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Plural assay systems derived from different cell lines and hepatitis C virus strains are required for the objective evaluation of anti-hepatitis C virus reagents

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ABSTRACT

Persistent hepatitis C virus (HCV) infection causes chronic liver diseases and is a global health problem. HuH-7 hepatoma-derived cells are widely used as the only cell-based HCV replication system for HCV research, including drug assays. Recently, using different hepatoma Li23-derived cells, we developed an HCV drug assay system (ORL8), in which the genome-length HCV RNA (O strain of genotype 1b) encoding renilla luciferase replicates efficiently. In this study, using the HuH-7-derived OR6 assay system that we developed previously and the ORL8 assay system, we evaluated 26 anti-HCV reagents, which other groups had reported as anti-HCV candidates using HuH-7-derived assay systems other than OR6. The results revealed that more than half of the reagents showed different anti-HCV activities from those in the previous studies, and that anti-HCV activities evaluated by the OR6 and ORL8 assays were also frequently different. In further evaluation using the HuH-7-derived AH1R assay system, which was developed using the AH1 strain of genotype 1b, several reagents showed different anti-HCV activities in comparison with those evaluated by the OR6 and ORL8 assays. These results suggest that the different activities of anti-HCV reagents are caused by the differences in cell lines or HCV strains used for the development of assay systems. Therefore, we conclude that plural HCV assay systems developed using different cell lines or HCV strains are required for the objective evaluation of anti-HCV reagents.

1. Introduction

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis, which often leads to liver cirrhosis and hepatocellular carcinoma. Since approximately 170 million people are infected with HCV worldwide, HCV infection is a serious global health problem [1]. Although the combination of pegylated-interferon (PEG-IFN) and ribavirin is the standard therapy worldwide, only half of the patients receiving this treatment exhibit a sustained virologic response [2]. HCV is an enveloped virus with a positive single-stranded RNA virus of the *Flaviviridae* family. The HCV genome encodes a large polyprotein precursor of approximately 3000 amino acids, which is cleaved into 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [3,4].

To date, HuH-7 hepatoma-derived cells are used as the only cell culture system for robust HCV replication in HCV research, including drug assays. We have also developed a HuH-7-derived drug assay system (OR6), in which genome-length HCV RNA (O strain of genotype 1b derived from an HCV-positive blood donor) encoding renilla luciferase (RL) efficiently replicates [5]. Recently, we found a new human hepatoma cell line, Li23, that enables robust HCV RNA replication [6], and we showed that the gene expression profile of Li23 cells was distinct from that of HuH-7 cells, although both cell lines had similar liver-specific expression profiles [7]. In that study, we identified three genes (New York esophageal squamous cell carcinoma 1, β-defensin-1, and galectin-3) showing Li23-specific expression profiles by a comparative analysis using several other hepatic cell lines [7]. We further developed Li23-derived drug assay systems (ORL8 and ORL11), which are relevant to the HuH-7-derived OR6 assay system [6]. During the process of evaluating the ORL8 and ORL11 assay systems using anti-HCV reagents such as IFNs, we noticed that these assay systems were frequently more sensitive to anti-HCV reagents than the OR6 assay system [6]. Furthermore, we recently found that ribavirin at clinically achievable concentrations (approximately 10 μM) effectively inhibited HCV RNA replication in both the ORL8 and ORL11 assay systems, but not in the OR6 assay system [8]. This finding led to the clarification of the anti-HCV mechanism of ribavirin, and we demonstrated that ribavirin's anti-HCV activity was mediated by the inhibition of inosine monophosphate dehydrogenase, a key enzyme in the guanosine biosynthetic pathway [8]. From these findings, we supposed that the anti-HCV reagents reported to date might show different activities among the different drug assay systems. To test this assumption, we evaluated 22 anti-HCV reagents that were reported using HuH-7-derived assay systems other than OR6, using the OR6 and ORL8 assay systems. Four additional

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reagents predicted by antiviral activity other than HCV were also evaluated. Furthermore, a recently developed HuH-7-derived AH1R assay system (AH1 strain of genotype 1b derived from a patient with acute hepatitis) (Mori et al., in preparation) was also used for the evaluation. Here, we report that plural assay systems derived from different cell lines and different HCV strains are required for the objective evaluation of anti-HCV reagents.

2. Materials and methods

2.1. Cell cultures

HuH-7-derived OR6 and AH1R cells were maintained in medium containing G418 (0.3 mg/ml) as described previously [5]. Li23-derived ORL8 cells were also maintained in medium containing G418 (0.3 mg/ml) as described previously [6].

2.2. Reagents

Acetylsalicylic acid, cephalotaxine, clemizole, crucumin, isoliquiritigenin, nitazoxanide, and tizoxanide were purchased from Sigma–Aldrich (St. Louis, MO). Cantharidin, 2′-deoxy-5-fluorouridine, griseofulvin, guanazole, homoharringtonine, resveratrol, and Y7632 were purchased from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan). Artemisinin and bisindoly maleimide 1 were purchased from Alexis Biochemicals (San Diego, CA). Artesunate and silibinin A were purchased from Lkt Laboratories (St. Paul, MN). Esomeprazole and nelfinavir were purchased from Toronto Research Chemicals (North York, ON, Canada). Cinanserin hydrochloride and HA1077 were purchased from Tocris Bioscience (Bristol, UK). 6-Azauridine was purchased from MP Biomedicals (Solon, OH). Carvedilol was purchased from Calbiochem (San Diego, CA). Hemin was purchased from Alfa Aesar (Ward Hill, MA). Methotrexate was purchased from Tokyo Chemical Industry (Tokyo, Japan). Cinanserin hydrochloride, guanazole, HA11077, and Y27632 were dissolved in the culture medium for Li23-derived cells. Artesunate was dissolved in 0.5% $NaHCO_3$ solution. Other reagents were dissolved in dimethyl sulfoxide.

2.3. RL assay

RL assay was performed as described previously [6]. Briefly, the cells were plated onto 24-well plates (2×10^4 cells per well) in triplicate and then treated with each reagent at several concentrations for 72 h. After treatment, the cells were subjected to luciferase assay using the RL assay system (Promega, Madison, WI). From the assay results, the 50% effective concentration (EC_{50}) of each reagent was determined.

2.4. WST-1 cell proliferation assay

The cells were plated onto 96-well plates (1×10^3 cells per well) in triplicate and then treated with each reagent at several concentrations for 72 h. After treatment, the cells were subjected to the WST-1 cell proliferation assay (Takara Bio, Otsu, Japan) according to the manufacturer's protocol. From the assay results, the 50% cytotoxic concentration (CC_{50}) of each reagent was determined.

2.5. Western blot analysis

The preparation of cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting analysis were performed as previously described [9]. The antibodies used in this study were those against HCV Core (CP11; Institute of Immunology, Tokyo, Japan) and β-actin (AC-15, Sigma–Aldrich) as the control for the amount of protein loaded per lane. Immunocomplexes were detected with the Renaissance enhanced chemiluminescence assay (Perkin–Elmer Life Sciences, Boston, MA).

2.6. Selective index (SI)

The SI value of each reagent was determined by dividing the CC_{50} value by the EC_{50} value.

3. Results

3.1. Evaluation of 26 reagents for anti-HCV activity using OR6 and ORL8 assay systems

To obtain candidates for the evaluation of anti-HCV activity using OR6 and ORL8 assay systems, we first searched the literature in the PubMed database using the key words (HCV or hepatitis C) and (inhibit or antiviral or suppress or block); this yielded approximately 4500 reports published between January 2003 and April 2010. From these results, we further selected the reports in which the EC_{50} values of reagents were determined or estimated by the HuH-7-derived HCV assay systems using the Con-1 strain (genotype 1b) [10], N strain (genotype 1b) [11], or HCV JFH-1 strain (genotype 2a) [12]. We finally chose 22 commercially available reagents for the evaluation of anti-HCV activity using OR6 and ORL8 assay systems. Four reagents predicted from the antiviral activity (hepatitis B virus, cytomegalovirus, etc.) other than HCV were also included in the evaluation study. The 26 selected reagents and their references are listed in Supplementary Table S1.

For each of the 26 reagents, we determined the EC_{50} value by RL assay and the CC_{50} value by WST-1 assay using the OR6 or ORL8 assay system, and calculated the SI value by dividing the CC_{50} value by the EC_{50} value. For each reagent, we first compared the EC_{50} value obtained from the OR6 or ORL8 assay with that of the previous study. Consequently, we classified the 26 reagents into five classes, A to E (Table 1). Eight reagents (methotrexate, artemisinin, artesunate, clemizole, hemin, 6-azauridine, acetylsalicylic acid, and isoliquiritigenin with the order of the SI value in the ORL8 assay) belonged to class A, in which the EC_{50} value obtained by either the OR6 or ORL8 assay was less than one-third of that in the previous study (Supplementary Table S1 and Table 1). Artesunate, an artemisinin-derivative possessing antiviral activity against cytomegalovirus, herpesvirus, Epstein-Barr virus etc., was included in class A by the comparison with the data on anti-cytomegalovirus activity. In this class, we especially noticed that methotrexate (an anti-cancer drug) showed very strong anti-HCV activity (EC_{50} 0.1 μM; $CC_{50} > 200$ μM; SI > 2000) in the ORL8 assay (upper panel in Fig. 1A and Table 1), whereas methotrexate showed very weak anti-HCV activity ($EC_{50} > 200$ μM; $CC_{50} > 200$ μM) in the OR6 assay as well as in a previous report [13] (upper panel in Fig. 1A and Table 1). This drastic difference was confirmed by Western blot analysis (lower panels in Fig. 1A). These results indicate that only the ORL8 assay is drastically sensitive to methotrexate, and suggest that the anti-HCV activity of methotrexate depends on the types of hepatic cells. The comparison of the EC_{50} values of other reagents belonging to class A revealed that the ORL8 assay was more sensitive than the OR6 assay (1.9–15-fold) to artemisinin, artesunate, clemizole, acetylsalicylic acid, and 6-azauridine, and conversely the OR6 assay was more sensitive than the ORL8 assay (2–2.5-fold) to hemin and isoliquiritigenin (Table 1). Furthermore, the CC_{50} values of clemizole and 6-azauridine also differed more than twofold between the OR6 and OR8 assays (Table 1). These results suggest that the anti-HCV activities of these reagents are affected by the kind of assay systems used. Especially, we noticed that artemisinin and artesunate (antimalarial drugs) showed higher SI values in the

Table 1
Anti HCV activities of 26 reagents evaluated in this study.

