Scientific Consulting), respectively. The primary antibodies used to detect NS5B of O strain and JFH1 strain were anti-NS5B (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science) and anti-NS5B (Murakami et al., 2008), respectively. The secondary antibody was Cy2-conjugated anti-mouse secondary antibody or FITC-conjugated anti-rabbit secondary antibody (for NS5B of JFH1) (Jackson ImmunoResearch). The nuclei were stained with 4′,6-diamidino-2-phenylindole (Sigma). The coverslips were mounted on glass slides by PermaFluor Aqueous Mountant (ThermoFisher) and then the cells were photographed under a confocal laser scanning microscope (LSM510; Carl Zeiss).

2.11. cDNA microarray analysis

HuH-7, Oc, OAc, Li23, OL8, OL11, OL8c, and OL11c cells (1×10^6 each) were plated onto 10-cm diameter dishes and cultured for 2 days in the absence of G418. Total RNAs from these cells were prepared using the RNeasy extraction kit (Qiagen). cDNA microarray analysis was performed by Dragon Genomics Center of Takara Bio. (Otsu, Japan) through an authorized Affymetrix service provider. cDNA was synthesized by the GeneChip T7-Oligo(dT) Promoter Primer Kit (Affymetrix) and TaKaRa cDNA Synthesis Kit (Takara Bio) from 3 μg total RNA. Biotinylated complementary RNA (cRNA) was synthesized by the IVT Labeling Kit (Affymetrix). Following fragmentation, 10 μg of cRNA was hybridized for 16 h at 45 °C on the GeneChip Human Genome U133 Plus 2.0 Array. GeneChips were washed and stained in the Affymetrix Fluidics Station 450, and then were scanned using GeneChip Scanner 3000 7G. Single array analysis was calculated by Microarray Suite version 5.0 (MAS5.0) with the Affymetrix setting. Differentially expressed genes were selected by comparing HuH-7-derived cells and Li23-derived cells.

2.12. RT-PCR

RT-PCR was performed to detect cellular mRNA as described previously (Dansako et al., 2003). Briefly, total RNA (2 μg) was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen) using an oligo dT primer (Invitrogen) according to the manufacturer's protocol. One-tenth of synthesized cDNA was used for PCR. The primers and PCR cycles used in this study are listed in Supplemental Table 1.

2.13. Quantification of HCV core protein

The levels of the core protein in the culture supernatants were determined by enzyme-linked immunosorbent assay (Mitsubishi Kagaku Bio-Clinical Laboratories).

2.14. Renilla luciferase assay for anti-HCV reagents

To monitor the effects of anti-HCV reagents, the cells were plated onto 24-well plates (2×10^4 cells per well) and cultured with the medium for Li23-derived cells in the absence of fungizone and G418 for 24 h. The cells were then treated with anti-HCV reagent at several concentrations for 72 h (sometimes 24 or 48 h), or the cells were treated with a combination of IFN-α and another anti-HCV reagent at several concentrations for 72 h. After treatment, the cells were subjected to luciferase assay using the renilla luciferase assay system according to the manufacturer's protocol (Promega). A manual Lumat LB 9501/16 luminometer (EG&G Berthold) was used to detect luciferase activity. The experiments were performed in at least triplicate.

2.15. Cell viability

To examine the cytotoxic effects of anti-HCV reagents on the cells, the cells were plated onto 24-well plates (2×10^4 cells per well) and cultured for 24 h. They were then treated with or without anti-HCV reagents for 72 h in the absence of G418. The viable cells were then counted in an improved Neubauer-type hemotocytometer after Trypan blue dye (Invitrogen) treatment. The experiments were performed in triplicate.

2.16. Infection of cells with secreted HCV

The inoculum for HCV infection was the culture medium of RSc cells (Ariumi et al., 2007, 2008; Kuroki et al., 2009) at 145 days after transfection with JFH1 RNA *in vitro* synthesized from pJFH1 (Wakita et al., 2005). This inoculum was passed through a 0.2-μm filter after low-speed centrifugation before use for infection. We seeded cells 24 h before infection at a density of 2×10^4 cells per well in a 24-well plate. We infected cells with 100 μl (equivalent to $10^{4.3}$ $TCID_{50}$) of inoculum for 2 h, washed them, added complete medium and cultured them for a maximum of 30 days with adequate passage of the cells. In some cases, at 7 or 8 days p.i., supernatant was used as an inoculum for the next HCV infection. The cells at 7 or 14 days p.i. were used to detect HCV proteins by Western blot analysis, to quantify HCV RNA by quantitative RT-PCR or to analyze the immunofluorescence of HCV proteins or dsRNA.

