cell viability at 72 h after raloxifene treatment (0.15, 0.31, 0.625, 1.25, 2.5, 5, and 10 μM) was determined using WST-1 cell proliferation assay and is expressed as a percentage of control. (C) Raloxifene inhibited HCV proteins. OR6 cells were treated with IFN-α (10 IU/ml) or raloxifene (0, 0.625, 1.25, and 2.5 μM). After 72 or 120 h treatment, the production of Core and that of NS3 were analyzed by immunoblotting using anti-Core and anti-NS3 antibodies, respectively. OR6c cells were cured cells in which HCV RNA was eliminated using IFN-α, and were used as a negative control. β-actin was used as a control for the amount of protein loaded per lane.

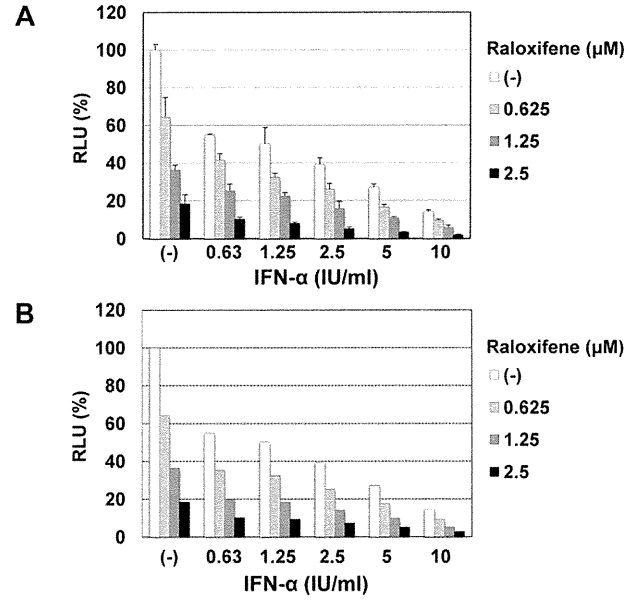

Fig. 2. Raloxifene enhanced the anti-HCV activity of IFN-α. (A) Anti-HCV activity of raloxifene in combination with IFN-α. OR6 cells were co-treated with raloxifene (0, 0.625, 1.25, and 2.5 μM) and IFN-α (0, 0.63, 1.25, 2.5, 5, 10 IU/ml). Relative RL activity is shown as a percentage of control. Each bar represents the average with standard deviations of triplicate data points. (B) Expected anti-HCV activity was calculated based on the results when the cells were treated with only raloxifene or IFN-α.

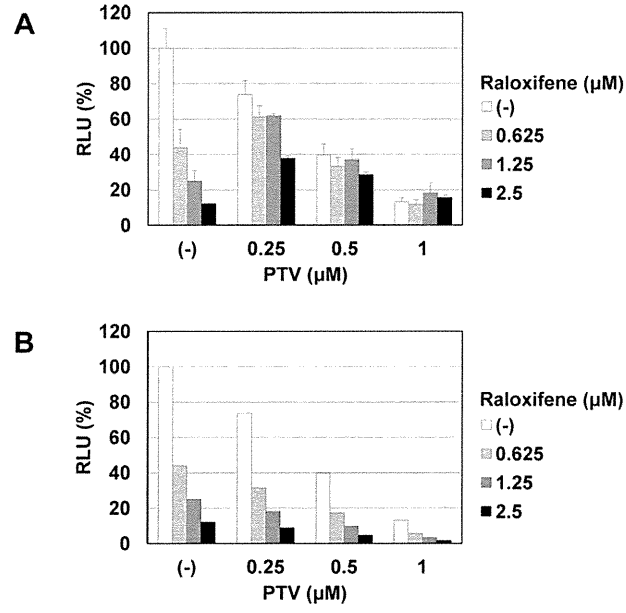

Fig. 3. Statin antagonized the anti-HCV activity of raloxifene. (A) OR6 cells were co-treated with raloxifene (0, 0.625, 1.25, and 2.5 μM) and PTV (0, 0.25, 0.5, and 1 μM). Relative RL activity was shown as a percentage of control. Each bar represents the average with standard deviations of triplicate data points. (B) Expected anti-HCV activity was calculated based on the results when the cells were treated with only raloxifene or PTV.

