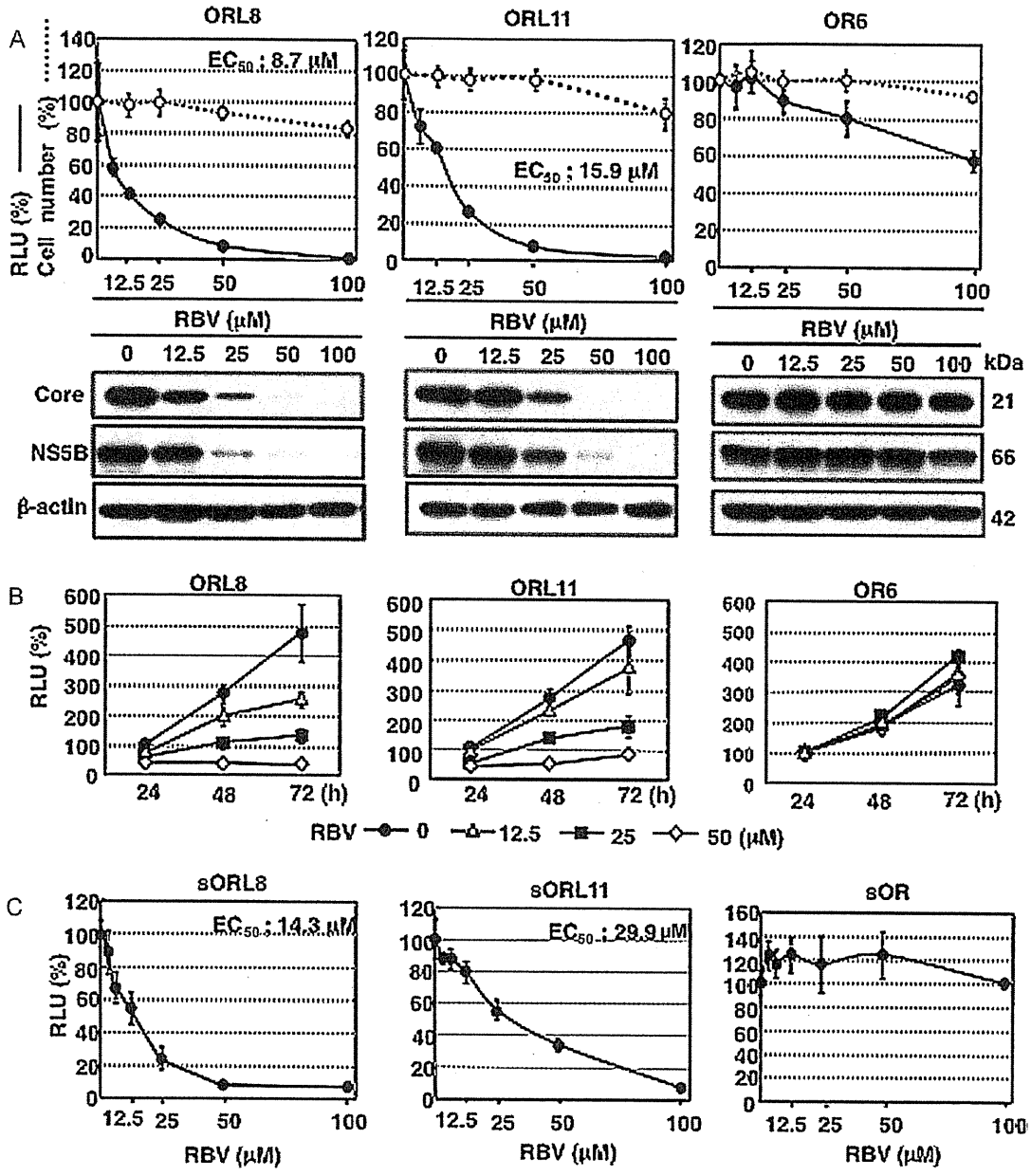

Fig. 1. Anti-HCV activity of RBV detected in the ORL8 and ORL11 system. (A) RBV sensitivities on genome-length HCV RNA replication in ORL8, ORL11, and OR6. The ORL8, ORL11 and OR6 cells were treated with RBV for 72 h, and then an RL assay (bold line in the upper panel) was performed. The relative luciferase activity (RLU) (%) calculated at each point, when the level of luciferase activity in non-treated cells was assigned to be 100% is presented here. The cell number (dotted line in the upper panel) at each concentration was determined as described in Section 2. Western blot analysis of RBV-treated ORL8, ORL11, and OR6 cells for HCV proteins, Core and NS5B, was also performed (lower panel). (B) Time-dependent anti-HCV activity of RBV. The ORL8, ORL11, and OR6 cells were treated with RBV, and an RL assay was performed at 24, 48, and 72 h after the treatment. The RLU (%) calculated at each time point, when the luciferase activity of non-treated cells at 24 h was assigned to be 100%, is shown. (C) Anti-HCV activity of RBV was observed in Li23-derived replicon assay systems (sORL8 and sORL11), but not in HuH-7-derived replicon assay system (sOR). RBV treatment and RL assay were performed as described for panel A.

two parts (6.0 kb covering 5′-UTR to NS3 and 6.1 kb covering NS3 to NS5B) and the two PCR products were subcloned for the sequence analysis as described above.

2.9. RNA interference and quantitative RT-PCR

SiRNA duplexes targeting the coding regions of human IMPDH1 (Dhamacon; catalog no. M-009687-01) and IMPDH2 (Dharmacon; catalog no. M-004330-02) were chemically synthesized. SiRNA duplex non-targeting (Dharmacon; catalog no. D-001206-13) was also used as a control. ORL8 cells were transfected with the indicated siRNA duplexes using Oligofectamine (Invitrogen) (Dansako et al., 2007). Extraction of total RNA and quantitative RT-PCR analysis for HCV RNA were performed by real-time LightCycler PCR as described previously (Ikeda et al., 2005).

2.10. Statistical analysis

Statistical comparison of the luciferase activities between the treatment groups and controls was performed using the Student's

t-test. *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. Anti-HCV activity of RBV was clearly observed in the Li23-derived assay systems, but not in the HuH-7-derived assay system

Recently we demonstrated that Li23-derived assay systems (ORL8 and ORL11), in which genome-length HCV RNA (O strain of genotype 1b) encoding RL robustly replicates, were frequently more sensitive to anti-HCV reagents such as IFNs and statins than the corresponding HuH-7-derived assay system (OR6) (Kato et al., 2009). Since we had observed a marginal anti-HCV activity of RBV in OR6 system, we assumed that the anti-HCV activity of RBV might also be illuminated by ORL8 or ORL11 system. Indeed, marked differences were observed between OR6 and both of the other assay systems: RBV at clinically achievable concentrations effectively inhibited HCV RNA replication in both ORL8 and ORL11, but not in OR6 (Fig. 1A). The EC_{50} values of RBV in ORL8, ORL11, and OR6 were 8.7, 15.9, and >100 μM, respectively, without suppression of cell growth (upper panels in Fig. 1A). These pronounced differences in the anti-HCV activity of RBV were confirmed by Western blot analysis (lower panels in Fig. 1A). In addition, time course assays revealed that the anti-HCV activity of RBV was dose- and time-dependent in ORL8 and ORL11, but not in OR6 (Fig. 1B). We next examined the activity of RBV using polyclonal cell-based assay systems (sORL8, sORL11, and sOR (Ikeda et al., 2005)) harboring HCV replicon RNA. The results revealed that the EC_{50} values of RBV in sORL8 and sORL11 were 14.3 and 29.9 μM, respectively, whereas RBV showed no anti-HCV activity in sOR (Fig. 1C), suggesting that the anti-HCV activity of RBV was not either a clone-specific or genome-length HCV RNA-specific phenomenon. Moreover, we demonstrated by Western blot (upper panel of Fig. 2) and quantitative RT-PCR (lower panel of Fig. 2) analyses that RBV suppressed HCV RNA replication in HCV-JFH1-infected ORL8c cells, but not in HCV-JFH1-infected RSc cells, which HCV could infect and efficiently replicate within (Ariumi et al., 2007; Kato et al., 2009). These results also indicate that only the Li23-derived assay system can illuminate the anti-HCV activity of RBV.