	Assay	[a]		OR6		ORL8		AHIR	
	Cell origin	HuH-7		HuH-7		Li23		HuH-7	
	HCV strain	Con-1, N, JFH-1, etc.		O		O		AH1	
Class	Reagent	$\frac{CC_{50}}{EC_{50}}$	SI	$\frac{CC_{50}}{EC_{50}}$	SI	$\frac{CC_{50}}{EC_{50}}$	SI	$\frac{CC_{50}}{EC_{50}}$	SI
A	Methotrexate	$\frac{> 100}{> 100}$	–	$\frac{> 200}{> 200}$	–	$\frac{> 200}{0.1}$	>2000	$\frac{170}{> 200}$	<0.9
A	Artemisinin	$\frac{> 177}{> 78}$	>2.3	$\frac{380}{81}$	4.7	$\frac{370}{23}$	16	$\frac{310}{5.3}$	58
A	Artesunate[b]	$\frac{> 15}{3.9}$	>3.8	$\frac{6.1}{2.3}$	2.7	$\frac{3.4}{0.22}$	15	$\frac{4}{0.81}$	4.9
A	Clemizole	$\frac{> 20}{8}$	>2.5	$\frac{11}{22}$	0.5	$\frac{22}{2.0}$	11	$\frac{7.3}{> 25}$	<0.3
A	Hemin	$\frac{> 52}{22}$	>2.4	$\frac{10}{1.2}$	8.3	$\frac{18}{2.4}$	7.5	$\frac{7.2}{1.1}$	6.5
A	6-Azauridine	$\frac{> 100}{100}$	>1.0	$\frac{10}{5.7}$	1.8	$\frac{1.5}{0.37}$	4.1	$\frac{14}{3.3}$	4.2
A	Acetylsalicylic acid	$\frac{8^{d}}{4^{d}}$	2.0	$\frac{2.6^{d}}{1.6^{d}}$	1.6	$\frac{2.4^{d}}{0.83^{d}}$	2.9	ND	–
A	Isoliquiritigenin	$\frac{< 24}{24}$	<1.0	$\frac{12}{3.9}$	3.1	$\frac{15}{9.8}$	1.5	ND	–
B	Nelfinavir	$\frac{> 10}{9.9}$	>1.0	$\frac{26}{11}$	2.4	$\frac{68}{12}$	5.7	ND	–
B	2′-Deoxy-5-fluorouridine	$\frac{< 15}{15}$	<1.0	$\frac{31}{32}$	1.0	$\frac{36}{14}$	2.6	$\frac{13}{86}$	0.2
B	Resveratrol	$\frac{> 10}{10}$	>1.0	$\frac{35}{4.3}$	8.1	$\frac{42}{16}$	2.6	$\frac{76}{9.9}$	7.7
B	Cantharidine[c]	$\frac{3.5}{0.3}$	12	$\frac{1.5}{0.28}$	5.4	$\frac{1.8}{0.69}$	2.6	ND	–
B	Homoharringtonine[c]	$\frac{0.5}{30^{e}}$	17	$\frac{38^{e}}{18^{e}}$	2.1	$\frac{0.11}{45^{e}}$	2.4	$\frac{22^{e}}{19^{e}}$	1.2
B	Crucumin	$\frac{> 15}{15}$	>1.0	$\frac{18}{14}$	1.3	$\frac{19}{11}$	1.7	ND	–
B	Griseofulvin	$\frac{207}{6.1}$	34	$\frac{16}{4.4}$	3.6	$\frac{14}{8.6}$	1.6	ND	–
B	Cinanserin hydrochloride	$\frac{> 10}{> 10}$	–	$\frac{33}{25}$	1.3	$\frac{39}{35}$	1.1	ND	–
B	Cephalotaxine[c]	$\frac{> 100}{60}$	>1.7	$\frac{35}{29}$	1.2	$\frac{38}{47}$	0.8	$\frac{4.8}{41}$	0.1
C	Tizoxanide	$\frac{15}{0.15}$	100	$\frac{11}{2.4}$	4.6	$\frac{24}{9.6}$	2.5	ND	–
C	Nitazoxanide	$\frac{38}{0.21}$	181	$\frac{11}{2.8}$	3.9	$\frac{17}{9.2}$	1.8	$\frac{7.2}{2.2}$	3.3
D	Guanazole	$\frac{< 100}{> 100}$	<1.0	$\frac{200}{> 200}$	<1.0	$\frac{170}{> 200}$	<0.9	$\frac{173}{> 200}$	<0.9
D	HA1077	$\frac{50}{15}$	3.3	$\frac{> 50}{> 50}$	–	$\frac{> 50}{> 50}$	–	$\frac{> 50}{> 50}$	–
E	Bisindoly maleimide 1	$\frac{ND}{5}$	–	$\frac{8.1}{6.2}$	1.3	$\frac{15}{15}$	1.0	$\frac{14}{9.1}$	1.5
E	Esomeprazole	$\frac{ND}{> 10}$	–	$\frac{67}{67}$	1.0	$\frac{27}{27}$	1.0	$\frac{20}{25}$	0.8
E	Y27632	$\frac{> 50}{50}$	>1.0	$\frac{> 80}{> 80}$	–	$\frac{> 80}{> 80}$	–	$\frac{39}{> 80}$	<0.5
E	Carvedilol	$\frac{17}{4.5}$	3.8	$\frac{4.4}{3.7}$	1.2	$\frac{6.6}{8.8}$	0.8	$\frac{6.3}{6.2}$	1.0
E	Silibinin A	$\frac{ND}{23}$	–	$\frac{12}{85}$	0.1	$\frac{26}{89}$	0.3	$\frac{28}{96}$	0.3

ND, not determined.

[a] Assay used in previous reports.

[b] Reported as anti-cytomegalovirus reagent.

[c] Reported as anti-hepatitis B virus reagent. EC_{50} and CC_{50} values are indicated by the order of μM except 'd' (μM) and 'e' (nM).

ORL8 assay than previously reported [14,15]. The anti-HCV profiles of artemisinin and artesunate in the OR6 and ORL8 assays are shown in Fig. 1B and Supplementary Fig. 1A, respectively. In addition, the comparison of SI values revealed that the OR6 assay was more sensitive to hemin and isoliquiritigenin than the HuH-7-derived assays (Con-1 and N strains) used in the previous reports (Supplementary Table S1), suggesting that the HCV strains used in the assay systems affect the evaluation of anti-HCV reagents.

Nine reagents (nelfinavir, 2′-deoxy-5-fluorouridine, resveratrol, cantharidin, homoharringtonine, crucumin, griseofulvin, cinanserin hydrochloride, and cephalotaxine with the order of SI value in the ORL8 assay) were placed in class B, in which the EC_{50} values obtained by the OR6 and ORL8 assays were similar (more than one-third to less than threefold) to those in the previous study (Table 1 and Supplementary Table S1). Cantharidin, homoharringtonine, and cephalotaxine, all of which possess anti-hepatitis B virus activity, were placed in class B by the comparison with the data on anti-hepatitis B virus activity (Supplementary Fig. 1).

Tizoxanide and nitazoxanide belonged to class C, in which the EC_{50} values obtained by both the OR6 and ORL8 assays were more than threefold higher than in the previous study (Table 1 and Supplementary Table S1). Guanazole and HA1077 were placed in class D, in which there was no anti-HCV activity in both the OR6 and ORL8 assays (Table 1). No anti-HCV activity of guanazole and HA1077 was also confirmed by Western blot analysis (data not shown). Lastly, five reagents (Bisindoly maleimide 1, esomeprazole, Y27632, carvedilol, and silibinin A) were placed in class E, in which pro-HCV activity was exhibited in both OR6 and ORL8 assays. We unexpectedly observed that these reagents enhanced the HCV RNA replication level. As a

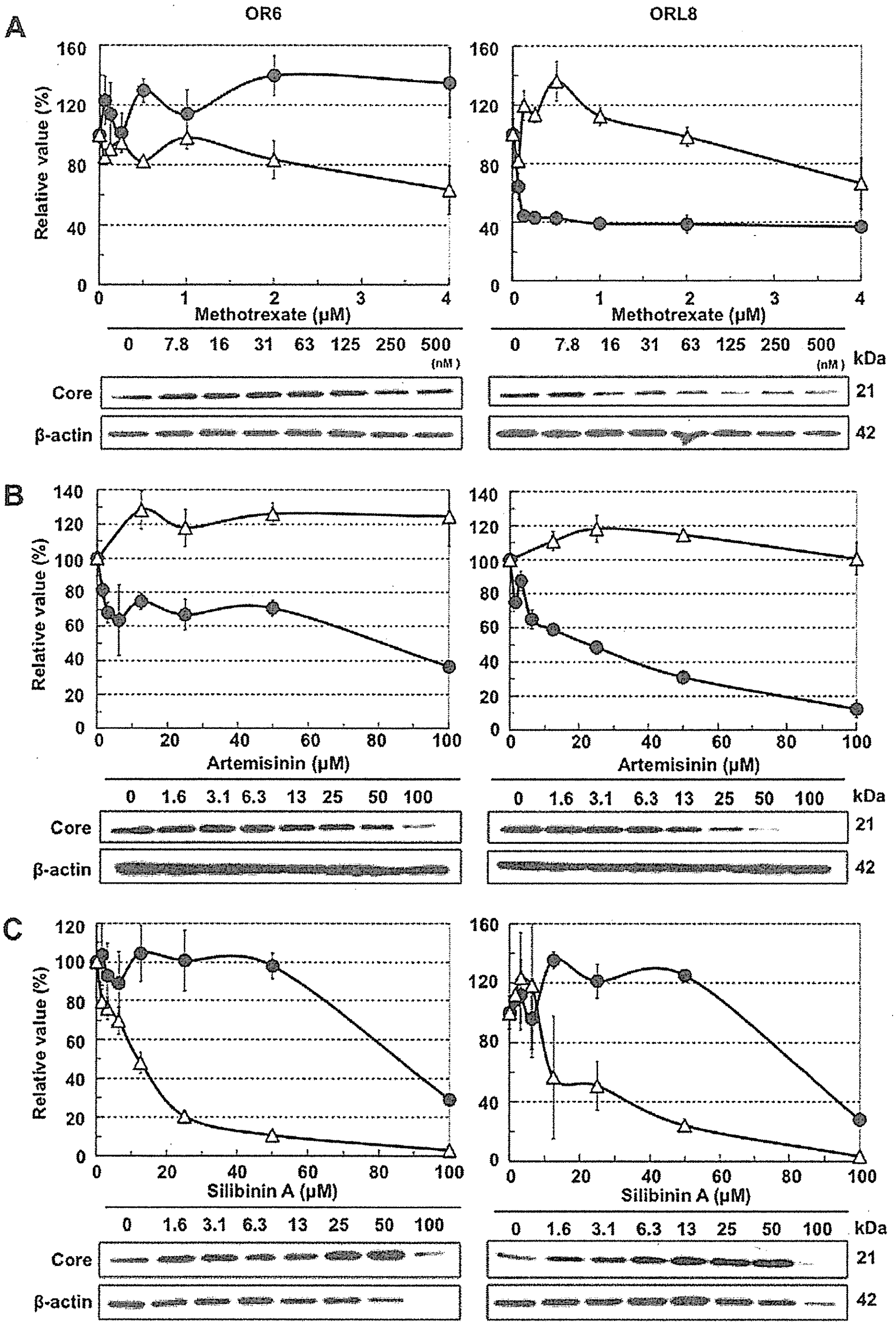

Fig. 1. Anti-HCV profiles of representative reagents in the OR6 and ORL8 assay systems. (A) Methotrexate sensitivities on genome-length HCV RNA replication in the OR6 and ORL8 assay systems. OR6 and ORL8 cells were treated with methotrexate for 72 h, followed by RL assay (black circle in the upper panel) and WST-1 assay (open triangle in the upper panel). The relative value (%) calculated at each point, when the level in nontreated cells was assigned to 100%, is presented here. Western blot analysis of the treated cells for the HCV Core was also performed (lower panel). (B) Artemisinin sensitivities on genome-length HCV RNA replication in the OR6 and ORL8 assay systems. RL assay, WST-1 assay, and Western blot analysis were performed as described in (A). (C) Silibinin A sensitivities on genome-length HCV RNA replication in the OR6 and ORL8 assay systems. RL assay, WST-1 assay, and Western blot analysis were performed as described in (A).

representative reagent, pro-HCV profiles of silibinin A are shown in the upper panel of Fig. 1C. These pro-HCV profiles were confirmed by Western blot analysis (lower panels in Fig. 1C for silibinin A and data not shown for the other reagents). Since the anti-HCV activity of silibinin A was detected by the HCV replicon assay system using the Con-1 strain [14], the converse effects obtained by our assay systems using the O strain may be due to the difference in HCV strains. In summary, the differences in anti-HCV activities observed among HuH-7- and Li23-derived assay systems used in this study and the other HuH-7-derived assay systems used in the previous studies suggest that the activities of anti-HCV reagents differ depending on which HCV strains and cell lines are used in the evaluation assays.