the infection was monitored with RL activity at 48 h after infection. As shown in Fig. 4A, raloxifene inhibited HCV infection in RSc cells in a dose-dependent manner. Next we examined the effect of raloxifene after HCV infection. RSc cells were inoculated with HCV JFH-1 virion with *RL*. After HCV infection, the cells were treated with raloxifene for 72 h and raloxifene's inhibitory effect on post-infection was assessed using the RL assay. Raloxifene inhibited HCV proliferation in a dose-dependent manner when it was added to the cells after infection in RSc cells, although inhibitory effect of raloxifene on JFH-1 HCV RNA replication seemed to be weak compared to the genotype 1b HCV-O RNA replication (Fig. 4B). Raloxifene did not exhibit cytotoxicity to RSc cells until 2.5 μM (Fig. 4C). We found that raloxifene could not inhibit subgenomic JFH-1 HCV (JRN/35B) RNA replication (Fig. 4D). We further examined the inhibitory action of raloxifene around infection step. RSc cells were treated for short time with raloxifene around infection step: for 1, 4, and 4 h before, during, and after inoculation, respectively (Fig. 4E). Raloxifene inhibited JFH-1 infection, when it was treated during inoculation but not just before or after inoculation. In case of genotype 2a JFH-1, raloxifene's anti-HCV activity is mainly due to the inhibition of infection. These results indicate that raloxifene inhibits JFH-1 infection but not its RNA replication.

4. Discussion

In this study, we demonstrated that raloxifene, an osteoporotic reagent, inhibited the replication of genotypes 1b HCV RNA replication and inhibited genotype 2a HCV JFH-1 infection. Raloxifene additively enhanced the anti-HCV activity of IFN-α. On the other hand, raloxifene exhibited an antagonistic effect on statins.

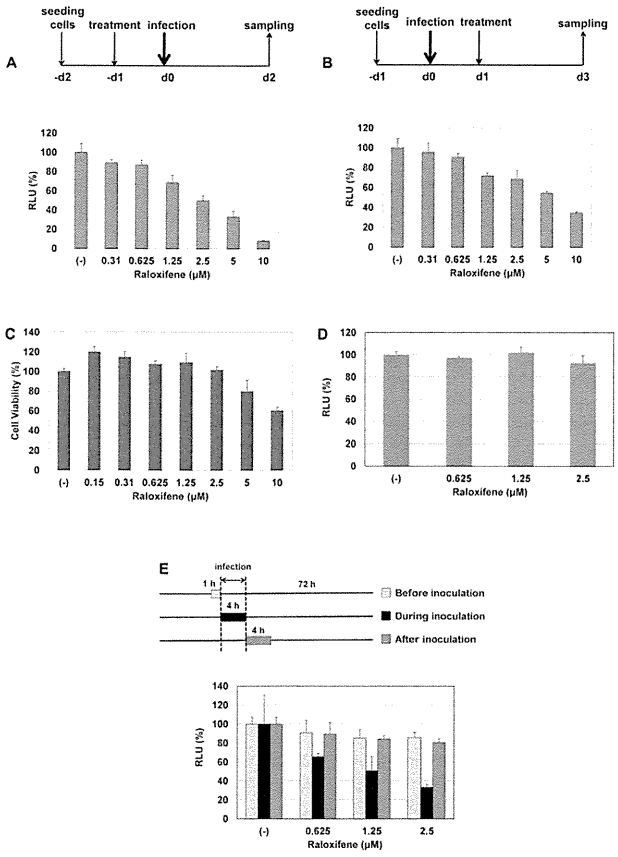

Fig. 4. Raloxifene inhibited genotype 2a HCV infection. (A) Raloxifene inhibited HCV JFH-1 infection. RSc cells were treated with raloxifene (0, 0.31, 0.625, 1.25, 2.5, 5, and 10 μM) 24 h before infection. HCV JFH-1 reporter virion was used as an inoculum after removal of raloxifene. The cells were then infected with reporter JFH-1 virion and cultured for 48 h. The inhibition of HCV infection was assessed by relative RL activity and expressed as a percentage of control. (B) Raloxifene inhibited HCV JFH-1 proliferation after infection. RSc cells were inoculated with HCV JFH-1 reporter virion and cultured for 24 h. Then the cells were treated with raloxifene (0, 0.31, 0.625, 1.25, 2.5, 5, and 10 μM) for 48 h. The inhibitory effect on HCV proliferation after infection was assessed by relative RL activity and expressed as a percentage of control. Each bar represents the average with standard deviations of triplicate data points. (C) Effect of raloxifene on RSc cells viability. Cell viability at 72 h after raloxifene treatment (0.15, 0.31, 0.625, 1.25, 2.5, 5, and 10 μM) was determined using WST-1 cell proliferation assay and is expressed as a percentage of control. (D) Subgenomic JFH-1 RNA (JRN/35B) replicating RSc cells were treated with raloxifene (0, 0.625, 1.25, and 2.5 μM) for 72 h. Relative RL activity for HCV RNA replication is expressed as a percentage of control. Each bar represents the average with standard deviations of triplicate data points. (E) Raloxifene (0, 0.625, 1.25, and 2.5 μM) was treated for 1, 4, and 4 h before, during, and after JFH-1 inoculation to RSc cells at MOI of 0.2, respectively. The cells were then cultured for 72 h. The inhibition of HCV infection was assessed by relative RL activity and expressed as a percentage of control.