3.2. An ENT inhibitor cancelled anti-HCV activity of RBV

As one possible explanation for the pronounced differences in RBV activity between the Li23- and HuH-7-derived assay systems, we considered that the efficiencies in the cellular uptake of RBV might have differed between the two types of cells. To date, two families of nucleoside transporter proteins have been identified: the ENT family (ENT1, ENT2, and ENT3) and the concentrative nucleotide transporter (CNT) family (CNT1, CNT2, and CNT3) (Pastor-Anglada et al., 2005). Two recent reports showed that ENT1 and CNT3 might be responsible for RBV uptake in HuH-7 cells (Ibarra and Pfeiffer, 2009), and that ENT1, but not ENT2 or CNTs, is a major RBV uptake transporter in human hepatocytes (Fukuchi et al., 2010). To test these points, we first examined the effects of an ENT inhibitor, NBMPR, and a CNT inhibitor, phloridzin dihydrate, on the anti-HCV activity of RBV (50 μM; 90% effective concentration [EC_{90}]) in ORL8 system. The results revealed that 5 μM NBMPR partially attenuated the anti-HCV activity of RBV in ORL8 (Fig. 3A). The marginal activity of RBV was also not changed in OR6 system treated with these transporter inhibitors (data not shown). A significant dose-dependency of the cancellation by NBMPR was also observed in ORL8 (Fig. 3B). Since we observed a lack of expression of CNT family members in ORL8 cells (data not shown), these

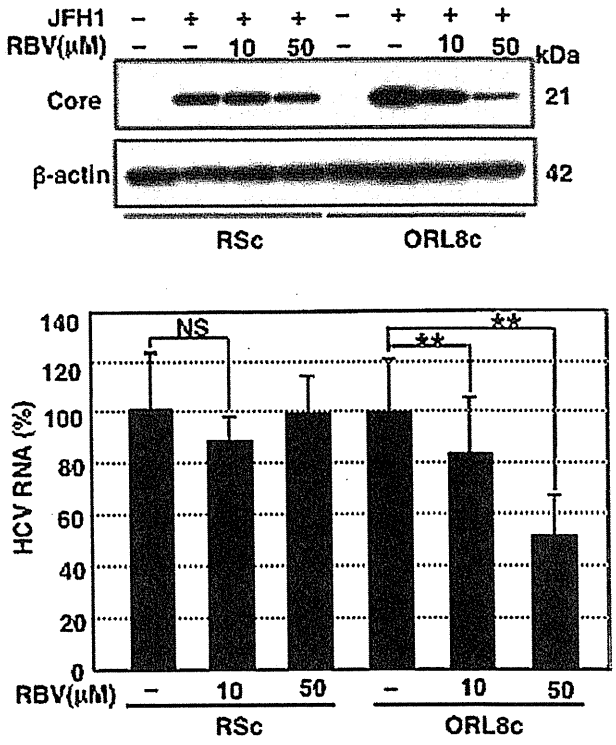

Fig. 2. RBV inhibited HCV production in JFH1-infected ORL8c cells, but not in JFH1-infected RSc cells. JFH1-infected ORL8c and RSc cells were treated with RBV for 72 h, and subjected to Western blot analysis using anti-Core or β-actin antibody (upper panel), and to quantitative RT-PCR analysis (lower panel). Asterisks indicate significant differences compared to the control treatment. $^{**}P < 0.01$; NS, not significant.

results suggest that cellular uptake of RBV is mediated by ENT member(s). Accordingly, we next examined the levels of ENT mRNAs in ORL8 and OR6 cells. However, the expression levels of ENT1, ENT2, and ENT3 mRNAs were comparable between ORL8 and OR6 cells (Fig. 3C). In addition, sequence analysis of ENT1, ENT2, and ENT3 mRNAs (data not shown) and Western blot analysis of ENT1 protein (Fig. 3D) revealed no differences between the two cell lines. These results suggest that the expression levels of ENT members are not associated with the differences in RBV activity.

3.3. RBV did not act as a mutagen in HCV RNA replication

Since the suppressive effect of RBV on HCV RNA replication was clearly observed in ORL8 system, we expected that ORL8 cells would be suitable for analysis of the anti-HCV mechanism of RBV. In regard to the anti-HCV mechanism of RBV, several groups have proposed that RBV (50–400 μM) acts as an RNA mutagen and induces error catastrophe in HCV RNA replication (Contreras et al., 2002; Zhou et al., 2003). Therefore, we first examined whether or not error catastrophe theory is involved in the anti-HCV activity of RBV observed in ORL8 system. To test the mutagenic effect of RBV, ORL8 cells were treated with or without RBV (50 μM; EC_{90} level in ORL8 system) for 72 h, and then genome-length HCV RNA from the ORL8 cells was amplified by RT-PCR. We performed HCV quasispecies analysis by sequencing of RL to the Neo^R, NS5A, and NS5B regions using at least 10 independent clones for each region. To estimate the mutation rate, the total number of mutations and the ratio of nonsynonymous to synonymous mutations in each region were determined by comparison with the parental HCV sequences (Kato et al., 2009). The results revealed that the overall mutation rate and the ratio of nonsynonymous to synonymous mutations in each

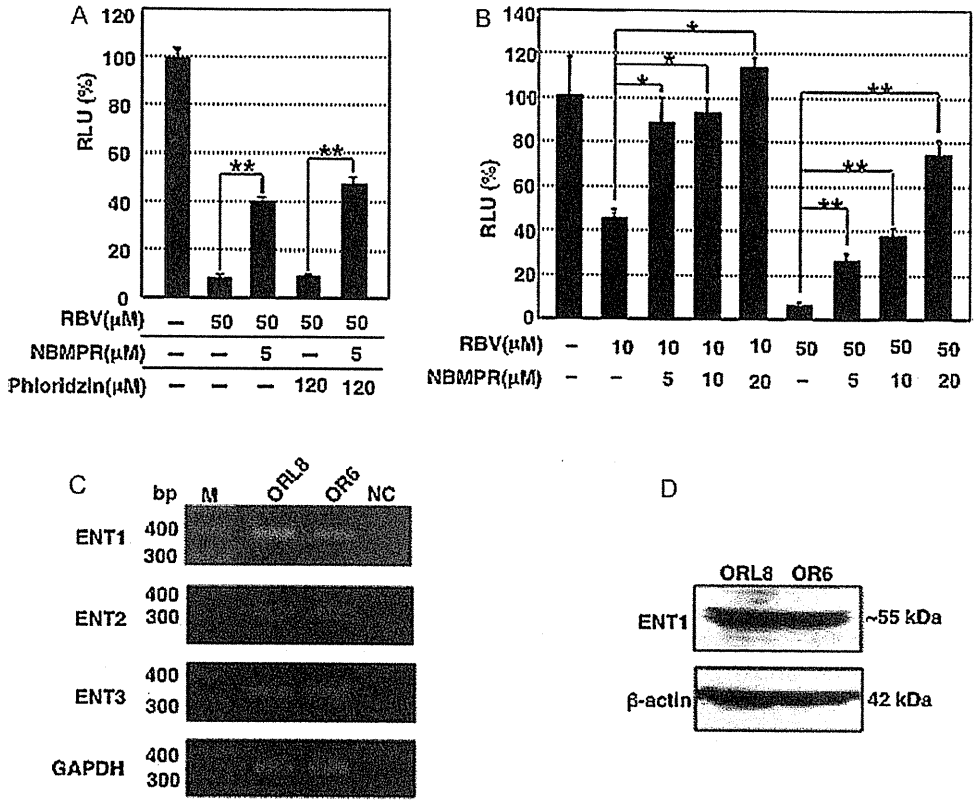

Fig. 3. An ENT inhibitor abolished anti-HCV activity of RBV. (A) An ENT inhibitor, NBMPR, canceled the anti-HCV activity of RBV in ORL8. ORL8 cells were pretreated with NBMPR and/or phloridzin dihydrate for 30 min, and then treated with RBV for 72 h, after which an RL assay was performed. Asterisks indicate significant differences compared to the control treatment. $^{**}P < 0.01$. (B) Dose-dependent cancellation by NBMPR of the activity of RBV. ORL8 cells were pretreated with NBMPR for 30 min, and then treated with RBV for 72 h, after which an RL assay was performed. Asterisks indicate significant differences compared to the control treatment. $^{*}P < 0.05$; $^{**}P < 0.01$. (C) RT-PCR analysis of ENTs. Total RNAs prepared from ORL8 and OR6 cells were subjected to RT-PCR using the primer sets for ENT1, ENT2, ENT3, and GAPDH as described in Section 2. RT-PCR products were detected by staining with ethidium bromide after 3% agarose gel electrophoresis. (D) Western blot analysis of ORL8 and OR6 cells for ENT1. The primary antibody used was ENT1. β-actin was used as a control for the amount of protein loaded per lane.