3. Results

3.1. Efficient replication system with HCV replicon or genome-length HCV RNA using human hepatoma Li23 cells

We previously established several genome-length HCV RNA (O strain of genotype 1b) replicating cell lines and found that a specific combination of adaptive mutations – either Q1112R, P1115L, and S2200R (QR,PL,SR) or Q1112R, K1609E, and S2200R (QR,KE,SR) – drastically enhanced the level of genome-length HCV RNA replication (Abe et al., 2007; Ikeda et al., 2005). This finding led us to hypothesize that such combinations of adaptive mutations may overcome the barrier that has made HuH-7 the only cell line thus far to allow the robust replication of genome-length HCV RNA. To test this hypothesis, HCV replicon RNA (ON/3-5B) possessing QR,PL,SR or QR,KE,SR (Supplemental Fig. 1) was transfected into various kinds of human cell lines (HuH-6, Li21, Li23, Li24, PH5CH, OUMS29, IHH10.3, IHH12 etc.), and the G418 selection was performed as described previously (Kato et al., 2003a). Although we failed to obtain the G418-resistant colonies in the most cell lines, fortunately, we found that the Li23 human hepatoma cell line gave only G418-resistant colonies (Fig. 1A). Approximately 200 and 700 colonies obtained from ON/3-5B/QR,PL,SR and ON/3-5B/QR,KE,SR-transfected cells, respectively, were pooled. Western blot analysis revealed that the expression levels of HCV proteins NS5A and NS5B were much higher in ON/3-5B/QR,KE,SR-derived colonies than in ON/3-5B/QR,PL,SR-derived colonies (Fig. 1B). We used the former for further analysis and referred to them as sOL cells. We demonstrated that the replicon in sOL cells showed a high level of sensitivity to anti-HCV reagents, similar to the level shown by the replicon (ON/3-5B/SR) in sO cells (Kato et al., 2003a) (Fig. 1C).

To obtain a source of cells with which to develop a genome-length HCV RNA replication system, we prepared cured cells (sOLc) from sOL cells by IFN-γ treatment, because cured cells are known to extremely enhance HCV RNA replication levels (Ikeda et al., 2005; Kato et al., 2003a). A genome-length HCV RNA (ON/C-5B/QR,KE,SR; Supplemental Fig. 1) was transfected into sOLc cells. Following G418 selection, many colonies were obtained (Fig. 2A). Fourteen