PEG-IFN/RBV therapy led to a 40–50% SVR rate among patients with CH–C. Telaprevir with PEG-IFN/RBV increases the effect of PEG-IFN/RBV therapy by 10–20%. However, the major complication of anemia in PEG-IFN/RBV therapy increased when telaprevir was added. Considering that PEG-IFN/RBV-based therapy is less effective on postmenopausal women, an alternative therapy with minimal side effects is needed. Add-on therapy for postmenopausal women may be a candidate for improving the SVR in these patients. We focused on the reagents, which compensate for the lack of estrogen function. Dyslipidemia and osteoporosis are the major complications in postmenopausal women, and these complications are attributable to the decrease in estrogen. Statins are clinically used reagents for dyslipidemia; they inhibit HCV RNA replication in vivo as well as in vitro [13–17]. Therefore, we investigated whether or not raloxifene exhibits anti-HCV activity using genotype 1b HCV RNA replication and genotype 2a infection systems. In the HCV life cycle, raloxifene inhibited genotype 2a HCV infection and genotypes 1b HCV RNA replication. Raloxifene may be a potential reagent with different anti-HCV mechanisms in the HCV life cycle. Further study is needed to clarify these underlying mechanisms.

Recently it was reported that vitamin D3, an osteoporotic reagent, inhibited HCV production in cell culture systems [20,21]. Furthermore, it was reported that vitamin D3 was associated with the effect of therapy for patients with CH–C [18,19]. Statins inhibited HCV RNA replication by suppressing geranylgeranyl pyrophosphate (GGPP) production [14]. Another osteoporotic reagent, bisphosphonate, may possess anti-HCV activity, because it also inhibited the biosynthesis of GGPP in the mevalonate pathway by inhibiting farnesyl pyrophosphate synthetase. Taken together, these findings indicate it is likely that the HCV life cycle is associated with osteoporosis.

Raloxifene and tamoxifen are SERMs for osteoporosis and breast cancer, respectively. Tamoxifen is used for estrogen receptor-positive breast cancer, and it inhibits HCV RNA replication in cell culture [23]. Tamoxifen's anti-HCV activity is associated with ERα. In our study, raloxifene inhibited HCV infection as well as replication. To clarify the multi-potential effects of raloxifene, further study is needed. The incidence of side effects including uterine cancer is lower in raloxifene therapy than in tamoxifen therapy [25]. This is another advantage of raloxifene in clinical use for patients with CH–C.

As for the precise role of ERα or ERβ on the HCV life cycle, we could not reach a clear conclusion because microarray analysis revealed an absence of expression for both ERα and ERβ in OR6 cells (data not shown). Hayashida et al. [22] reported that the most potent physiological estrogen, 17-β-estradiol, inhibited infectious HCV production using HuH-7.5 cells, and that ERα-selective agonist inhibited infectious HCV production whereas ERβ-selective agonist did not. Watashi et al. [23] reported that RNA interference-mediated knockdown of ERα reduced HCV RNA replication. In our study, the anti-HCV activity of raloxifene in infection and replication did not seem attributable to ERα or ERβ. It is not clear why our HuH-7-derived OR6 cells did not express ERα or ERβ. HuH-7 cells were developed in 1982 at Okayama University and distributed worldwide [26]. Recently, Bensadoun et al. [27] reported that the genetic background of the IL28B genotype of HuH-7 cells differed among different laboratories. This may be a consequence of the polyploidal nature of hepatoma cells. A similar mechanism might cause the different expression levels of ERα and ERβ. Another ER, GPR30 [28], was expressed in OR6 cells (data not shown; from microarray analysis). GPR30 may be the responsible host factor for anti-HCV activity in OR6 cells. Further study is needed to clarify this issue.

In conclusion, we found that raloxifene inhibited HCV RNA replication in genotype 1b and infection in genotype 2a. Raloxifene additively enhanced the anti-HCV activity of IFN-α. The antagonistic effects of statins and raloxifene will yield information on the clinical use of these reagents. Our results, as well as the reports of vitamin D3's anti-HCV activity, will open new fields of treatment for both osteoporosis and HCV infection.