3.2. Evaluation of 18 reagents for anti-HCV activity using AH1R assay system

We previously established a HuH-7-derived cell line (AH1), which harbors genome-length HCV RNA (AH1 strain of genotype 1b) derived from a patient with acute hepatitis [16]. To further examine the effect of the HCV strain on anti-HCV reagent activity, we developed an AH1R assay system that is based on the AH1 cell line and that corresponds to the OR6 assay system (Mori et al., in preparation).

Using the AH1R assay system, we further evaluated the anti-HCV activities of 18 reagents, which showed differential anti-HCV activity between the OR6 and ORL8 assays, or showed either no anti-HCV activity or pro-HCV activity in both the OR6 and ORL8 assays. The results of the evaluation are shown in Table 1. The comparisons of the data obtained by the OR6 and AH1R assays revealed that the difference in the EC_{50} value from reagent to reagent was held within the range of one-third to threefold. However, we noticed that the EC_{50} value (5.3 μM) of artemisinin in the AH1R assay was remarkably lower than that (81 μM) in the OR6 assay (Supplementary Fig. 2 and Table 1), suggesting that artemisinin's anti-HCV activity differs depending on the HCV strain. Furthermore, the results of the AH1R assay revealed that cephalotaxine, belonging to class B, would be recategorized into class D. In summary, some reagents showed differential anti-HCV activities between the HuH-7-derived OR6 (O strain) and AH1R (AH1 strain) assay systems, although most of the reagents showed similar levels of anti-HCV activity in both assays. Taking together the results of the previous and present studies, we conclude that plural assay systems derived from different cell lines and HCV strains are needed for the objective evaluation of anti-HCV reagents.

4. Discussion

In the present study, we demonstrated for the first time that a Li23-cell-derived drug assay system, not a HuH-7-derived system, was important to use for the objective evaluation of anti-HCV reagents. In addition, we demonstrated that assay systems derived from different HCV strains were also necessary for the objective evaluation of anti-HCV reagents.

Among the 26 reagents evaluated by our assay systems, methotrexate showed the most drastic differences between the HuH-7- and Li23-derived assay systems in terms of anti-HCV activity. Although methotrexate showed very weak anti-HCV activity in the HuH-7-derived assay (Con-1 strain) used in a previous study [13] as well as in our OR6 and AH1R assays (O and AH1 strains), the ORL8 assay revealed very strong anti-HCV activity ($SI > 2000$). Such drastic differences in both assays suggest that some host factor or factors required for HCV RNA replication are different between these two cell lines, although the anti-HCV target of methotrexate is unclear. Since methotrexate is currently used as an anti-cancer drug or anti-rheumatic drug and its EC_{50} value for HCV RNA replication is 0.1 μM, it may be a potential candidate for enhancing the effects of the current combination therapy of PEG-IFN and ribavirin.

The anti-HCV activities of two antimalarial drugs, artemisinin and its derivative artesunate, are interesting. Although Paeshuyse et al. [14] showed that artemisinin possessed weak or moderate anti-HCV activity using a HuH-7- or HuH-6-derived subgenomic HCV replicon system, artemisinin's anti-HCV mechanism was unclear. On the other hand, Efferth et al. [15] reported that artesunate, the most studied artemisinin-derivative for the treatment of severe malaria, possessed antiviral activity against Epstein–Barr virus, human cytomegalovirus, human herpesvirus 6A, herpes simplex virus 1, and so on, except for HCV with the low micromolar range, although artesunate's precise antiviral mechanism was ambiguous. Therefore, we supposed, and our assay systems clearly detected, that both artemisinin and artesunate possess anti-HCV activity. Especially, the AH1R assay was the most sensitive to artemisinin (EC_{50} 5.3 μM), and the ORL8 assay was the most sensitive to artesunate (EC_{50} 0.22 μM). Preliminary experiments for the anti-HCV mechanisms of these reagents showed that they did not activate the IFN-signaling pathway (data not shown), and that they did not induce the oxidative stress (data not shown) as observed in the treatment with a broad range of anti-HCV reagents, including cyclosporine A [8,17]. Further studies are needed to clarify the anti-HCV mechanisms of these reagents. Since the largest SI value of artemisinin was 58 in the AH1R assay and that of artesunate was 16 in the ORL8 assay, these reagents may be also useful for the treatment of patients with chronic hepatitis.

In this study, we demonstrated that many anti-HCV reagents showed differential anti-HCV activities among different assay systems (OR6, ORL8, and AH1R) on HCV RNA replication. These results suggest that reliance on only a single assay system may lead to an incorrect evaluation of anti-HCV candidates. Therefore, we propose that plural assay systems derived from different cell lines and HCV strains should be used in order to evaluate anti-HCV candidates. Furthermore, plural assay systems derived from at least two different cell origins would be also useful for the screening of anti-HCV candidates.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.05.061.

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ORIGINAL ARTICLE

Establishment of infectious HCV virion-producing cells with newly designed full-genome replicon RNA

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Abstract Hepatitis C virus (HCV) replicon systems enable in-depth analysis of the life cycle of HCV. However, the previously reported full-genome replicon system is unable to produce authentic virions. On the basis of these results, we constructed newly designed full-genomic replicon RNA, which is composed of the intact 5′-terminal-half RNA extending to the NS2 region flanked by an extra selection marker gene. Huh-7 cells harboring this full-genomic RNA proliferated well under G418 selection and secreted virion-like particles into the supernatant. These particles, which were round and 50 nm in diameter when analyzed by electron microscopy, had a buoyant density of 1.08 g/mL that shifted to 1.19 g/mL after NP-40 treatment; these figures match the putative densities of intact virions and nucleocapsids without envelope. The particles also showed infectivity in a colony-forming assay. This system may offer another option for investigating the life cycle of HCV.

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Introduction

Hepatitis C virus (HCV) is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. With over 170 million people currently infected [2], HCV is a growing public-health burden.

The life cycle of HCV has been difficult to study because cell culture and small animal models of HCV infection are not available. The recent development of HCV replicon systems has permitted the study of HCV translation and RNA replication in human hepatoma-derived Huh-7 cells in vitro [17]. However, these replicon systems cannot produce authentic virions because they lack the infection steps, and analysis of these infection steps is very important for understanding HCV pathogenesis.

Recently, some groups have successfully established in vitro infection systems [16, 21, 26, 28–30]. The strategies of these systems are basically the same as the ones used for transfection of Huh-7 cells or their derivatives with in vitro-generated HCV genome RNA [1]. The non-structural regions used in those studies were from the 2a genotype JFH (Japan Fulminant Hepatitis)-1 clone or the 1a genotype H77 clone. The former is known for its exceptionally vigorous amplification and broad permissiveness in cultured cells other than Huh-7 [3, 12, 13], while the latter shows only poor replication ability. Another group reported a newly established immortalized hepatocyte cell line that is susceptible to HCV infection, but only modest improvement was achieved [10]. There are also reports of a system using a full-genome replicon that has the entire coding region under the control of the internal ribosomal entry site of encephalomyocarditis virus, EMCV-IRES; however, this system also failed to show infectivity in the G418 selection assay [7, 20], and secretion of particles with the putative characteristics of HCV virions could not be confirmed [4].

We now report the establishment of infectious virion-producing replicon cells that utilize an ordinary genotype 1b replicon strain. In order to address the contribution of structural and non-structural gene products to the maturation of HCV particles in vitro, we partitioned these regions in the same cistron of the full genomic sequence, thereby enabling the functions of these structural and non-structural genes to be studied separately. Thus, we termed this construction "divided open reading frame carrying" full genome replicon, or dORF replicon.

Virus particles secreted from cells containing dORF replicon RNA, as confirmed morphologically using electron microscopy, were shown to be able to infect Huh-7 cells. Replication of dORF replicon RNA was so efficient that infected cells could survive and proliferate under G418 selection to form colonies, as seen after transfection with replicon RNA. In addition, a reporter gene was successfully inserted into the construct, and activity of the reporter gene could be transmitted to naive Huh-7 cells by infection.

We believe that the success of this system is due to the difference in the construction of the replicon, namely, having the intact 5′ half extending to NS2 instead of being divided at the beginning of the core region. Although further investigation is required to elucidate whether the encapsidation signal of HCV is located in the region that is divided in the full-genome replicon, this is the first report to describe genome-length replicon-containing cells that can produce virus particles that have the putative characteristics of the HCV virion, in terms of both morphology and biological properties.

Results

dORF replicon RNA can replicate in Huh-7 cells

We began this study with transfection with the dORF replicon RNAs (Fig. 1A). When 30 μg of each RNA was electroporated into 4×10^6 Huh-7 cells, the dORF and dORF bla RNA-transfected cells formed 20 and 5 colonies, respectively, after 3 weeks of G418 selection. No colonies appeared as a result of transfection with polymerase-defective mutants (data not shown). Two colonies were picked, amplified, and designated as dORF replicon cell #1 and #2, and dORF bla replicon cell #1 and #2. Some of these cells were then used for quantification of HCV RNA and northern blot analysis (Fig. 1B). Northern blot analysis showed that these clones contained HCV RNAs of the expected size and that the HCV RNA copy numbers of these clones did not differ substantially from that of the subgenomic replicon, indicating that replication ability had not been hampered by insertion of the structural genes, which is counter to what was expected. Western blot analysis showed that these clones express both structural and non-structural proteins (Fig. 1C). These results confirmed that transfected dORF HCV RNAs can replicate in Huh-7 cells, just as authentic subgenomic replicon RNAs do.

dORF replicon cells secrete virus particles

In a previous study, HCV subgenomic replicon cells secreted RNase-resistant subgenomic RNA into the culture supernatant [4, 7, 20]. We also detected a similar amount of RNase-resistant HCV RNA in the culture supernatant of our dORF replicon cells, as well as of the subgenomic and full-genome replicon cells. These supernatants showed no significant differences in terms of distribution of HCV RNA in buoyant density gradient analysis (Figs. 2A, B, open square). In contrast, there was a clear difference between these supernatants after NP-40 treatment. While almost all of the HCV RNA in the supernatant of the subgenomic replicon cells was eliminated by NP-40 treatment (Fig. 2A, filled triangle), there remained a peak of HCV RNA at a density of 1.18 g/mL in the supernatant of the dORF replicon cells (Fig. 2B, filled triangle). These results were confirmed in the same experiment, using concentrated culture supernatant (Figs. 2C, D). We also confirmed the results of previous reports [7, 20], which showed no genomic RNA resistant to NP-40 treatment in the supernatant of full-genome replicon cells (Fig. 2E). Secreted core proteins in the concentrated supernatant showed a different density gradient distribution compared to genomic RNA (Fig. 2F, open circle) in that the core proteins were present at densities of 1.1–1.2 g/mL, while HCV RNA was more broadly distributed in the range of 1.06–1.22 g/mL. Thus, HCV RNA and core proteins were not always associated with each other. However, after NP-40 treatment, core proteins were found only in the same fraction as HCV RNA, at 1.19 g/mL (Fig. 2F, filled triangle). Taken together with the results of the report mentioned above [20], our replicon cells harboring dORF RNA appeared to secrete particles with core proteins that were assembled into nucleocapsids as well as particles without core proteins that were sensitive to NP-40 treatment, like the ones from subgenomic and full-genome replicon cells. We concluded that the broader distribution of the HCV genome RNA in the density gradient than that of the core protein was caused by the overlapping distribution of these two particle types, and that the remaining peaks of genome RNA and core protein after NP-40 treatment were of nucleocapsids that had had their envelopes stripped off by NP-40 [11].