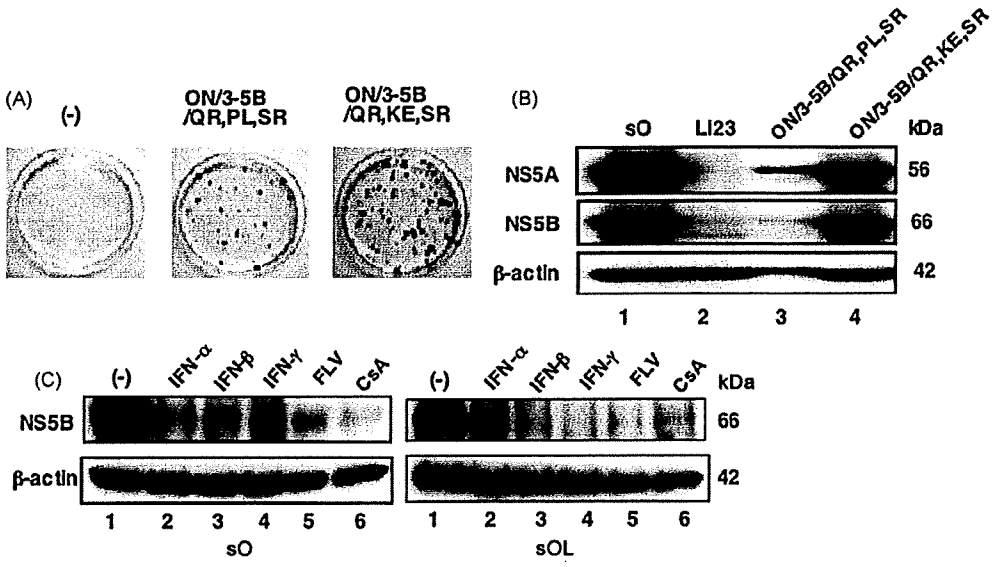

Fig. 1. Li23-derived cells harboring HCV replicon. (A) G418-resistant colonies from Li23 cells transfected with replicon RNA. ON/3-5B RNA with three additional mutations (ON/3-5B/QR,PL,SR or ON/3-5B/QR,KE,SR) was transfected into Li23 cells. The panels show G418-resistant colonies (57 colonies/μg RNA for ON/3-5B/QR,PL,SR and 132 colonies/μg RNA for ON/3-5B/QR,KE,SR) that were stained with Coomassie brilliant blue at 3 weeks after RNA transfection. (B) Western blot analysis of Li23-derived G418-resistant cells for HCV proteins NS5A and NS5B. Lane 1, sO (HuH-7-derived cell line harboring HCV replicon, ON/3-5B/SR); lane 2, Li23 as a negative control; lane 3, polyclonal G418-resistant cells obtained by transfection with ON/3-5B/QR,PL,SR RNA; lane 4, polyclonal G418-resistant cells (sOL) by transfection with ON/3-5B/QR,KE,SR RNA. (C) Sensitivity of sOL replicon to anti-HCV reagents. sOL cells were treated with IFN-α (lane 2, 20 IU/ml), IFN-β (lane 3, 20 IU/ml), IFN-γ (lane 4, 20 IU/ml), fluvastatin (FLV) (lane 5, 5 μM), or cyclosporine A (CsA) (lane 6, 0.5 μg/ml) for 5 days. Lane 1 shows no treatment. For comparison, sO cells were treated as well as sOL cells. NS5B was detected by Western blot analysis.

colonies (referred to as OL1–OL14) and a mixture of approximately 200 other colonies (referred to as OL) were successfully proliferated as cell lines. Using quantitative RT-PCR, we selected OL8, OL11, and OL14 because of their high levels (>9 × 10^6 copies/μg total RNA) of HCV RNA, although the titer of HCV RNA from genome-length HCV RNA replicating HuH-7-derived O cells (Ikeda et al., 2005) was approximately 4.5 × 10^7 copies/μg total RNA (Supplemental Fig. 2). We also demonstrated that the HCV sequence was not