Acknowledgments

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Supplementary Material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fob.2012.08.003.

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Article

Inhibition of Hepatitis C Virus Replication and Viral Helicase by Ethyl Acetate Extract of the Marine Feather Star *Alloeocomatella polycladia*

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Abstract: Hepatitis C virus (HCV) is a causative agent of acute and chronic hepatitis, leading to the development of hepatic cirrhosis and hepatocellular carcinoma. We prepared extracts from 61 marine organisms and screened them by an *in vitro* fluorescence assay targeting the viral helicase (NS3), which plays an important role in HCV replication, to identify effective candidates for anti-HCV agents. An ethyl acetate-soluble fraction of the feather star *Alloeocomatella polycladia* exhibited the strongest inhibition of NS3 helicase activity, with an IC_{50} of 11.7 μg/mL. The extract of *A. polycladia* inhibited interaction between NS3 and RNA but not ATPase of NS3. Furthermore, the replication of the replicons derived from three HCV strains of genotype 1b in cultured cells was suppressed by the extract with an EC_{50} value of 23 to 44 μg/mL, which is similar to the IC_{50} value of the NS3 helicase assay. The extract did not induce interferon or inhibit cell growth. These results suggest that the unknown compound(s) included in *A. polycladia* can inhibit HCV replication by suppressing the helicase activity of HCV NS3. This study may present a new approach toward the development of a novel therapy for chronic hepatitis C.

Keywords: marine organism; *Alloeocomatella polycladia*; hepatitis C virus; NS3 helicase

1. Introduction

Hepatitis C virus (HCV) is an etiological agent of liver disease including steatosis, cirrhosis, and hepatocellular carcinoma, and has infected over 170 million individuals worldwide [1,2]. HCV belongs to the genus *Hepacivirus* of the *Flaviviridae* family. The genome of HCV is a single positive-strand RNA composed of 9.6 kb flanked by 5' and 3'-untranscribed regions (UTRs) and encodes a polyprotein consisting of approximately 3000 amino acids [3]. The polyprotein is translated from a viral genome by an internal ribosome entry site (IRES), which is localized in 5'-UTR [4]. The translated polyprotein is cleaved by host and viral proteases into 10 proteins. The structural proteins consisting of core, E1, and E2 and a viroporin p7, which has not yet been classified as either a structural or nonstructural protein, are located in the *N*-terminal quarter of the polyprotein. The nonstructural proteins including

NS2, NS3, NS4A, NS4B, NS5A, and NS5B occupy the remaining portion of the polyprotein and form a replication complex with several host factors.

HCV NS3 is well known to play a crucial role in viral replication because it possesses helicase and protease activities [5,6]. The *N*-terminal third of NS3 forms a complex with the NS4A protein and exhibits serine protease activity (NS3-4A protease) to cleave the viral polyprotein for the maturation of viral proteins [7]. The remaining portion of NS3 occupies the RNA helicase domain, characterized by the activities of ATPase and RNA binding, both of which contribute to the unwinding of duplex RNA [8,9]. The helicase activity is needed to separate duplex RNA during viral RNA replication [10]. A negative-strand RNA is synthesized based on a viral genome (positive strand) after the uncoating of a viral particle in the infected cells and then is itself used as a template to synthesize a positive-strand RNA packaged into the viral particle. Thus, helicase as well as protease activities of NS3 can be targeted for use in the development of antiviral agents against HCV.

The current therapy, which combines pegylated interferon with ribavirin, is effective in only about half of patients infected with the most common genotype worldwide, genotype 1 [11–13]. However, this therapy has side effects including influenza-like symptoms, cytopenias, and depression [11]. Furthermore, no effective vaccines for HCV have been developed yet. Biotechnological advances of the past decade have led to the development of novel therapies using anti-HCV agents that directly target HCV proteins or host factors required for HCV replication. This approach has been named either "specifically targeted antiviral therapy for hepatitis C" (STAT-C) or "directed-acting antiviral agents" (DAA) [14–16]. Several compounds of STAT-C or DAA have proceeded to clinical trials. Telaprevir and boceprevir, which are categorized as advanced NS3/4A protease inhibitors, were recently approved for the treatment of chronic hepatitis C patients infected with genotype 1 in the US, EU, Canada, and Japan [17,18]. However, the emergence of drug-resistant viruses is the major problem for therapies using antiviral compounds [19,20]. Accordingly, several kinds of drugs targeting various molecules or positions will be required for the complete eradication of the virus from hepatitis C patients.