region were not increased irrespective of the presence or absence of RBV treatment (Table 1). To confirm that mutation frequencies given in Table 1 are overwhelmingly above the error level associated with the PCR, we sequenced independent five clones (6.0 kb covering 5′-UTR to NS3 and 6.1 kb covering NS3 to NS5B) obtained by PCR using KOD-plus DNA polymerase and a plasmid containing the parental HCV sequences (Kato et al., 2009) as a template. No mutations were detected in these sequenced clones, indicating that KOD-plus DNA polymerase possesses extremely high fidelity, and suggesting that the mutations obtained in the present study are not produced by the errors associated with the PCR. Therefore, these results indicate that RBV does not act as a mutagen in HCV RNA replication in ORL8 cells, and suggest that the anti-HCV activity of RBV (EC_{50}; 8.7 μM) observed in ORL8 system is not due to the induction of error catastrophe in the HCV RNA genome.

3.4. RBV did not activate the IFN-signaling pathway

Regarding HCV, Liu et al. (Liu et al., 2007) have reported that RBV (40–500 μM) enhances the IFN-signaling pathway in in vitro cell culture systems. Furthermore, a recent report showed that RBV improved early responses to PEG-IFN through enhanced IFN signaling in the treatment of patients with chronic hepatitis (Feld et al., 2010). In that study, it was shown that the RBV concentration in patients at day 3 was correlated with IP-10 induction at 12 h, but only in patients with an adequate first phase viral decline (Feld et al., 2010). Therefore, we expected that RBV would enhance the IFN-signaling pathway in our new cell culture system. Accordingly, we first examined the effect of RBV in combination with IFN-α on HCV RNA replication using ORL8 system. OR6 system was also used for purpose of comparison. The results showed that RBV had an additive effect in decreasing HCV RNA replica-

Table 1
Mutation frequencies in RL-Neo^R, NS5A, and NS5B regions.

Region	Condition	Total no. of clones	Total no. of mutations	Nonsynonymous/synonymous substitutions (ratio)
RL-Neo^R (1953 nts)	Control	12	59	39/20 (1.95)
	RBV (50 μM)	12	49	31/18 (1.72)
NS5A (1341 nts)	Control	10	35	24/11 (2.18)
	RBV (50 μM)	10	36	24/12 (2.00)
NS5B (1773 nts)	Control	10	10	3/7 (0.43)
	RBV (50 μM)	10	9	2/7 (0.29)

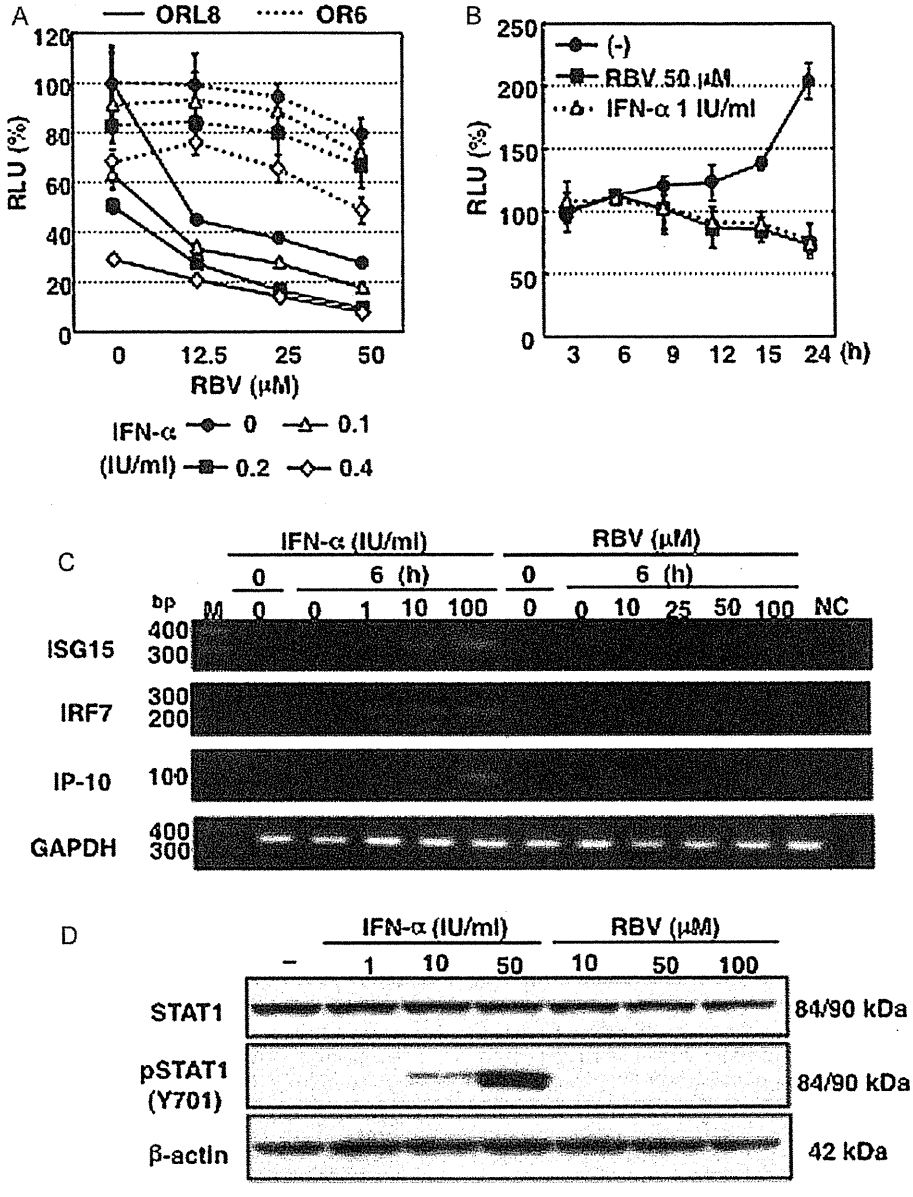

Fig. 4. RBV did not activate the IFN-signaling pathway in ORL8 cells. (A) Additive effect of RBV in combination with IFN-α. ORL8 and OR6 cells were treated with a combination of IFN-α and RBV for 72 h, after which the RL assay was performed. (B) Time course assay of the anti-HCV activity of RBV or IFN-α. ORL8 cells were treated with RBV or IFN-α, and an RL assay was performed at 3, 6, 9, 12, 15, and 24 h after treatment. Presented here is the RLU (%) calculated at each point, when the RL activity of non-treated cells at 3 h was assigned to be 100%. (C) ISGs were not induced by RBV treatment. ORL8 cells were treated with IFN-α or RBV for 6 h, and then the total RNAs extracted from the cells were subjected to RT-PCR using the primer sets for ISG15, IRF7, IP-10, and GAPDH as described in Section 2. RT-PCR products were detected by staining with ethidium bromide after 3% agarose gel electrophoresis. (D) Phosphorylation of STAT1 was not induced by RBV treatment. ORL8 cells were treated with IFN-α or RBV for 30 min, and subjected to Western blot analysis using anti-STAT1, anti-phospho-STAT1(Y701), and anti-β-actin antibodies.

tion in both assay systems, but its activity was greater in ORL8 than in OR6 (Fig. 4A). A comparative time course assay using RBV or IFN-α demonstrated that RBV- and IFN-α-treated ORL8 cells had the same anti-HCV kinetics, leading to decreased RL activity at 9 h after treatment (Fig. 4B). These results suggest that RBV induces some anti-HCV signaling pathway, such as an IFN-signaling pathway, rather than inducing IFN or directly inhibiting RNA replication.