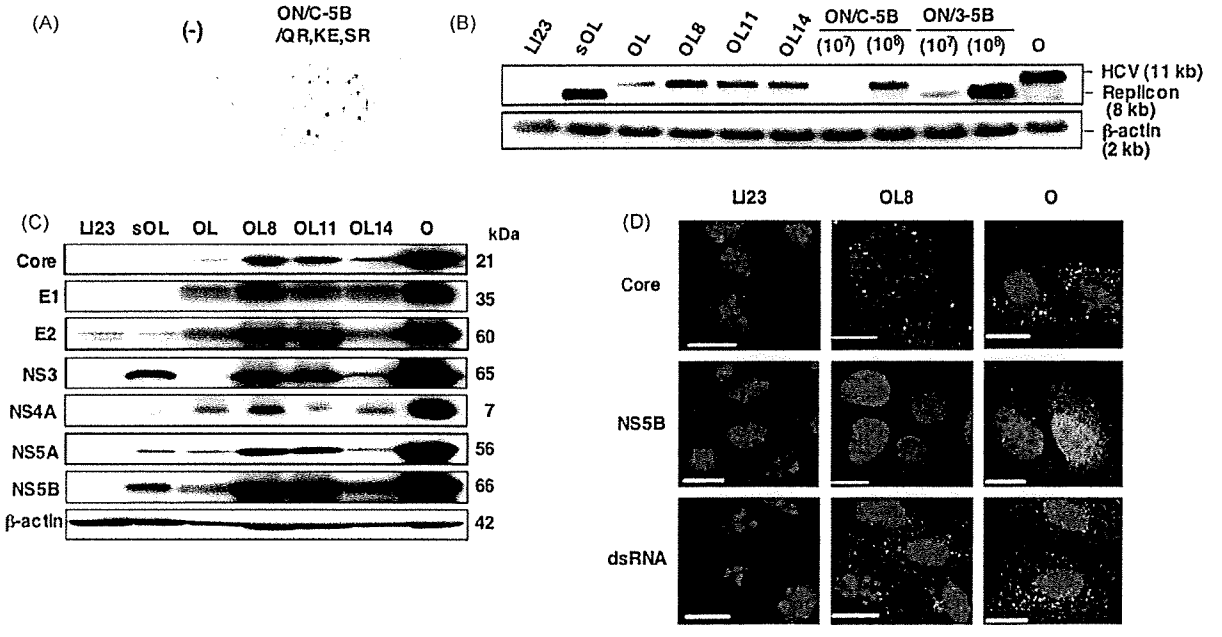

Fig. 2. Establishment of Li23-derived cell lines harboring replicative genome-length HCV RNA. (A) G418-resistant colonies from sOLc cells transfected with genome-length HCV RNA (ON/C-5B/QR,KE,SR). The panels show G418-resistant colonies (100 colonies/μg RNA) that were stained with Coomassie brilliant blue at 3 weeks after RNA transfection. (B) Northern blot analysis of total RNA prepared from sOL cells and genome-length HCV RNA-replicating cells (OL, OL8, OL11, and OL14). Synthetic RNA, given number of ON/C-5B or ON/3-5B RNA. HuH-7-derived O cells harboring replicative genome-length HCV RNA (ON/C-5B/KE,SR) and Li23 cells served as positive and negative controls, respectively. (C) Western blot analysis of sOL and genome-length HCV RNA-replicating cells (OL, OL8, OL11, and OL14) for HCV proteins, core, E1, E2, NS3, NS4A, NS5A, and NS5B. O cells and Li23 cells served as positive and negative controls, respectively. (D) Immunofluorescence analysis of OL8 cells. The cells were processed and stained with anti-core, anti-NS5B, or anti-dsRNA antibodies and Cy2-conjugated secondary antibody. The O cells and Li23 cells served as positive and negative controls, respectively. Bar, 20 μm.

integrated into the genomic DNA in OL, OL8, OL11, OL14, or sOL cells (data not shown). Northern and Western blot analyses also showed that the levels of HCV RNA and proteins in OL8 and OL11 cells were somewhat lower than those in O cells (Fig. 2B and C). Immunofluorescence analysis of the intracellular localization of HCV proteins and dsRNA, which is an intermediate of RNA replication, showed that the staining levels of HCV proteins and dsRNA located in the cytoplasm of OL8 cells, were also comparable to those in O cells (Fig. 2D). Both OL8 and O cells had two types of core protein staining patterns (detergent-resistant dots or patches and detergent-sensitive ring-like structures), as described previously (Matto et al., 2004) in HuH-7 cells harboring the genome-length HCV RNA (Con1 strain of genotype 1b) (Fig. 2D). These results suggest that robust replication of genome-length HCV RNA occurs in OL8 and OL11 cells. We performed sequence analysis of HCV RNAs derived from OL8, OL11, and OL14 cells, but no additional mutations were detected commonly among the three independent clones sequenced (data not shown). This suggested that no mutations other than Q1112R, K1609E, and S2200R are needed for genome-length HCV RNA replication in Li23-derived cells.