According to our hypothesis, the distribution of core proteins in the density gradient represented that of the

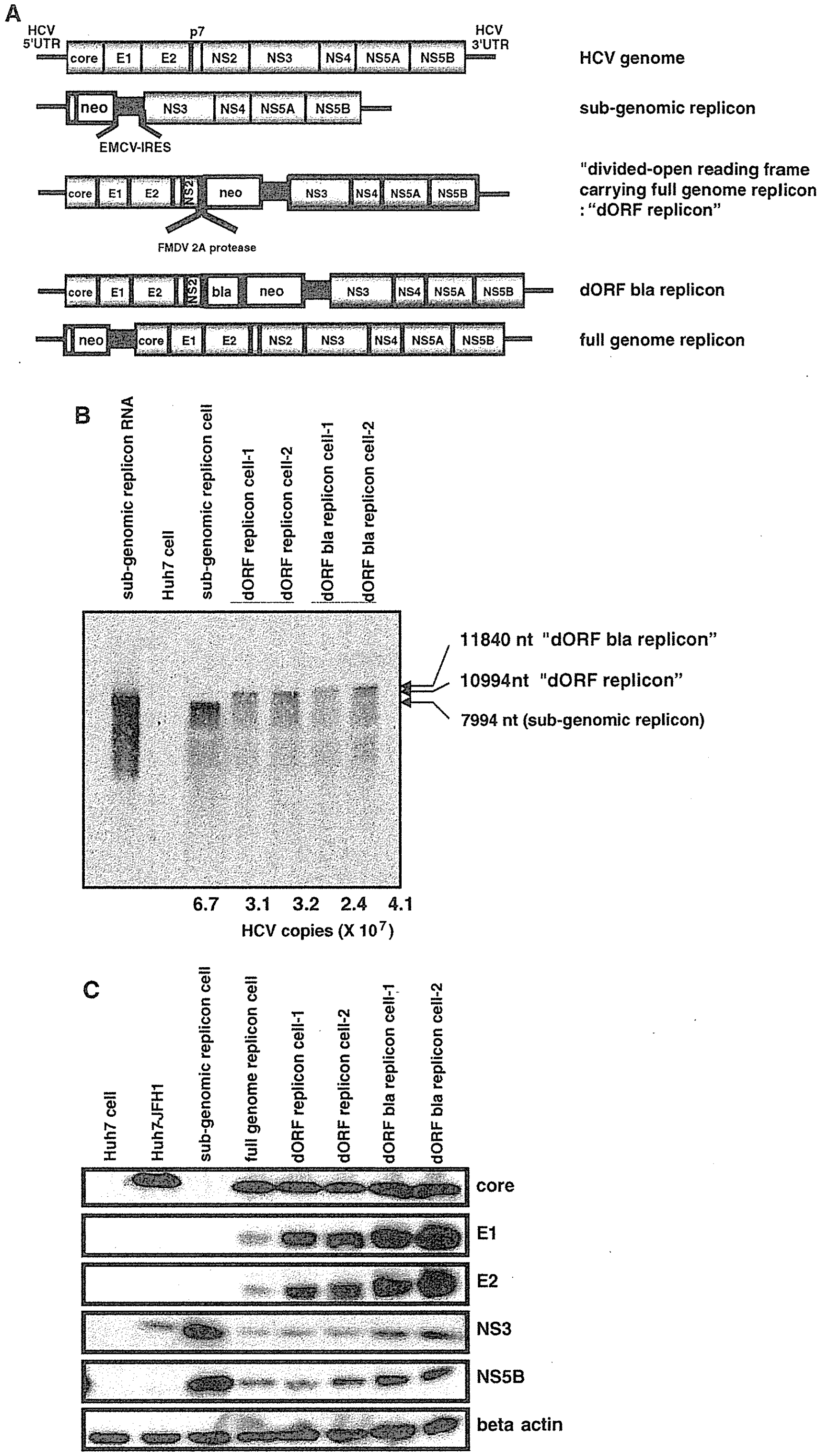


Fig. 1 Confirmation of "divided open reading frame carrying" (dORF) replicon cells. (A) Schematic representations of replicon RNAs used in this study. All the replicon constructs contained inserts just after the T7 promotor. UTR, untranslated region; NS, non-structural protein; neo, neomycin phosphotransferase II; EMCV, encephalomyocarditis virus; IRES, internal ribosomal entry site; FMDV, foot-and-mouth disease virus; bla, beta-lactamase. (B) Northern blot analysis. A 10-μg amount of total RNA from each cell sample was loaded. Subgenomic replicon RNA: 10^8 copies of in vitro-generated subgenomic RNA. Numbers below the lanes are the HCV copy number per microgram of total RNA. Huh-7 cell, subgenomic replicon cell, dORF replicon cell #1, #2, dORF bla replicon cell #1, #2. (C) Western blot analysis. A 10-μg amount of each cell lysate was loaded. Huh-7 cell, Huh-7-JFH1: Huh-7 cell transfected with JFH1 viral RNA, subgenomic replicon cell, full-genome replicon cell, dORF replicon cell #1, #2, dORF bla replicon cell #1, #2

intact virion, and we therefore tried to observe virions directly by electron microscopy, using the fraction in which the core protein was present. We easily identified numerous round-shaped virus particles approximately 50 nm in diameter by scanning electron microscopy (Fig. 3A). Furthermore, when the immunogold method using anti-E2

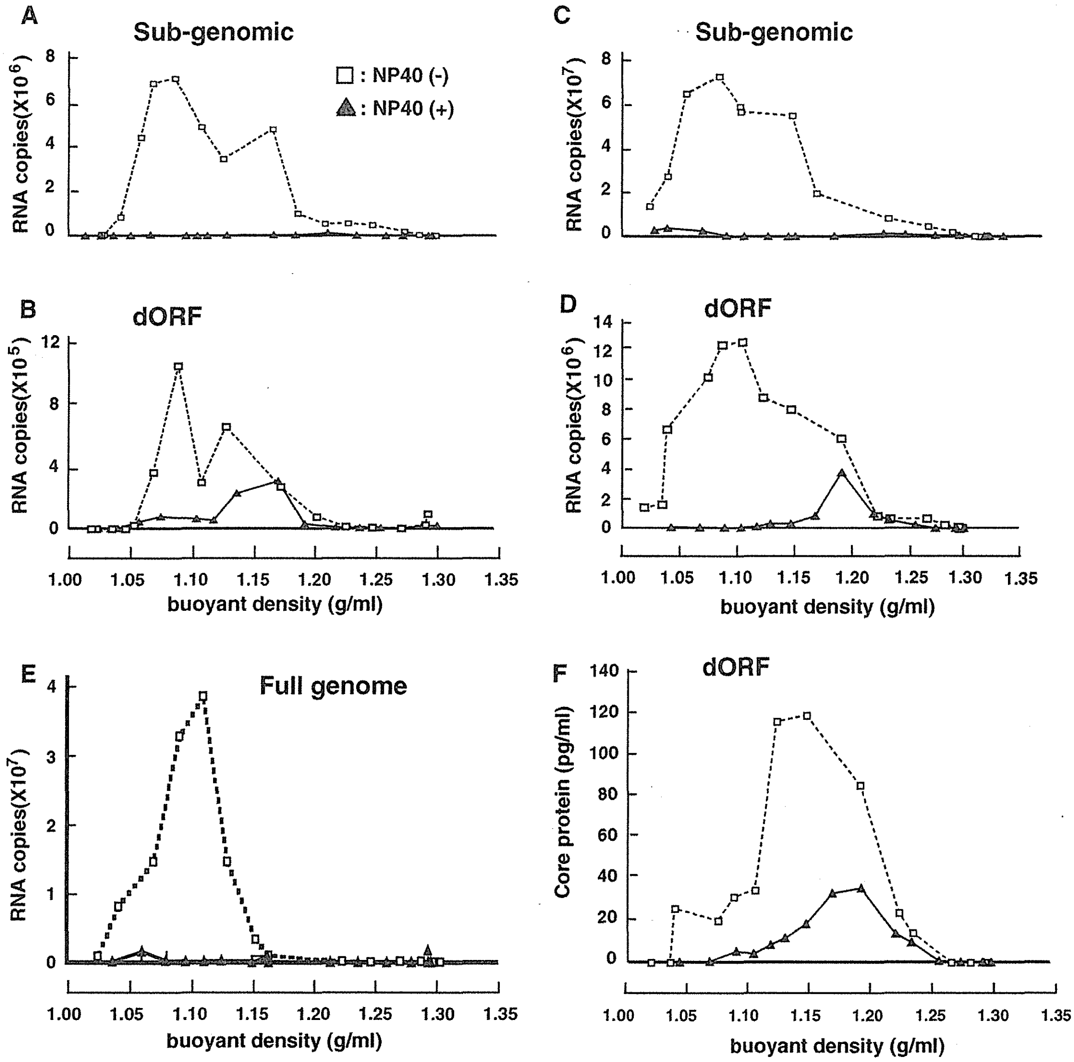


Fig. 2 Density gradient analysis of supernatants. Culture supernatants were treated with RNaseA and loaded directly onto a sucrose density gradient without treatment (open square) or after NP-40 treatment (filled triangle). Quantification of HCV RNA in each fraction of supernatant from the subgenomic replicon (A) and dORF replicon (B). Analysis of concentrated culture supernatant from the subgenomic replicon (C) and dORF replicon (D). Concentrated culture supernatant from the full-genome replicon NNC#2 was also analyzed (E). Quantification of HCV core protein in each fraction of supernatant from the dORF replicon (F)

RR6 antibody was applied to samples fixed on the mesh, transmission electron microscopy could be used to visualize virus particles labeled with colloidal gold (Fig. 3B). These findings provide evidence of intact virion production from our dORF replicon cells.