We next examined the ability of RBV to activate ISGs. RT-PCR analysis revealed that RBV treatment (6 h) did not cause an induction of representative ISGs, ISG15, IRF7, and IP-10, in ORL8 cells, although even treatment (6 h) with 1 IU/ml (ISG15 and IRF7) or 10 IU/ml (IP-10) of IFN-α could induce these ISGs (Fig. 4C). Similar results were also obtained in OR6 cells and Huh7.5 cells (data not shown). In addition, enhancement of these ISGs was also not observed in the ORL8 cells co-treated with IFN-α and RBV (data not shown). Furthermore, we examined the phosphorylation status of STAT1 after RBV treatment. The results revealed that RBV treatment (up to 100 μM for 30 min) did not induce the phosphorylation of STAT1 in ORL8 cells, although phosphorylation of STAT1 was observed even after the treatment with 10 IU/ml of IFN-α (Fig. 4D). Together, these results indicate that RBV does not activate the IFN-signaling pathway.

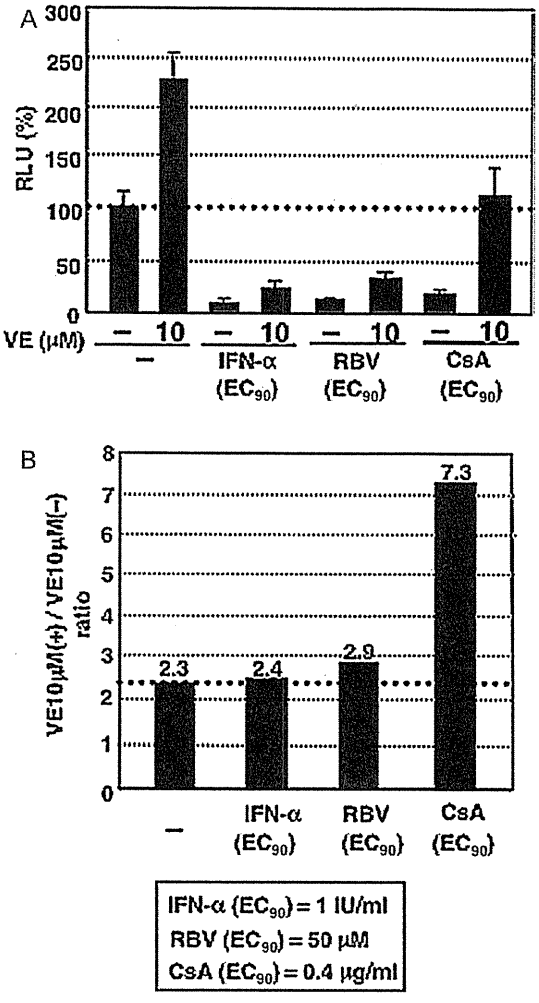

Fig. 5. The anti-HCV activity of RBV was not canceled by addition of VE. (A) Effects of VE on IFN-α, RBV, and CsA at the EC_{90}. ORL8 cells were treated with control medium (–), IFN-α, RBV, or CsA in either the absence or presence of VE for 72 h, and then an RL assay was performed. (B) The ratio of RL activity in the presence of VE to the RL activity in the absence of VE. The above ratio was calculated from the data of panel A. The horizontal line indicates the promotive effect of VE alone on HCV RNA replication as a baseline.

3.5. RBV did not induce the oxidative stress or subsequent anti-HCV status

Recently we reported that the antioxidant VE negated the antiviral activities of a broad range of anti-HCV reagents, including CsA, and demonstrated the involvement of the MEK-ERK1/2-signaling pathway in the anti-HCV status induced by oxidative stress (Yano et al., 2007, 2009). Therefore, we next expected that RBV induces oxidative stress. Accordingly, we examined the effect of VE on RBV, IFN-α, or CsA at the EC_{90} level in ORL8 system. Although the anti-HCV activity of CsA was canceled to a significant level by VE, the inhibitory effects of RBV and IFN-α were hardly influenced by co-treatment with VE (Fig. 5A). We normalized these results by dividing the RL value obtained in the presence of VE by that in the absence of VE as described previously (Yano et al., 2007) (Fig. 5B). The value of RBV was almost the same as that of IFN-α or control, although the value of CsA was somewhat higher (7.3) which was consistent with previous findings (Yano et al., 2007). These results suggest that induction of oxidative stress is not associated with the activity of RBV detected in ORL8 system.

3.6. Guanosine dose-dependently attenuated the anti-HCV activity of RBV

Previously, using a qualitative colony-forming efficiency (CFE) assay of an HCV RNA replicon, Zhou et al. (2003) showed that RBV (50 μM) reduced the CFE by 2-fold in HuH-7 cells, although 10 μM RBV did not result in a significant change in CFE. In that study, when exogenous guanosine, but not adenosine, which would replenish GTP pools via the salvage pathway, was co-administered with RBV, the RBV-induced CFE reduction was partially cancelled (Zhou et al., 2003). From this result, the authors suggested that IMPDH inhibition and subsequent lowering of GTP pools contribute to the observed reduction in CFE. However, they failed to observe the any suppressive effects of the IMPDH inhibitors MPA and Merimepodib (MMPD)/VX-497 on HCV RNA replication (Zhou et al., 2003). Conversely, Henry et al. showed that MPA exerted anti-HCV activity on HCV RNA replication in HuH-7-derived cells (Henry et al., 2006). Therefore, in order to resolve these controversial results, we initially examined the anti-HCV activity of MPA in ORL8 and OR6 systems. The results revealed that MPA strongly inhibited HCV RNA replication in both systems without suppression of cell growth. The EC_{50} values of MPA in the ORL8 and OR6 were 0.29 and 0.57 μM, respectively (Fig. 6A). Dose-dependent cancellation by guanosine, but not by adenosine, of the activity of MPA, was observed in both systems (Fig. 6B and data not shown for OR6 system). These results suggest that the depression of GTP induced by inhibition of IMPDH decreases the level of HCV RNA replication. From these results, we expected that anti-HCV activity of RBV observed in ORL8 might also have been associated with the inhibition of IMPDH. Indeed, significant dose-dependent cancellation by guanosine, but not by adenosine, of the anti-HCV activity of RBV (10 μM) was observed in ORL8 (Fig. 6C). ORL11 also showed a similar cancellation by guanosine (data not shown). The suppressive effect of guanosine on the activity of RBV in ORL8 was confirmed by Western blot analysis (Fig. 6D). These results suggest that the anti-HCV activity of RBV at clinically achievable concentrations in ORL8 is mediated through the inhibition of IMPDH by RBV.

3.7. IMPDH is required for HCV RNA replication

To confirm the involvement of IMPDH on HCV RNA replication, the endogenous expression of IMPDH was suppressed by siRNA specific to IMPDH. Since IMPDH has two isoforms, IMPDH1 and IMPDH2, which share 84% amino-acid homology (Wang et al., 2008), we prepared IMPDH1- and/or IMPDH2-knockdown ORL8 cells. The effective knockdown of IMPDH1 and/or IMPDH2 in ORL8 cells was confirmed by quantitative RT-PCR (Fig. 7A). We observed that the levels of HCV RNA replication in these knockdown cells were notably reduced compared with the control cells without suppression of cell growth (Fig. 7B). These results suggest that IMPDH is crucial for the maintenance of HCV RNA replication. Taken together, these results indicate that the inhibitory activity of RBV on HCV RNA replication in Li23-derived cells is mediated through the inhibition of IMPDH by RBV.

4. Discussion

In this study, using novel Li23-derived cell culture assay systems, we demonstrated for the first time that RBV at clinically achievable concentrations efficiently inhibited HCV RNA replication, and clarified that its anti-HCV activity was mediated by the inhibition of IMPDH.