The helicase activity of NS3 could be targeted by development of anti-HCV compound in addition to its protease activity. Belon *et al.* reported that 1-*N*,4-*N*-*bis*[4-(1*H*-benzimidazol-2-yl)phenyl] benzene-1,4-dicarboxamine, designated as $(BIP)_2B$, is a potent and selective inhibitor of HCV NS3 helicase [21]. $(BIP)_2B$ could not affect ATP hydrolysis without RNA or at a saturated concentration of RNA. QU663 inhibits the unwinding activity of NS3 helicase by binding to the RNA-binding groove irrespective of its own ATPase activity [22]. Compound QU663 may competitively bind the RNA-binding site of NS3 but not affect ATPase activity, resulting in the inhibition of unwinding activity.

Various drugs have been generated from natural products, especially those from terrestrial plants and microbes. The development of drugs from natural products has declined in the past two decades by the emergence of high-throughput screening of synthetic chemical libraries. However, recent technical advances in the determination of molecular structures and in the synthesis of chemical compounds have raised awareness about natural products as a resource for drug development [23–25]. Several groups recently reported natural products that inhibit HCV replication *in vitro*. For instance, silbinin, which is identified from the milk thistle [26,27], epigallocatechin 3-gallate, which is from green tea [28], and proanthocyanidins, which are from blueberry leaves [29], can inhibit HCV replication in cultured cells. Marine organisms including plants and animals were recently established as a representative natural resource library for drug development, since there are estimated to be more than 300,000

species of marine organisms. The products isolated from the marine organisms often possess potent biological activities corresponding to the organisms' own novel molecular structures. Thus, marine natural products are considered to include highly significant lead compounds for drug development [30,31]. For example, trabectedin (Yondelis), cytarabine (Ara-C), and eribulin (Halaven) are approved anticancer drugs developed from marine organisms [32]. However, marine organisms have not yet been screened for development into anti-HCV agents.

In this study, we screened extracts of marine organisms by using an *in vitro* fluorescence NS3 helicase assay and HCV replicon system to find candidates for safe and effective anti-HCV agents. The marine feather star *Alloeocomatella polycladia* may produce anti-HCV helicase agents that suppress HCV replication.

2. Results and Discussion

2.1. Primary Screening of Marine Organism Extracts on HCV NS3 Helicase Activity

We employed high-throughput screening using a photoinduced electron transfer (PET) assay to identify inhibitors of HCV NS3 helicase activity from extracts of marine organisms (Figure 1). The EtOAc- and MeOH-soluble extracts were prepared from marine organisms obtained from the sea around Okinawa Prefecture, Japan. We identified 16 extracts possessing an arbitrary level of inhibitory activity, which is defined as below 60% of the control in this study (Table 1). Five extracts exhibited high inhibition levels (<30%), and eleven extracts exhibited intermediate inhibition levels (30% to 60%). The EtOAc extract prepared from the feather star *Alloeocomatella polycladia* (Figure 2) exhibited the strongest inhibitory activity among them, and was designated SG1-23-1 in this study. Treatment with SG1-23-1 inhibited the helicase activity in a dose-dependent manner (Figure 3A). The value of IC_{50} is calculated as 11.7 ± 0.7 μg/mL. We confirmed the effect of SG1-23-1 on NS3 helicase unwinding activity by the RNA helicase assay using ^{32}P-labeled double-stranded RNA (dsRNA) as a substrate. Treatment with SG1-23-1 inhibited dsRNA dissociation at concentrations of 16 μg/mL and above (Figure 3B). These results suggest that treatment with SG1-23-1 inhibits the unwinding ability of HCV NS3 helicase.

Table 1. Inhibitory effects of marine organism extracts on hepatitis C virus (HCV) NS3 helicase activity.

Sample	Helicase Activity (% of control)	Specimen	Phylum	Extract	Collection Site
OK-99-2	78	*Agelas* sp.	Porifera	EtOAc	Shimoji Island
OK-99-3	73	*Plakortis* sp.	Porifera	EtOAc	Shimoji Island
OK-99-4	60	*Dysidea arenaria*	Porifera	EtOAc	Shimoji Island
OK-99-5	96	*Theonella cupola*	Porifera	EtOAc	Shimoji Island
OK-99-6	52	*Theonella conica*	Porifera	EtOAc	Shimoji Island
OK-99-7	85	*Epipolasis kushimotoensis*	Porifera	EtOAc	Shimoji Island
OK-99-9	51	*Hyrtios* sp.	Porifera	EtOAc	Shimoji Island