Secreted virus particles can infect naive Huh-7 cells

Next, we examined the infectivity of these virus particles. The culture supernatants of these dORF replicon cells were collected, and 3 kinds of naive Huh-7 cells, one purchased from the J.C.R.B. (Japanese Collection of Research Bioresources) and the other two, designated as the cured cells F2 and K4, generated by IFN-α treatment of 1bneo/delS replicon cells, were infected with these supernatants. After two sequential passages and three weeks of G418 selection as described above, a number of colonies appeared, as shown in Fig. 4A. The largest number of colonies was produced from the cured cells K4, and slightly fewer colonies were produced from the cured cells F2, while no colonies appeared when normal Huh-7 cells were used (data not shown). The same infection experiment carried

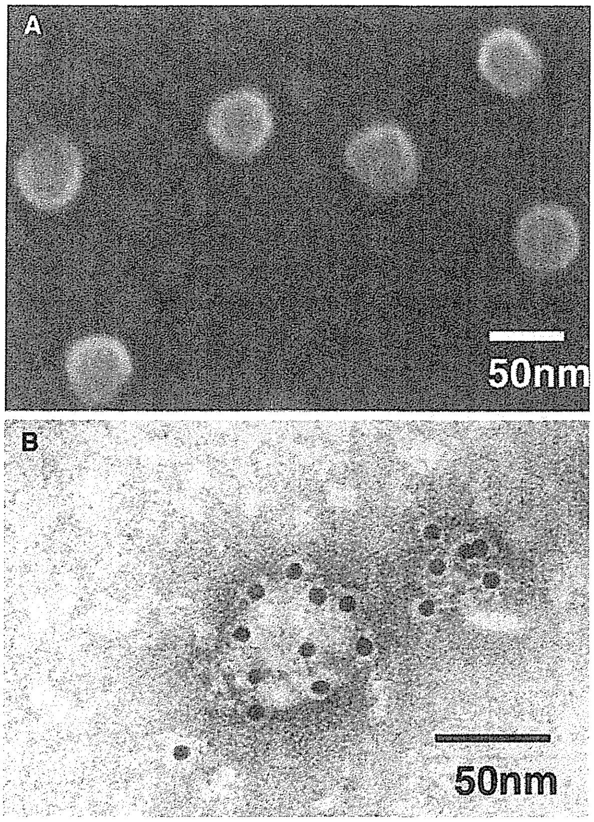


Fig. 3 Electron microscopy analysis of virus-like particles. The core-protein-rich fraction collected from the density gradient was further concentrated by ultracentrifugation and observed by scanning electron microscopy (A). The same fraction attached to formvar-coated grids was incubated with rabbit anti-E2 RR6 antibody, treated with goat anti-rabbit IgG coupled to 10-nm colloidal gold, negatively stained with uranyl acetate, and then examined by transmission electron microscopy (B)

out with full-genome replicon cells produced no infectivity in the supernatant (data not shown). Under the most efficient conditions, the titer of the supernatant reached as high as 20 cfu (colony-forming units) per milliliter when the putative doubling time of these cells was approximately 24 h. Furthermore, the appearance of colonies was abolished by addition of the antibody JS-81 (BD Pharmingen), an antibody to CD81, a possible co-receptor of HCV [22] (Fig. 4B).

Next, we propagated some of these colonies for further analysis. Northern blot analysis showed that these clones carry HCV RNAs of reasonable size (Fig. 5A), including subgenomic RNA (7994 bases), dORF RNA (10994 bases), and dORF bla RNA (11840 bases). Western blot analysis revealed that the cell clones that were infected with supernatant from Huh-7 cells containing the dORF replicon expressed structural proteins (Fig. 5B), indicating that the colonies were not just the reappearance of subgenomic replicons hidden in the cured cells.

Together, our findings indicate that these particles in the supernatant infected the Huh-7 cells through a CD81-associated pathway and that infected cells formed colonies after G418 selection, similar to what was observed with electroporation with subgenomic RNA.

A reporter gene inserted into the dORF replicon RNA can be transmitted through infection

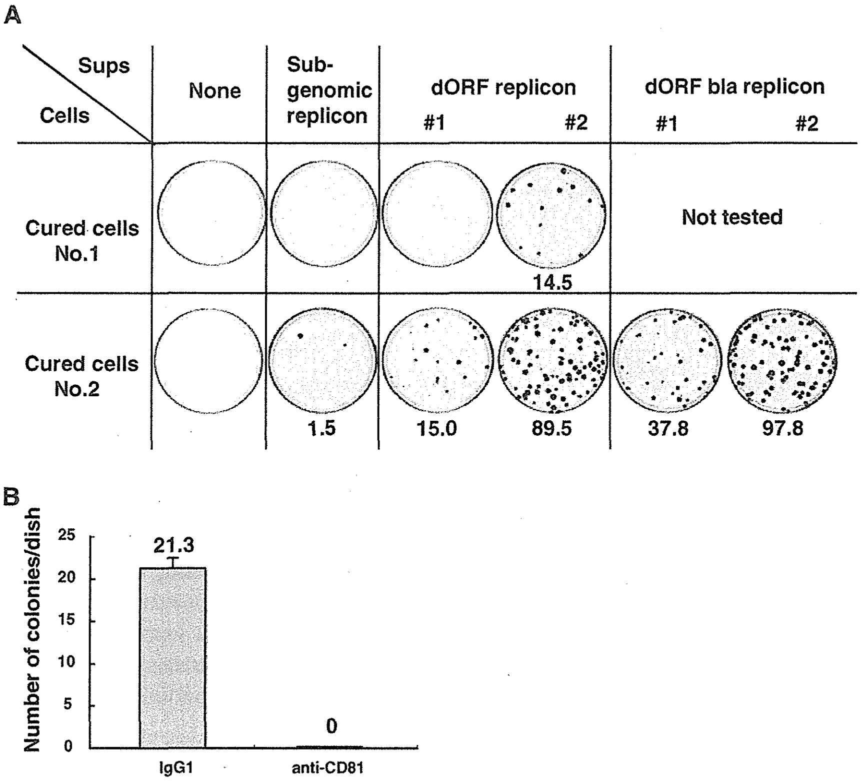
First, we confirmed that the beta-lactamase gene in the dORF bla replicon RNA was active in established replicon cell clones and able to process the green fluorescent substrate into blue fluorescent product (Fig. 6A). Next, we attempted to detect the activity of the beta-lactamase gene in the cloned infected colonies. Three clones grown from cells infected with the dORF bla supernatant were treated using a GeneBLAzer In Vivo Detection Kit. One clone was positive for blue fluorescence (Fig. 6B), demonstrating that a reporter gene inserted into the dORF replicon could be transmitted to naive Huh-7 cells through secreted virus particles in the culture supernatant.

Discussion

There have been several previous reports of full-genome HCV replicons that can replicate well in Huh-7 cells and express sufficient amounts of structural proteins [1, 4, 7, 14, 20]. Pietschmann et al. (2002) observed the secretion of an RNase-resistant HCV genome into the supernatant from both full-genome and subgenomic replicon cells and nonspecific uptake of these genomes by naive Huh-7 cells. Ikeda et al. (2002) were also unable to detect any infectivity in the supernatant of their full-genome replicon cells. They assumed that the reason for this failure was the inability of Huh-7 cells to release intact virions or to be infected by the virus, although this was later demonstrated not to be the case by a series of reports on infection using the JFH-1 clone [16, 26, 30].

First, we attempted to improve the efficiency of the full-genome replicon in two ways, namely, by modifying the construct and reducing the genome size. Numerous studies have examined the encapsidation signal in the genomic RNA of positive-sense single-stranded viruses [5, 8, 9]. Frolova et al. [5] showed that the encapsidation signal of Sindbis virus lies in the nsP1 gene and is 132 nucleotides long. Johansen et al. [9] found that the IRES of poliovirus had the ability to enhance the efficiency of packaging of the polio subgenomic replicon. We think that these findings indicate that the construction of the genome could affect the efficacy of encapsidation, and we therefore decided to

Fig. 4 Infectivity of supernatants from various replicon cells. Colonies of cells infected with the indicated supernatant. Numbers shown below the plates are the average of a total of four plates per condition (A). Inhibition of infection by anti-CD81 antibody. Cured cell K4 cells (No.2 in Fig. 4A) were treated with mouse IgG1 as the negative control or anti-CD81 before infection (B)



change the site of genome division from the beginning of the core region to the middle of the NS2 region. Regarding the size of the genome, there have been reports that the insertion of a foreign gene of significant size can result in the deletion of a portion of the chimeric genome during replication [18, 19]. We therefore removed the second half of the NS2 region, because this region appears to be unnecessary for both replication and packaging in Huh-7 cells, and this deletion was found to have no influence on the efficacy of encapsidation, as there were no apparent differences between the NS2-deleted construct and the one containing the entire NS2 region (data not shown).

Our established dORF replicon was able to replicate well in Huh-7 cells and express sufficient amounts of structural proteins, similar to the previously reported full-genome replicon. Although both the dORF replicon cells and the previously reported full-genome replicons secreted RNase-resistant genomes, there was a striking difference between these two full-genome replicons when NP-40 treatment was carried out on their supernatants. There was no RNase-resistant genome left in the NP-40-treated supernatant of full-genome replicons, although density gradient analysis of the NP-40-treated supernatant of dORF replicon cells clearly showed the coexistence of the HCV genome and core proteins at a peak of 1.18 g/mL. This peak may represent NP-40-resistant nucleocapsids. The distribution of core proteins in the density gradient analysis of the concentrated supernatant of the dORF replicons did not match that of the HCV genome. A reasonable explanation for this mismatch is that the lighter side of the broad peak of the HCV genome was not representative of intact virions and is instead an indication of secretion by a pathway used in subgenomic replicon cells, which differs from the natural process. The fact that the peak of the HCV genome of full-genome replicons was located in a narrow range on the lighter side compared to that of the dORF replicons supports this hypothesis. We observed round particles in the concentrated core protein fraction using electron microscopy, and those particles also seemed to contain core proteins. These findings indicate that our dORF replicon cells produced both intact virions and artificial membranous particles, with the former having the morphological and biophysical characteristics of putative virions.

The colony-forming assay clearly demonstrated the ability of the supernatants of our dORF replicon cells to infect Huh-7 cells efficiently. The reason for the difference in efficacy between the two cured cells is uncertain but may involve the ability to support replication or the level of receptor expression. This needs to be clarified in order to improve the efficiency of HCV infection in vitro. Differences in the efficiency of infection were also noted between

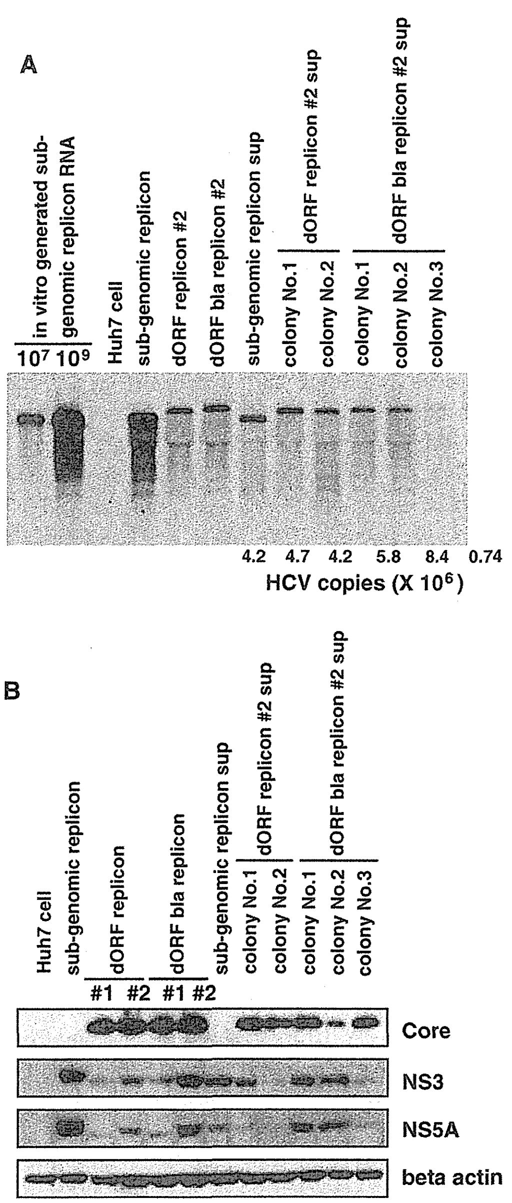


Fig. 5 Northern blot analysis of colonies formed after infection. 10^7, 10^9: amounts of in vitro-generated subgenomic replicon RNA loaded. Numbers below the lanes are the HCV copy number per μg of total RNA (A). Huh-7 cells, subgenomic replicon cells, dORF replicon cell #2, dORF bla replicon cell #2, subgenomic replicon sup: colony from cells transduced with subgenomic replicon supernatant, colony No.1, 2 of dORF replicon #2 sup: colonies from cells infected with dORF replicon #2 supernatant, colony No.1, 2, and 3 of dORF bla replicon #2 sup: colonies from cells infected with dORF bla replicon #2 supernatant. Western blot analysis of colonies formed after infection (B). The order of the lanes is identical to that for the northern blot, except for the dORF and dORF bla replicons, which represent two clones in this figure