To date, several mechanisms as described above have been proposed based on the results of studies using an HuH-7-derived cell culture system (Feld and Hoofnagle, 2005; Feld et al., 2010; Lau

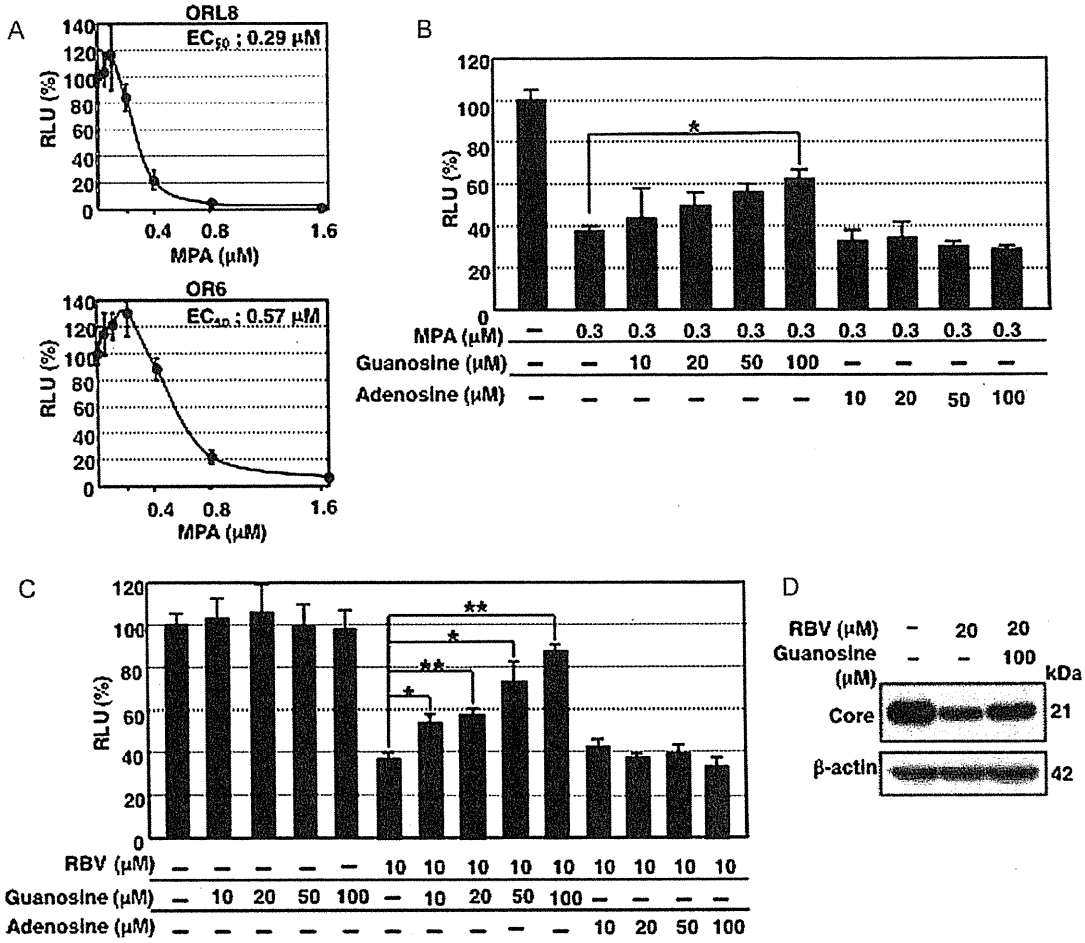

Fig. 6. Guanosine canceled the anti-HCV activity of RBV in ORL8 system. (A) Anti-HCV activity of MPA in ORL8 and OR6. The ORL8 and OR6 cells were treated with MPA for 72 h, and then RL assay was performed. (B) Effect of guanosine or adenosine on MPA in ORL8 system. ORL8 cells were treated with MPA alone or in combination with guanosine or adenosine for 72 h, and then RL assay was performed. Asterisk indicates a significant difference compared to the control treatment. *$P<0.05$. (C) Effect of guanosine or adenosine on RBV in ORL8 system. ORL8 cells were treated with RBV alone or in combination with guanosine or adenosine for 72 h, and then the RL assay was performed. Asterisks indicate significant differences compared to the control treatment. *$P<0.05$; **$P<0.01$. (D) Effect of guanosine on RBV in ORL8 system. ORL8 cells were treated with RBV alone or in combination with guanosine for 72 h, and subjected to Western blot analysis using anti-Core and β-actin antibodies.

et al., 2002; Thomas et al., 2011; Zhou et al., 2003). Although the effective concentrations (50–1000 μM) of RBV in those studies were much higher than the clinically achievable concentrations (5–14 μM) (Feld et al., 2010; Pawlotsky et al., 2004; Tanabe et al., 2004), the effective concentration of RBV in this study was close to the clinically achievable concentrations. Furthermore, it is noteworthy that the replication of a different HCV strain (JFH1 of genotype 2a) in the Li23-derived cell culture system, but not in the HuH-7-derived cell culture system, was also suppressed with RBV at the concentration of 10 μM (Fig. 1C). These results demonstrate that the Li23 cell-derived assay system is a more sensitive biosensor of RBV than the HuH-7 cell-derived assay system.

The finding that RBV remarkably inhibited HCV RNA replication in our new assay systems led us to analyze the anti-HCV mechanism of RBV. In this study, we evaluated several possible anti-HCV mechanisms of RBV, as described above. Regarding the induction of error catastrophe by RBV, we obtained no evidence that RBV (even at 50 μM) acted as a mutagen in HCV RNA replication. Therefore, we could not explain the mechanism underlying the suppression of HCV RNA replication by RBV according to the theory of error catastrophe. In addition, no increasing mutation rate of HCV RNA in patients receiving RBV monotherapy or a combination of RBV plus IFN-α was observed in a previous clinical study (Chevaliez and Pawlotsky, 2007). In consideration of all these findings, we suggest that the clinically achievable concentrations of RBV do not act as a mutagen in HCV RNA replication. Indeed, our previous study using the replicon cell culture system demonstrated that RBV treatment (6 months at 5 and 25 μM) did not accelerate the mutation rate or increase the genetic diversity of the HCV replicon (Kato et al., 2005). In regard to the effect of RBV on the IFN system, we obtained no evidence that RBV (even at 50 μM) induced ISGs (ISG15, IRF7, and IP-10) or phosphorylation of STAT1 even in the cells co-treated with IFN-α and RBV (data not shown). On the other hand, very recently Thomas et al. (Thomas et al., 2011) reported that RBV treatment (500 μM) resulted in the induction of a distinct set of ISGs including ISG15, IRF7, and IRF9, using HuH-7-derived cell line Huh7.5.1. In that study, they demonstrated that the induction of these ISGs was mediated by a novel mechanism different from those associated with IFN signaling and double stranded RNA sensing pathway, and concluded that the effect of RBV on ISG regulation is IFN-independent. However, in our cell culture system, which is highly sensitive to RBV, the induction of ISG15 and IRF7 by RBV was not observed (Fig. 4C). This kind of controversial results may be dependent on the difference of cell lines used in both studies, since recent microarray analysis revealed that the expression profiles of Li23 and HuH-7 cells, both of which possess an environment