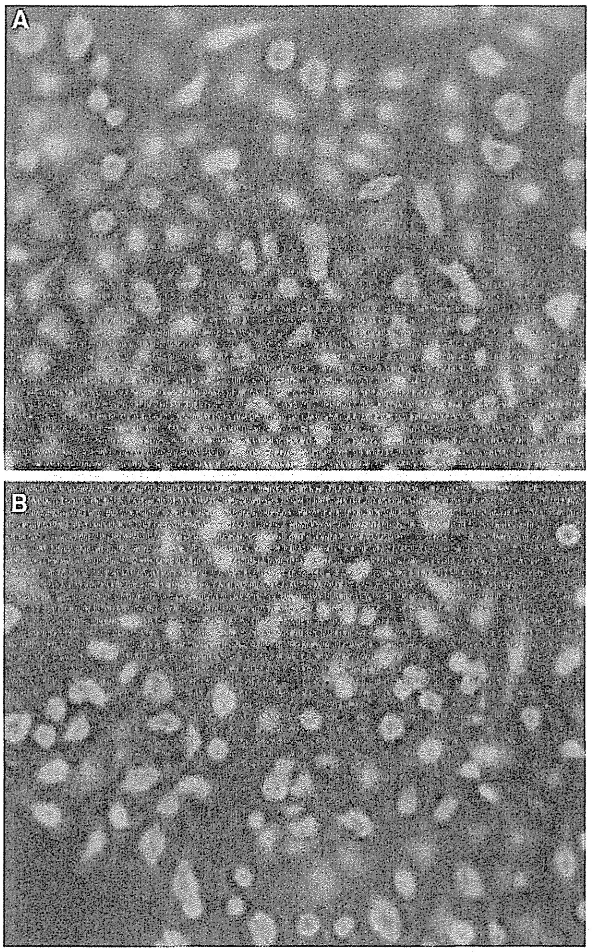


Fig. 6 Detection of beta-lactamase activity in dORF replicon cells. Parental dORF bla replicon #2 cell (A) and colony no. 3 cloned from cells infected with dORF bla replicon #2 cell supernatant (B). Blue fluorescence shows high beta-lactamase activity, indicating that the reporter gene functioned normally after infection

clones of the same dORF replicon cells, which may have been due to the accumulation of different mutations in the structural region, although we have not yet confirmed this hypothesis. We also observed colonies being formed by cells that were treated with supernatant containing subgenomic replicons, and these colonies most likely represent the so-called "non-specific transduction" of the subgenomic replicon. Although this dORF supernatant infection could be blocked by the anti-CD81 antibody reported previously [30], we cannot exclude the possibility that the infection we observed was due to highly efficient "non-specific transduction," as we could not determine whether "non-specific transduction" also could be affected by the anti-CD81 antibody because of the low colony-forming ability of the supernatant of subgenomic replicons.

We also demonstrated that the reporter gene that was inserted in addition to the neomycin resistance gene could be transmitted to the new generation of viruses. This finding raises the possibility of producing sufficient amounts of reporter virus constitutively.

In summary, we established an infectious-particle-producing HCV replicon system. This achievement should yield more precise information about the encapsidation signal of HCV, which was kept intact despite the partitioning of the genome. This system also allows analysis of the pathway of HCV infection, including adsorption of virions to cell-surface receptors, penetration, uncoating, virus particle assembly, and HCV release. Moreover, the dORF replicon system may be used as a convenient tool to investigate the utility of the newly established siRNA system [14, 27] and evaluation of compounds that are effective against subgenomic replicons.

Although we believe that the reason for our success is our new construct, further examination is necessary to verify our findings.

Materials and methods

Construction and RNA transcription

To construct dORF replicon RNA, the second half of the NS2 region of the HCV-R6 strain [25] was replaced in frame with the foot and mouth disease virus (FMDV) 2A protease gene, the neomycin resistance gene, and the encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES). In addition, the region from NS3 to the beginning of NS5B was replaced with the 1bneo/delS replicon sequence from the N strain of genotype 1b [6](kindly provided by Dr. Seeger). This construct was designated as the "divided open reading frame carrying full genome" (dORF) replicon. The subgenomic replicon construct was also prepared from the R6 strain and also contained the 1bneo/delS replacement. For the reporter assay, the FMDV 2A protease gene and beta-lactamase gene (bla; Invitrogen) were inserted after the remaining NS2 gene to produce the dORF bla replicon construct. Replication-deficient versions of these three replicons were also prepared by deleting 27 nucleotides, including the GDD motif of NS5B polymerase.

In vitro transcription of these replicon RNAs was performed using the MEGAscript kit (Ambion).

Cell culture and electroporation

Huh-7 cells were cultured in DMEM (SIGMA) with 10% fetal bovine serum. Replicon cells were maintained in the same medium supplemented with 300 μg/mL G418 (Invitrogen). These cells were passaged 3 times a week at a 4:1 splitting ratio. Electroporation of replicon RNA was performed as described previously [17]. The subgenomic replicon (1bneo/delS replicon) cells were treated with 1000 IU of IFN-α for 2 months and cloned by the limited dilution method. Two of these clones were designated as HCV replicon-cured Huh-7 cells F2 and K4. The cell line containing the full-genome replicon of genotype1b, namely the NNC#2 clone [15], was a kind gift from Dr. Shimotohno of Keio University.

Northern blot analysis and quantification of HCV RNA

Total RNA was purified from cells using ISOGEN (Nippon Gene) for northern blot analysis or ABI prizm6100 (Applied Biosystems) for real-time RT-PCR. Purified RNAs were quantified by absorbance at 260 nm. For northern blot analysis, 30 μg of each total RNA was used with a Northern Max Kit (Ambion) according to the manufacturer's instructions. The probe for detection of HCV RNA was a PCR fragment of the NS5B region (nucleotide numbers 7629–7963) that had been biotin-labeled using a BrightStar Psoralen-Biotin Kit (Ambion) according to the manufacturer's instructions. Following hybridization of the membranes, the probe was detected using a BrightStar BioDetect Kit (Ambion) according to the manufacturer's instructions, and luminescence was detected using the LAS1000 detection system (Fujifilm). Measurement of the HCV RNA copy number by real-time RT-PCR was performed using an ABI PRISM 7900 system (Applied Biosystems) as described previously [24].

Western blot analysis

Western blot analysis was carried out using the conventional semi-dry blot method. Cells were lysed with buffer containing 100 mM Tris-HCl (pH 7.4) and 4% sodium dodecyl sulfate. A 10-μg amount of protein from each sample was separated by SDS-PAGE through a 4–20% gradient gel (Invitrogen) and transferred to the membrane according to the gel manufacturer's protocol. The antibodies used in this study were anti-core mouse monoclonal antibody (MAb), anti-E1 MAb, anti-E2 MAb (reported previously; [25]), anti-NS3 antiserum (reported previously; [25]), anti-NS5B antiserum (Upstate), and anti-beta-actin MAb (Abcam). Horseradish peroxidase-labeled anti-mouse and anti-rabbit IgG goat antibodies (Santa Cruz Biotechnology and DAKO, respectively) were used as the secondary antibody. The membranes were treated using an ECL Plus kit (Amersham) according to the manufacturer's instructions, and luminescence was detected using an LAS1000 system (Fujifilm).

Density gradient analysis and core ELISA

Culture supernatants from replicon cells were loaded onto 10–60% sucrose density gradient tubes with or without 10-fold concentration in an Amicon-100 (Millipore). The tubes were then ultracentrifuged at 100,000 *g* for 16 h and fractionated. NP-40 was added to the culture supernatants to a final concentration of 0.5%, and they were then incubated at 4°C for 30 min. For electron microscopy, the culture supernatant was concentrated, loaded onto a 60% sucrose cushion, and ultracentrifuged at 100,000 *g* for 4 h. The interface between the concentrated medium and the sucrose cushion was collected and separated by the density gradient method described above. A 2-mL fraction from 5 ml to 7 mL from the bottom, with a density of 1.1–1.2 g/mL, was examined by electron microscopy after further concentration by the sucrose cushion ultracentrifugation method described above. The amount of core protein in the fractions was quantified using an Ohso ELISA kit in accordance with the manufacturer's instructions.

Electron microscopy

The concentrated fraction of core protein was observed by scanning and transmission electron microscopy. For scanning electron microscopy, the sample was allowed to settle on the surface of a poly-L-lysine-coated glass cover slip for 30 min, and the attached sample was then fixed with 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min, washed three times with 0.1 M phosphate buffer, and post-fixed with 1% osmium tetroxide in the same buffer for 10 min. After dehydration through a graded series of ethanol, the samples were dried in a freeze dryer (Hitachi ES-2020, Hitachi) using t-butyl alcohol, coated with osmium tetroxide, approximately 2 nm thick, using an osmium plasma coater (NL-OPC80; Nippon Laser and Electronics Laboratory), and then examined using a Hitachi S-4800 field emission scanning electron microscope at an accelerating voltage of 10 kV [23]. For transmission electron microscopy, the sample was allowed to settle on a formvar-coated nickel grid for 10 min, dried in air, incubated with rabbit anti-E2RR6 antibody (prepared as described in the supplementary information), washed with PBS, and then incubated with goat anti-rabbit IgG coupled to 10-nm colloidal gold (British BioCell). After negative staining with 2% uranyl acetate, the sample was examined using a JEM 1200EX transmission electron microscope (JEOL) at an accelerating voltage of 80 kV.

Rabbit anti-E2 RR6 antibody to the HCV-E2 protein was prepared as follows: The E2 gene of HCV type 1b [25] was cloned under the control of the ATI-P7.5 hybrid promoter of vaccinia virus vector pSFB4 and allowed to recombine with the Lister strain of the vaccinia virus to give vector RVV. Rabbits were infected intradermally with 10^8 p.f.u. of RVV, and 2 months later, they received two booster injections with the purified E2 protein. HCV-E2 protein was expressed from the RVV vector and purified by lentil lectin column chromatography and affinity chromatography using an anti-E2 monoclonal antibody [25].

Infection

A 2.5-ml aliquot of cleared culture supernatants from replicon cells was added to approximately 70% confluent of Huh-7 cells in 25-cm^2 flasks, and the same amount of complete DMEM was added 2 h later. Infected cells were transferred to 75-cm^2 flasks the next day and to four 10-cm dishes 2 days later. G418 at a concentration of 300 μg/mL was added to the medium immediately after the second passage. The three types of Huh-7 cells used in this study included the one purchased from J.C.R.B. and the 2 IFN-cured replicon cell lines F2 and K4 described above. The medium was changed every other day. For the blocking experiment, cells were treated with the anti-CD81antibody as described previously [30]. Cells were fixed with 10% formalin/PBS(-) for 10 min after washing with PBS(-) and staining with 1% crystal violet/PBS(-) for 1 h before washing with water.

Beta-lactamase detection assay

Beta-lactamase activity was detected using a GeneBLAzer In Vivo Detection Kit (Invitrogen) according to the manufacturer's instructions and observed using a fluorescence microscope (Nikon) with UV light excitation.