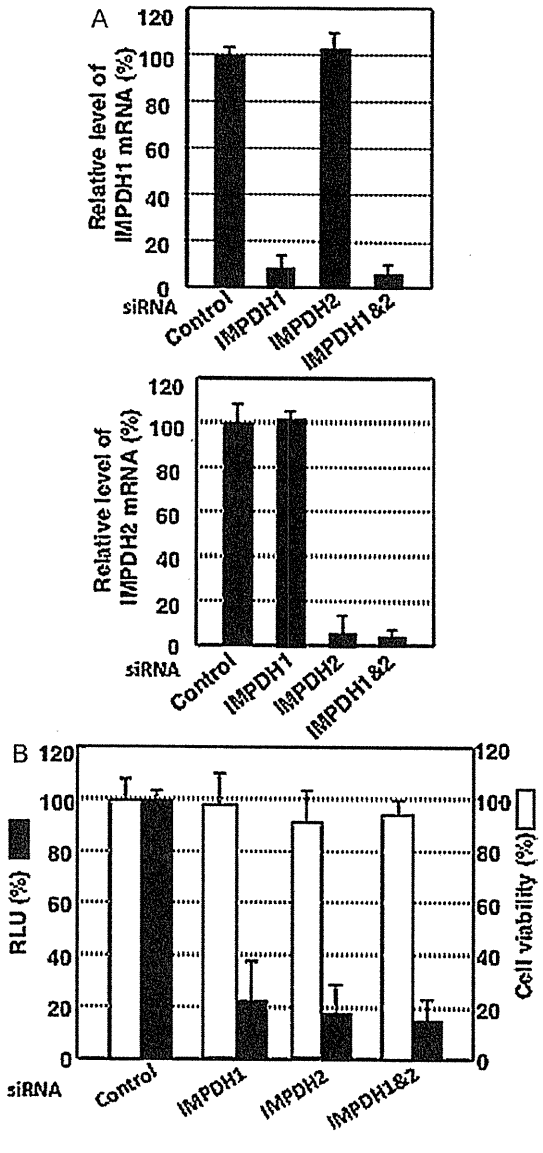

Fig. 7. IMPDH is required for HCV RNA replication. (A) Inhibition of IMPDH1 and IMPDH2 expression by siRNA in ORL8 cells. ORL8 cells were transfected with 8 nM siRNA targeting for IMPDH1 and/or IMPDH2. After 72 h, the expression levels of IMPDH1 and IMPDH2 mRNAs were determined by the quantitative RT-PCR. Experiments were done in triplicate. (B) Suppression of HCV RNA replication in IMPDH1- and/or IMPDH2-knockdown ORL8 cells. The RLU (%) calculated, when the luciferase activity of the cells treated with control siRNA was assigned to be 100%, is shown. The cell viability was determined as described in Section 2.

for robust HCV replication, differed considerably (Kato et al., 2009; Mori et al., 2010). However, Thomas et al. (2011) observed that the addition of guanosine to the medium could block RBV-induced ISGs induction. Therefore, further additional studies would be needed to resolve the differences of results obtained from both studies.

The highlight in this study is that a Li23-derived cell culture system clearly demonstrated an association between the suppression of HCV RNA replication by RBV and IMPDH inhibition by RBV. Although RBV is known to be an IMPDH inhibitor (Lau et al., 2002), it had been considered that such inhibitory activity would not contribute to the anti-HCV activity of RBV, because of the marginal antiviral effect of RBV in HuH-7-derived HCV RNA replicating cells (Naka et al., 2005; Tanabe et al., 2004; Zhou et al., 2003). Although Zhou et al. (2003) previously showed that exogenous guanosine cancelled the RBV-induced CFE reduction using an HuH-7-based HCV replicon system, they did not observe any dose-dependent reversion of the adverse effect of RBV by the addition of guanosine. However, in our Li23-based HCV replication assay system, we observed a near complete cancellation of the activity of RBV in the dose-dependent manner of guanosine (Fig. 6C). This finding indicated that anti-HCV activity of RBV might be mediated through the inhibition of IMPDH by RBV. Indeed, we could demonstrate that HCV RNA replication was notably suppressed in IMPDH-knockdown ORL8 cells (Fig. 7B). Taken together, these results revealed that the Li23-derived assay system was superior to HuH-7-derived assay system in order to clarify the anti-HCV mechanism of RBV.

The remarkable effect of RBV observed in this study was considered to be due to the difference in the cell lines used, because Li23-derived cells possessed rather different gene expression profiles from those in HuH-7-derived cells (Kato et al., 2009; Mori et al., 2010). As one of the possibilities, we examined the expression status of nucleoside transporters (ENT family) involved in cellular uptake of RBV or ATP-binding cassette transporters, including multidrug resistance 1, which is involved in cellular excretion. However, the mRNA levels of these transporters were almost the same in both types of cells (Fig. 3C). Although unfortunately we failed to clarify the mechanism underlying the remarkable differences in the activity of RBV in both types of cells, we observed that the anti-HCV activity of RBV was completely canceled by NBMPR, an ENT inhibitor, suggesting that RBV is taken by ENT member(s) at least in ORL8 cells. This finding supports the recent report describing the involvement of ENT1 on cellular uptake of RBV (Fukuchi et al., 2010; Ibarra and Pfeiffer, 2009). Therefore, a comparative analysis regarding the functions of ENT member(s) derived from both types of cells will be needed. As the other possibility, the differences of activities or expression levels of IMPDH in OR6 and ORL8 cells may contribute to the remarkable effect of RBV observed in ORL8 cells.

On the other hand, it has been known that rapid reduction of the intracellular level of GTP occurs when RBV inhibits IMPDH (Feld and Hoofnagle, 2005). Therefore, it is assumed that the decrease of GTP would lead to a suppression of HCV replication. To date, several studies (Lohmann et al., 1999; Luo et al., 2000; Simister et al., 2009) have shown that high concentration of GTP (approximately 500 μM corresponding to the intracellular concentration) is required for the efficient de novo initiation of RNA synthesis by HCV NS5B RdRp. In addition, Simister et al. (2009) showed that change from 500 μM to 100 μM of GTP concentration decreased a log of the NS5B RdRp activity. From these studies, we expect that the inhibition of IMPDH by RBV may cause rapid decrease of intracellular GTP concentration, resulting in the suppression of de novo RNA synthesis by NS5B. Before our assumption, MMPD/VX-497 has developed as an inhibitor of IMPDH, and it has been shown to exert anti-HCV activity (EC_{50}; 0.39 μM) in an HCV replicon system (Marcellin et al., 2007). However, MMPD/VX-497 monotherapy of patients with chronic hepatitis C had no effect on HCV RNA levels (Marcellin et al., 2007) just as, in another study, RBV monotherapy had no effect on HCV RNA levels in patients with chronic hepatitis C (Di Bisceglie et al., 1995). Although we showed that the EC_{50} value of RBV in this study was equivalent to the clinically achievable concentrations (Feld et al., 2010; Pawlotsky et al., 2004; Tanabe et al., 2004), we considered that the effective concentration for a reduction of HCV RNA levels in monotherapy would be less than the EC_{90} value. However, an IMPDH inhibitor at EC_{50} would be effective in combination with IFN-α as an adjuvant. Indeed, combination therapy with IFN-α and MMPD/VX-497 was effective in previously untreated patients with chronic hepatitis C (McHutchison et al., 2005). However, a recent study (Rustgi et al., 2009) showed that the addition of MMPD/VX-497 to PEG-IFN-α and RBV combination

therapy in patients who had been nonrespondent to PEG-IFN-α and RBV combination therapy did not increase the proportion of patients who achieved an SVR. Since we showed that RBV also acted as an IMPDH inhibitor in the present study, it would seem to be a reasonable result that MMPD/VX-497 had no significant effect on patients who were nonresponsive to combination therapy with PEG-IFN-α and RBV.

In conclusion, we clarified the anti-HCV mechanism of RBV in a new HCV cell culture system. The fact that anti-HCV activity of RBV was mediated by the inhibition of IMPDH would provide a clue to the mechanism of the increase of SVR by the current standard combination therapy with PEG-IFN-α and RBV. In addition, our findings should also be useful for the screening and development of new anti-HCV drugs, which inhibit IMPDH, with reduced side effects, including anemia.