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Augmentation of DHCR24 expression by hepatitis C virus infection facilitates viral replication in hepatocytes

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Background & Aims: We characterized the role of 24-dehydrocholesterol reductase (DHCR24) in hepatitis C virus infection (HCV). DHCR24 is a cholesterol biosynthetic enzyme and cholesterol is a major component of lipid rafts, which is reported to play an important role in HCV replication. Therefore, we examined the potential of DHCR24 as a target for novel HCV therapeutic agents.

Methods: We examined DHCR24 expression in human hepatocytes in both the livers of HCV-infected patients and those of chimeric mice with human hepatocytes. We targeted *DHCR24* with siRNA and U18666A which is an inhibitor of both DHCR24 and cholesterol synthesis. We measured the level of HCV replication in these HCV replicon cell lines and HCV infected cells. U18666A was administrated into chimeric mice with humanized liver, and anti-viral effects were assessed.

Results: Expression of DHCR24 was induced by HCV infection in human hepatocytes *in vitro*, and in human hepatocytes of chimeric mouse liver. Silencing of *DHCR24* by siRNA decreased HCV replication in replicon cell lines and HCV JFH-1 strain-infected cells. Treatment with U18666A suppressed HCV replication in the replicon cell lines. Moreover, to evaluate the anti-viral effect of U18666A *in vivo*, we administrated U18666A with or without pegylated interferon to chimeric mice and observed an inhibitory effect of U18666A on HCV infection and a synergistic effect with interferon.

Conclusions: DHCR24 is an essential host factor which augmented its expression by HCV infection, and plays a significant role in HCV replication. DHCR24 may serve as a novel anti-HCV drug target.

Introduction

Extensive epidemiological studies have identified multiple risk factors for hepatocellular carcinoma (HCC), including chronic infection with hepatitis C virus (HCV), and hepatitis B virus (HBV), and cirrhosis due to non-viral etiologies, such as alcohol abuse and aflatoxin B1 exposure [1,2]. Of these factors, HCV appears to be the dominant causative factor for HCC in many developed countries. The World Health Organization estimates that 170 million people worldwide are infected with HCV and are, therefore, at risk of developing liver cirrhosis and HCC [3]. The combination of pegylated interferon-α (PEG-IFN-α) and ribavirin is currently the standard treatment regimen for patients with chronic HCV infection. However, viral clearance is achieved in only 40% to 60% of patients and depends on the HCV genotype with which the patient is infected [4].

We previously established the RzM6 cell line, a HepG2 cell line in which the full-length HCV genome (HCR6-Rz) can be conditionally expressed under control of the Cre/*loxP* system and is precisely self-trimmed at the 5′ and 3′-termini by ribozyme sequences [5]. Anchorage-independent growth of these cells accelerates after 44 days of continuous passaging, during which the Cdk-Rb-E2F pathway is activated [5]. In a previous study, we developed monoclonal antibodies (MoAbs) against cell surface antigens on HCV-expressing cells that had been passaged for over 44 days [6]. One of the targets of these MoAbs was 24-dehydrocholesterol reductase (DHCR24 is also called 3-β-hydroxysterol-Δ-24-reductase, seladin-1, desmosterol delta-24-reductase), a molecule that is frequently overexpressed in the hepatocytes of HCV-infected patients.

Keywords: Hepatitis C virus; Replication; DHCR24; U18666A.

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Abbreviations: DHCR24, 24-dehydrocholesterol reductase; HCV, hepatitis C virus; MoAb, monoclonal antibody; HCC, hepatocellular carcinoma; HBV, hepatitis B virus.

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DHCR24 confers resistance to apoptosis in neuronal cells [7]. It also regulates the cellular response to oxidative stress by binding to the amino terminus of p53, thereby displacing mouse double minute 2 homolog isoform MDM2 (*Homo sapiens*) (MDM2) from p53 and inducing the accumulation of p53 in human embryonic fibroblasts [8].

DHCR24 is a cholesterol biosynthetic enzyme that is also called desmosterol reductase [9,10]. Cholesterol is a major component of lipid rafts, which are reported to play an important role in HCV replication [11]. Therefore, we characterized the role of DHCR24 in HCV replication and evaluated its potential as a target for novel HCV therapeutic agents. We also examined the synergistic antiviral effect of U18666A which is an inhibitor of both DHCR24 [12] and cholesterol synthesis [13] with IFN-α in the treatment of HCV.

Materials and methods

Cells and plasmids

Cell culture methods of the HuH-7 [14], HepG2 [15], hybridoma and myeloma PAI cells, RzM6 cells [5], and the HCV subgenomic replicon cells lines FLR3-1 (genotype 1b, strain Con-1; [16]), R6FLR-N (genotype 1b, strain N; [17]), and Rep JFH Luc3-13) genotype 2a, strain JFH-1 [18]) were utilized to evaluate HCV replication [19] are described in Supplementary data.

The *DHCR24* cDNA was synthesized and amplified by PCR using Phusion™ DNA polymerase (Finnzymes) and cloned into the pcDNA3.1 vector (Invitrogen) or lentivirus vector, as described previously [6].

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis

The detailed procedures are described in Supplementary data [20].

Immunohistochemistry and Western blot analysis

The detailed procedures are described in Supplementary data.

The antibodies used in this experiment were: anti-Core, anti-NS3, anti-NS4B, anti-NS5B [5], and anti-NS5A (kindly provided by Dr. Matsuura, Osaka University), and anti-actin (Sigma).

Inhibition of DHCR24 by siRNA

We synthesized two siRNAs that were directed against human *DHCR24* mRNA: siDHCR24-417 and siDHCR24-1024. The target sequence of siDHCR24-417 was 5′-GUACAAGAAGACACACAAATT-3′, while that of siDHCR24-1024 was 5′-GAGAACUAUCUGAAGACAATT-3′. Additionally, we used siRNAs targeted against the HCV genome (siE-R7 and siE-R5) [17,21]. The siCONTROL Non-Targeting siRNA #3 (Dharmacon RNA Technologies) was used as the negative control siRNA. The chemically synthesized siRNAs were transfected into cells using Lipofectamine RNAiMAX (Invitrogen) and Opti-MEM (Invitrogen) by reverse-transfection. Cells were characterized 72 h after transfection.

Inhibition of viral replication by U18666A

U18666A (Calbiochem) was utilized to treat HCV replicon cells at a concentration of 62.5–1000 nM and chimeric mice at a concentration of 10 mg/kg (i.p.).

To determine whether cholesterol can reverse the U18666A treatment by the addition of cholesterol, we performed the experiments using HCV replicon cells (4×10^3 cells/well in a 96-well white plate, SUMILON). Culture medium was replaced after the cells had spread (at 24 h), and LDL (Calbiochem) was added to reach a final cholesterol concentration of 50 μg/ml. After a 24 h-incubation, U18666A (62.5, 125, 250, 500, and 1000 nM) was added to each well, and the cells were incubated for an additional 48 h. HCV replication activity was measured by luciferase assay, and cell viability was measured with the WST-8 cell counting kit according to the manufacturer's instructions (Dojindo Laboratories). Cholesterol measurements are described in Supplementary data.

Inhibition assay of HCV replication in replicon cells and persistent infected cells

For evaluation of the anti-HCV replication effect of the inhibitor U18666A in replicon cells and HCV persistently infected cells are described in Supplementary data.

Real-time detection (RTD)-PCR

Total RNA was purified from JFH-K4 cells that had been treated with siRNA or U18666A by the acid guanidium–phenol–chloroform method. HCV RNA was quantified by RTD-PCR as previously described [22].

HCV infection of chimeric mice with humanized liver and mRNA quantification by RTD-PCR

We used chimeric mice that were created by transplanting human primary hepatocytes into severe combined immunodeficient mice carrying a urokinase plasminogen activator transgene [23,24] that was controlled by the albumin promoter. These hepatocytes had been infected with plasma from a HCV-positive patient HCR6 (genotype 1b) [19]. The HCV 1b RNA level reached 2.9–18.0×10^6 copies/ml in mouse sera after 1–2 months of infection. HCV RNA in the mouse serum or total RNA from liver tissue from humanized chimeric mice with/without HCV infection was extracted using the acid guanidium–phenol–chloroform method. HCV RNA and *DHCR24* mRNA levels were quantified by RTD-PCR [22]. The primers and probes for HCV were prepared as previously described [22], and the primers and probes for *DHCR24* were prepared using TaqMan® Gene Expression assays (Applied Biosystems) according to the manufacturer's instructions. PEG-IFNα-2a (Chugai) was administered subcutaneously at a concentration of 30 μg/kg, at day 1, 4, 8, and 11 (the amount of PEG-IFN-α administered to the chimeric mice was 20-fold relative to that used in humans), and U18666A was administered intraperitoneally at a concentration of 10 mg/kg, every day for 2 weeks (Fig. 6A). The protocols for the animal experiments were approved by the local ethics committee.

Human serum albumin in the blood of humanized chimeric mice was measured using a commercially available kit, according to the manufacturer's instructions (Alb-II kit; Eiken Chemical).

Results

Identification of DHCR24

We inoculated mice (BALB/c) with RzM6 cells that expressed HCV protein and had been cultured for over 44 days (denoted as RzM6-LC cells); mice were inoculated at least seven times over a 2-week period. We then fused the splenocytes from mice that had been immunized with RzM6-LC cells to myeloma cells to establish hybridomas. Characterization of the culture supernatant from more than 1000 hybridoma cells by ELISA (data not shown) revealed that one MoAb clone (2-152a) recognized a molecule of approximately 60 kDa in various cells (Supplementary Fig. 1A and B. This molecule was more highly expressed in RzM6-LC cells (Supplementary Fig. 1A), HeLa cells, and HCC cell lines (HepG2, HuH-7, Hep3B, and PLC/PRF/5) than in HEK293 cells and several normal liver cell lines (NKNT, TTNT, and WRL68) (Supplementary Fig. 1B). To further characterize this molecule, we performed matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and obtained seven peptide sequences (Supplementary Fig. 1C, underlined). These peptide sequences suggested that the molecule that was recognized by the 2-152a antibody was DHCR24. We constructed a lentivirus expression vector containing myc-tagged DHCR24 (DHCR24-myc) and transduced it into HepG2 cells. By western blot analysis with 2-152a and anti-Myc antibody, we then confirmed that DHCR24 was expressed in the transduced cells (Supplementary Fig. 1D). We found that the 2-152a antibody specifically recognized DHCR24.