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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

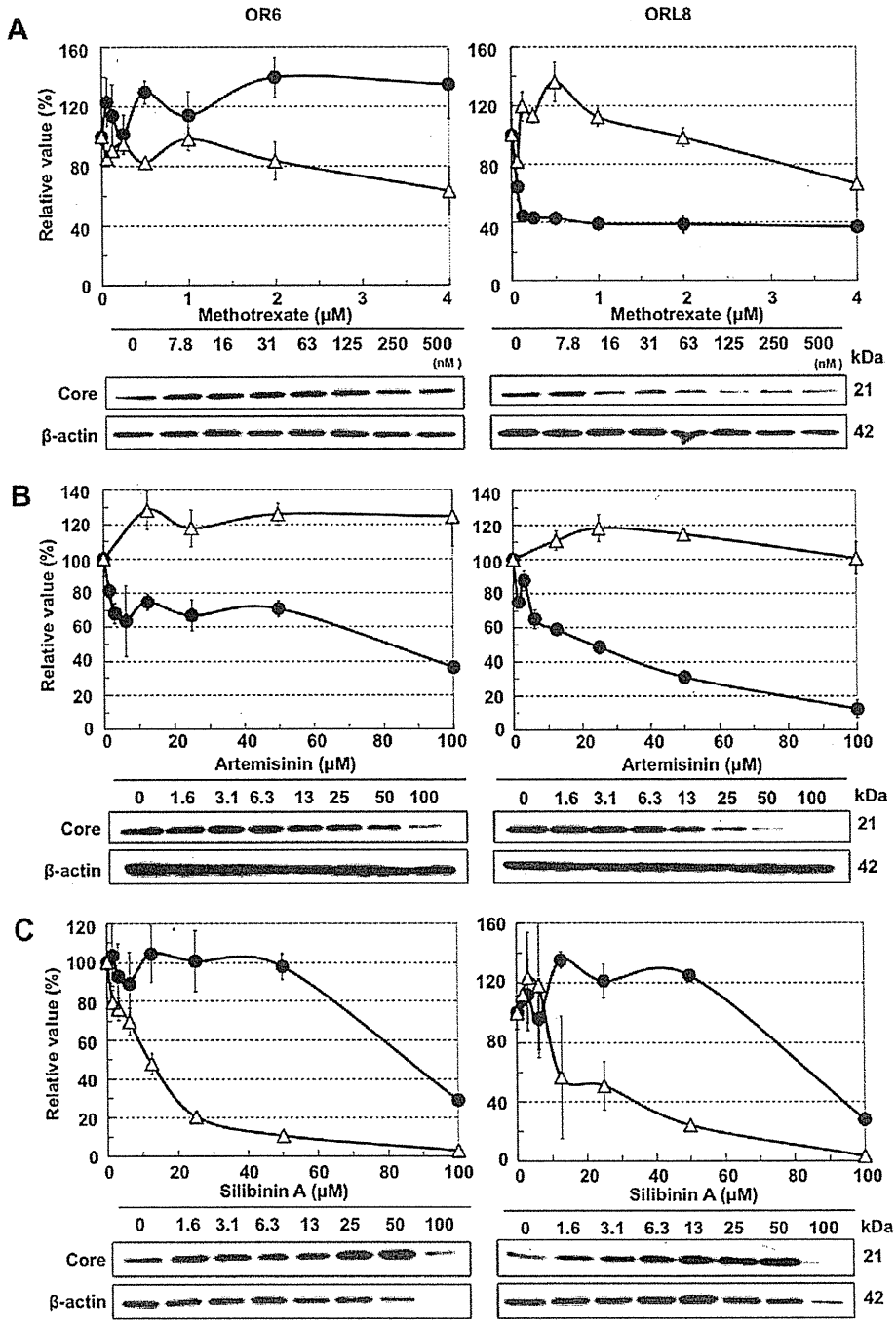

Fig. 1. Anti-HCV profiles of representative reagents in the OR6 and ORL8 assay systems. (A) Methotrexate sensitivities on genome-length HCV RNA replication in the OR6 and ORL8 assay systems. OR6 and ORL8 cells were treated with methotrexate for 72 h, followed by RL assay (black circle in the upper panel) 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

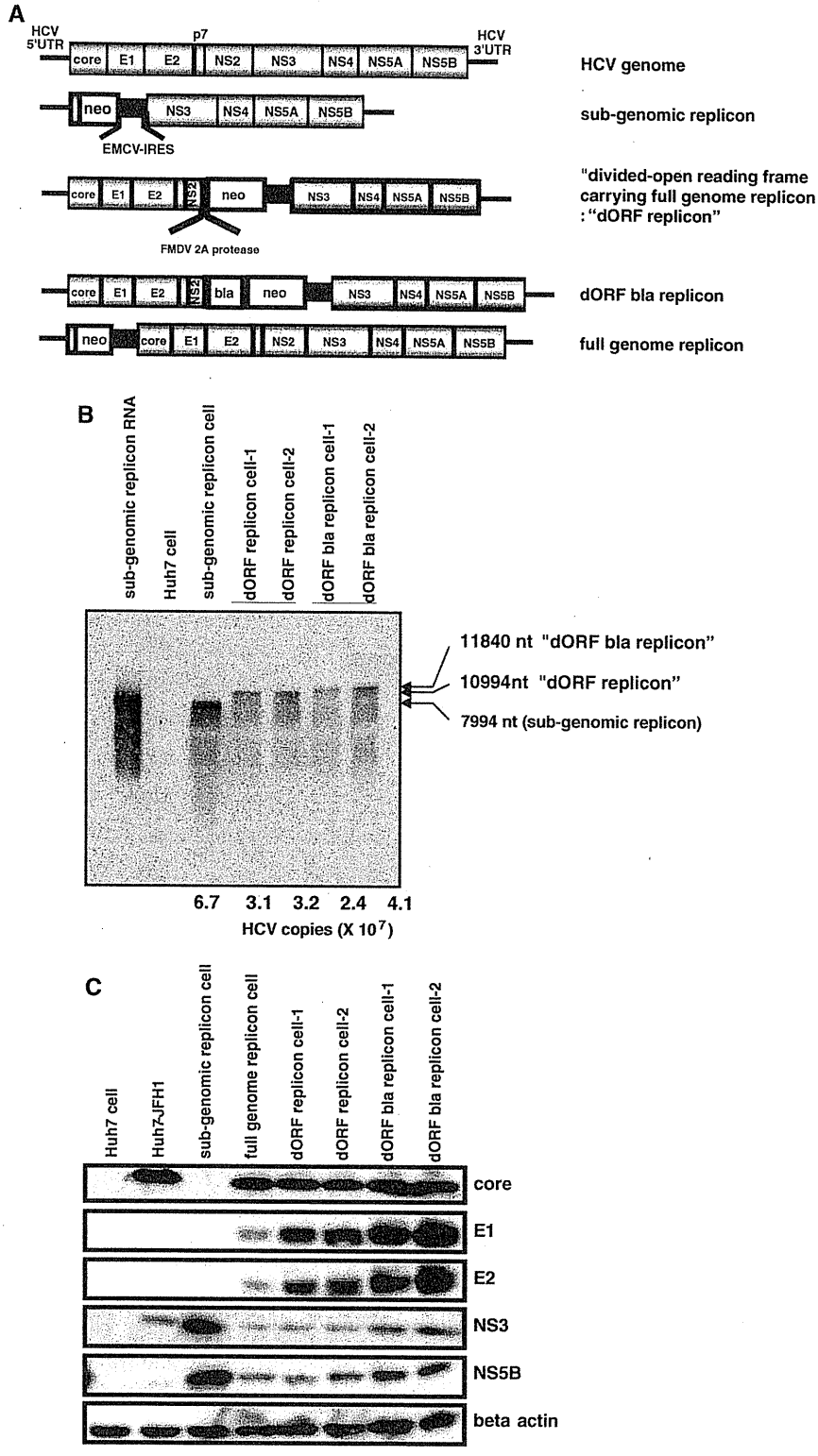

Fig. 1 Confirmation of "divided open reading frame carrying" (dORF) replicon cells. (A) Schematic representations of replicon RNAs used in this study. All the replicon constructs contained inserts just after the T7 promotor. UTR, untranslated region; NS, non-structural protein; neo, neomycin phosphotransferase 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