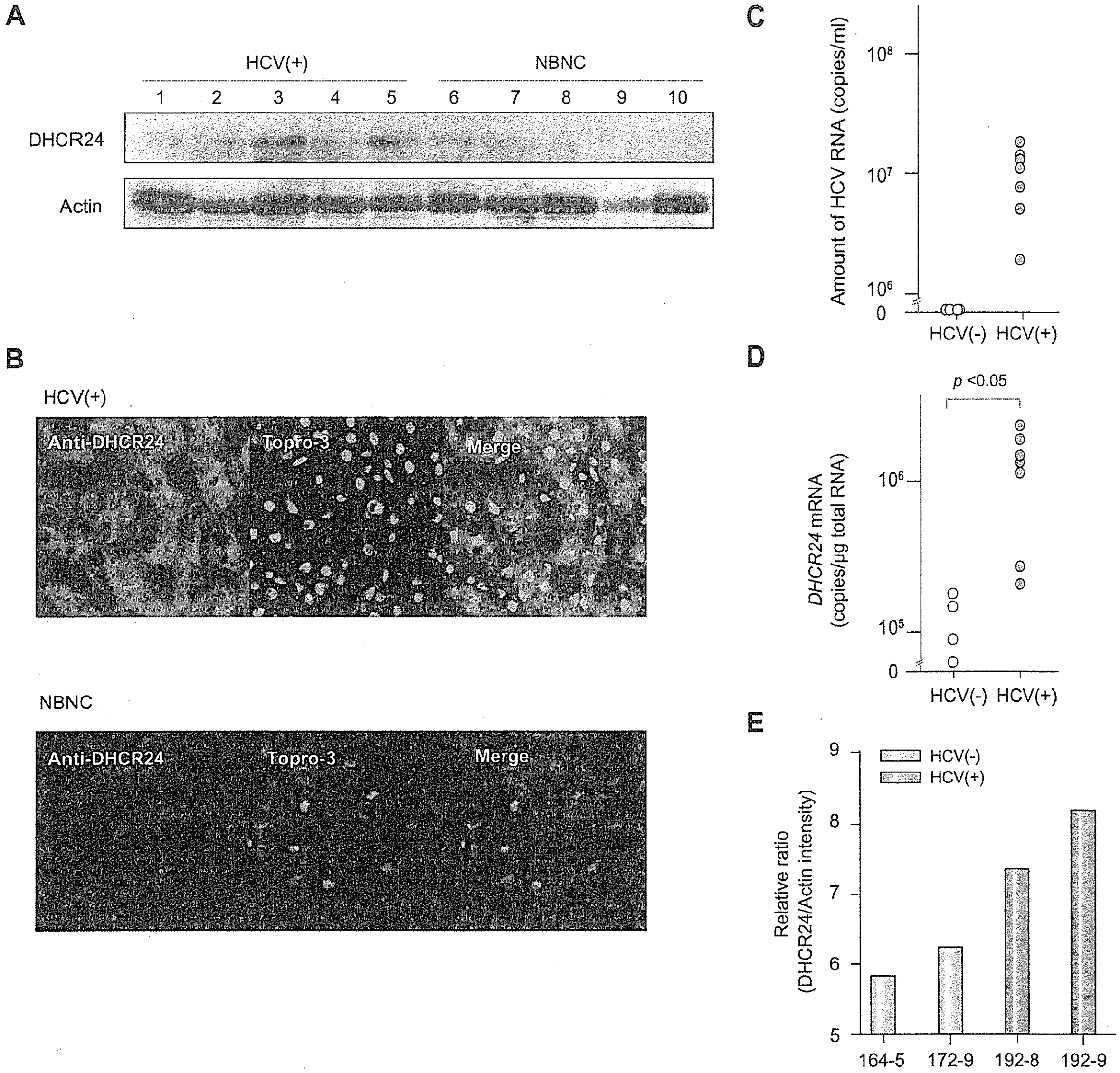

Fig. 1. HCV induces DHCR24 overexpression *in vitro* and *in vivo*. (A) Expression of DHCR24 in non-cancerous regions of livers of HCV-infected (+) and NBNC-HCC patients. Lysates (25 μg/lane) of non-cancerous liver tissues from HCC patients were analyzed by Western blot analysis using MoAb 2-152a. The patient numbers (Supplementary Table 1) are indicated at the top of the blot. (B) Immunohistochemical staining of HCV-infected non-cancerous tissues derived from an HCC patient using the monoclonal antibody 2-152a (Alexa488), anti-TO-PRO-3, or a merge (600× magnification) (upper panel). Tissues from an NBNC patient stained with the monoclonal antibody 2-152a (Alexa488) as well as TO-PRO-3 (640× magnification) (lower panel). (C) The amount of HCV RNA that was present in the HCV-R6 (genotype 1b)-infected chimeric mice with the humanized liver was quantified using RTD-PCR. The results of HCV uninfected ($n = 4$) and infected ($n = 7$) is indicated. (D) The amount of *DHCR24* mRNA present in total RNA isolates of HCV-R6 (genotype 1b)-infected chimeric mice with the humanized liver was quantified using RTD-PCR. $^*p < 0.05$ (Mann–Whitney test). The results of HCV uninfected ($n = 4$) and infected ($n = 7$) are indicated. (E) DHCR24 protein was detected by Western blot analysis using MoAb 2-152a as a probe, and quantitated by LAS3000. Protein levels are normalized to actin and ratio is indicated.

HCV infection in vivo induces persistent overexpression of DHCR24

We next examined whether HCV infection could induce DHCR24 expression in human hepatocytes. DHCR24 was overexpressed more frequently in liver tissues from HCV-positive patients than in tissues from HBV- and HCV-negative (NBNC) patients (Fig. 1A and Supplementary Table 1). The liver tissue from HCV-positive patients stained more strongly for DHCR24 expression than the liver tissue from NBNC patients (Fig. 1B). We inoculated chimeric mice [19,23,25] with HCV ($10^{6.2}$ copies/ml) that had been isolated from the plasma of HCV-infected patients (patient R6, HCV genotype 1b). The serum concentration of human albumin (Supplementary Fig. 2A) in the chimeric mice after transplantation of hepatocytes indicated that human hepatocytes had engrafted in the mouse livers. Thirty days after transplantation, mice were infected with HCV, and HCV and RNA titers were analyzed both before and after inoculation (Supplementary Fig. 2B). The average amount of HCV RNA that was present in the serum of the infected chimeric mice at 28 days post-infection was 1.1×10^7 copies/ml (Fig. 1C and Supplementary Fig. 2B). The *DHCR24* mRNA levels in the livers of the chimeric mice were also quantified at 28 days post-infection by real-time detection (RTD)-PCR [22]. The results revealed that there was a significant increase in *DHCR24* expression as measured by mRNA levels in HCV infected chimeric mice (Fig. 1D). Next, we examined the extent to which translation of DHCR24 occurred in the chimeric mice (Fig. 1E), higher DHCR24 protein levels were present in hepatocytes from HCV-infected mice (Nos. 192-8 and 192-9) than in those of uninfected mice (Nos. 164-5 and 172-9). These findings indicate that expression of DHCR24 is significantly up-regulated by HCV infection in human hepatocytes.

Role of DHCR24 in HCV replication

Since augmentation of DHCR24 expression was observed by HCV infection in humanized chimeric mice, we next examined whether DHCR24 was involved in HCV replication or not. We transfected siRNA into HCV replicon cell lines FLR3-1 (Fig. 2A and B) and R6FLR-N (Fig. 2C and D). Treatment with either two different *DHCR24* siRNA molecules (siDHCR24-417 or -1024) decreased HCV replication in a dose-dependent manner (Fig. 2A and C) but did not appear to have a significant effect on cell viability (Fig. 2B and D). Western blot analysis using HCV subgenomic replicon cell lines confirmed these findings (Fig. 2E and F). We also transfected the *DHCR24* siRNAs into HCV JFH-1 strain [18]-infected HuH7/K4 cell lines and found, by Western blot analysis, that the siRNAs inhibited HCV protein expression (Fig. 2G and H). These results indicate that DHCR24 may play a role in HCV replication.

The expression level of DHCR24 is linked to intracellular cholesterol levels

Human DHCR24 is involved in cholesterol biosynthesis [10]. It participates in multiple steps of cholesterol synthesis from lanosterol [26] (Fig. 3A). To examine the effect of cholesterol on the DHCR24 expression level in HuH-7 cells, we added cholesterol to cultured cells and determined the DHCR24 expression level (Fig. 3B). Expression levels of DHCR24 in HuH-7 cells were decreased approximately 50% by addition of cholesterol compared to that of the untreated control (Fig. 3B). On the other hand, that of DHCR24 in HepG2 cells was increased 2.5-fold by depletion of cholesterol using methyl-β-cyclodextrin (M-β-CD) (Fig. 3C).

These results indicate that the expression of DHCR24 in a cell correlates with the cholesterol level in that cell. Furthermore, silencing *DHCR24* reduced the cholesterol level in cells compared to control cells (Fig. 3D), suggesting that DHCR24 is essential for cholesterol synthesis.

Effect of U18666A on HCV replication in vitro

We further examined the role that DHCR24 plays in HCV replication by treating cells with U18666A. Treatment with U18666A (62.5, 125, 250, 500, and 1000 nM) of HCV replicon cells (FLR3-1) decreased HCV replication in a dose-dependent manner as shown by luciferase assay (Fig. 4A) and Western blot analysis (Fig. 4B). Notably, DHCR24 protein appeared as doublet bands in the absence of U18666A, but the lower band shifted to the upper band after treatment with U18666A (Fig. 4B). U18666A also suppressed HCV replication in other replicon cell lines (R6FLR-N and Rep JFH Luc 3-13; Fig. 4C and D). Treatment with U18666A (<250 nM) suppressed viral replication without producing significant cytotoxicity. We also examined the effect of 7-dehydrocholesterol reductase (DHCR7) (Fig. 3A) on HCV replication using the specific inhibitor BD1008 [26]. Treatment with BD1008 also suppressed HCV replication, but the concentration required was much higher than that needed in the U18666A assays (Fig. 4E); the concentration also greatly exceeded the intrinsic IC_{50} value for inhibition of σ-receptor binding (47 ± 2 nM) [27]. Therefore, DHCR24 may play a more significant role than DHCR7 in HCV replication. We next evaluated the compensatory effect that the addition of cholesterol had on cells treated with U18666A (Fig. 4F and G) by examining low density lipoprotein (LDL)-replaceable dissolved cholesterol levels as described in Supplementary data. Treatment with cholesterol led to partial restoration of HCV replication (Fig. 4F). These results suggest that U18666A suppresses HCV replication by depleting cellular cholesterol stores.

Next, we characterized the effect that U18666A had on HCV JFH-1 infection. Adding U18666A (62.5, 125, 250, and 500 nM) to HCV JFH-1-infected cell lines for 72 h, reductions of NS5B protein level were observed in cells treated more than 500 nM of U18666A (Fig. 5A and B). Additionally, the HCV RNA copy number in infected cells was suppressed by addition of 250 or 500 nM of U18666A (Fig. 5C). Examination of the cytotoxicity that U18666A (62.5–500 nM) had on infected cells revealed that it had little effect on cell viability (Fig. 5D). These results demonstrate that inhibition of DHCR24 by U18666A suppresses viral replication in HCV replicon cells and HCV-infected cells.

Evaluation of the anti-HCV effect of U18666A in vivo

To examine the effect of U18666A on HCV infection *in vivo*, we administered U18666A to HCV-infected chimeric mice with the humanized liver. The mice were infected with HCV via inoculation of patient serum HCR6 5 weeks after transplantation of human hepatocytes. U18666A (10 mg/kg) and PEG-IFN-α (30 μg/kg) were then administrated to these mice for 2 weeks (Fig. 6A). HCV RNA quantity (Fig. 6B) and serum human albumin levels (Fig. 6C) were measured in the mice after 1, 4, and 14 days of HCV infection. Treatment with U18666A alone significantly decreased HCV RNA levels in the serum (from 1×10^8 to 3×10^5 copies/ml) after 2 weeks, and its suppressive effect was more pronounced than that of PEG-IFN-α alone (8×10^5 copies/ml; Fig. 6B). Moreover, co-administration of U18666A and PEG-IFN-α synergistically (combination index <1) enhanced the antiviral effect of PEG-IFN-α (5×10^4 copies/ml). Treatment with these drugs did not significantly affect the serum human albumin concentrations in treated mice (Fig. 6C).

Discussion

The results of this study revealed that DHCR24, an enzyme that participates in cholesterol synthesis (last step; Fig. 3A), also plays a significant role in HCV replication. To our knowledge, this is the first report that this molecule is involved in HCV infection. The mevalonate route of the cholesterol synthesis pathway (starting from acetyl Co-A) has previously been reported to be involved in