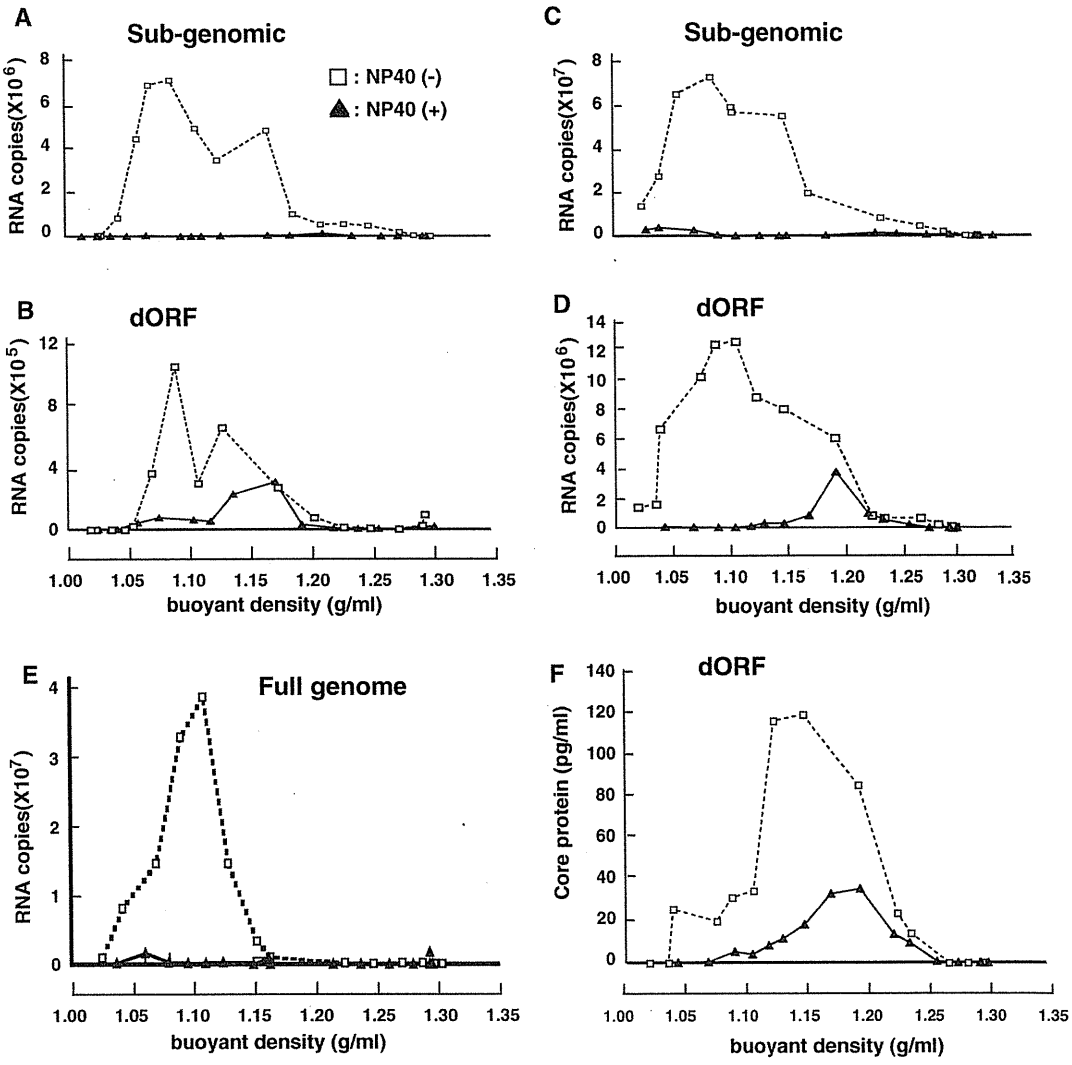

Fig. 2 Density gradient analysis of supernatants. Culture supernatants were treated with RNaseA and loaded directly onto a sucrose density gradient without treatment (open square) or after NP-40 treatment (filled triangle). Quantification of HCV RNA in each fraction of supernatant from the subgenomic 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

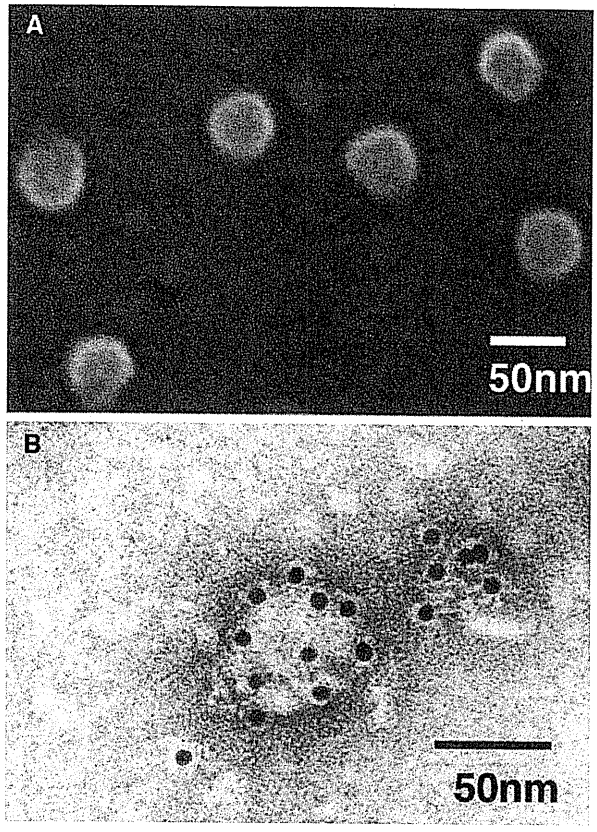

Fig. 3 Electron microscopy analysis of virus-like particles. The core-protein-rich fraction collected from the density gradient was further concentrated by ultracentrifugation and observed by scanning electron microscopy (A). The same fraction attached to formvar-coated grids was incubated with rabbit 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 non-specific 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

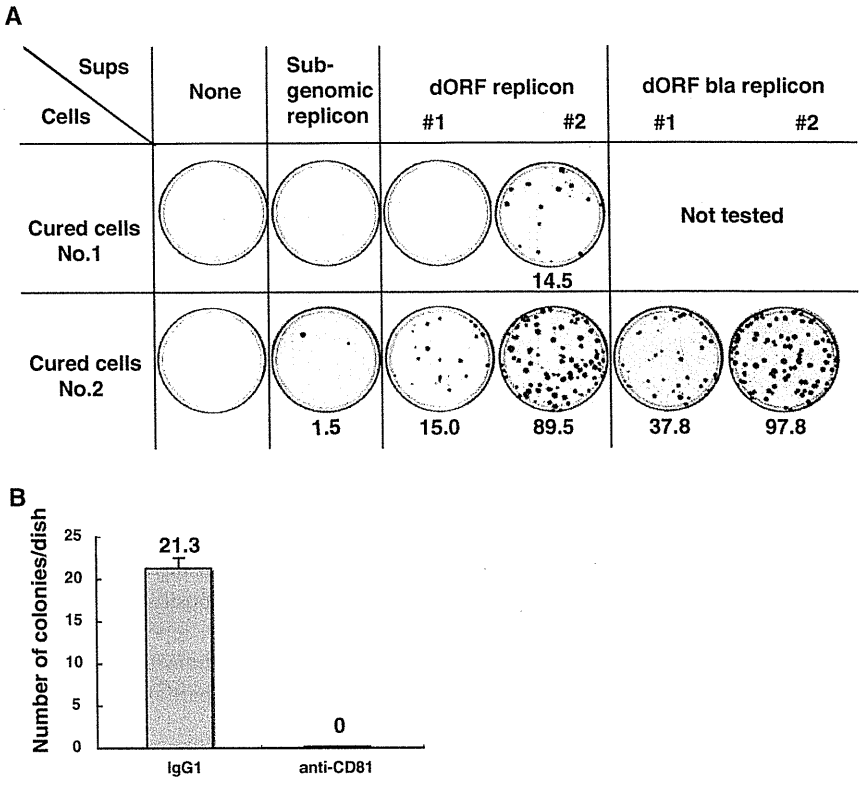

Fig. 4 Infectivity of supernatants from various replicon cells. Colonies of cells infected with the indicated supernatant. Numbers shown below the plates are the average of a total of four plates per condition (A). Inhibition of infection by anti-CD81 antibody. Cured cell K4 cells (No.2 in Fig. 4A) were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