3.2. Genes differentially expressed between Li23- and HuH-7-derived cells

RT-PCR analysis revealed that Li23 and HuH-7 cells had similar liver-specific gene expression profiles (Fig. 3A). However, there is no information regarding the Li23-specific gene expression profile. To address this, we performed cDNA microarray analysis using total RNAs prepared from Li23, OL8, OL11, cured OL8 (OL8c), and OL11c cells in addition to HuH-7, Oc (Ikeda et al., 2005), and OAc (Abe et al., 2007). As the first step in this analysis, we selected 206 and 326 genes whose expression levels were upregulated and downregulated at ratios of more than 2^5 and less than 2^{-5} in Li23 vs. HuH-7, respectively. Then, from among those selected in the first step, we performed an additional selection of genes whose expression levels were commonly upregulated or downregulated among Li23-derived cells when compared with HuH-7-derived cells, and each of several already-known genes were identified (data not shown). Fig. 3B shows the results of RT-PCR regarding the representative genes belonging to such a category in the expression levels between Li23- and HuH-7-derived cells. The most characteristic feature of Li23-derived cells was the high expression levels of cancer antigens (NY-ESO-1, MAGEA, etc.) compared with no expression in HuH-7-derived cells (Fig. 3B). We demonstrated that such drastic differences were not attributable to differences in culture media (Supplemental Fig. 3). These results exclude the possibility that OL8 and OL11 cells are derived from contamination of HuH-7-derived cells. On the other hand, this microarray analysis revealed that HuH-7- and Li23-derived cells showed similar expression levels of CD81, scavenger receptor class B type I (SR-BI), Claudin-1, and Occludin, which have been identified as the host factors for HCV entry (Burlone and Budkowska, 2009). RT-PCR analysis confirmed them (Fig. 3C).

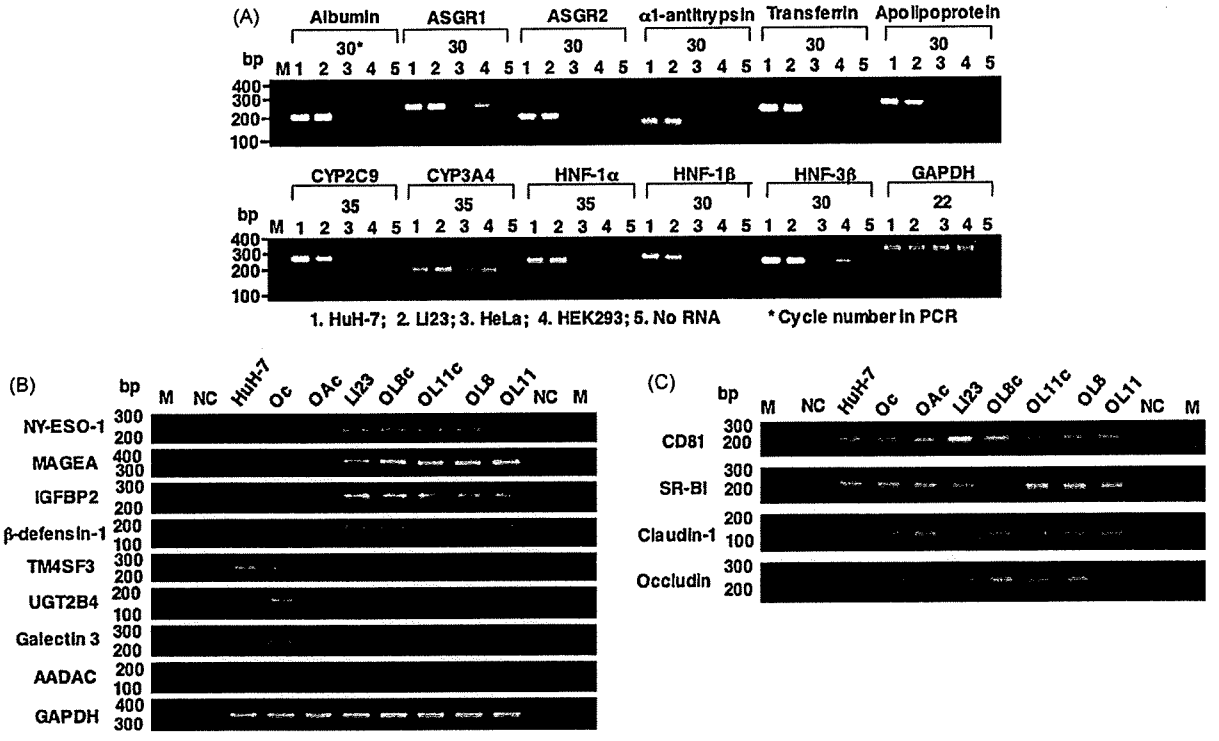

Fig. 3. Representative genes differentially expressed among Li23- and HuH-7-derived cells. (A) Li23 and HuH-7 cells showed similar liver-specific gene expression profiles. Total RNAs prepared from HuH-7, Li23, HeLa, and HEK293 cells were subjected to RT-PCR to detect liver-specific mRNAs using the primer sets listed in Supplementary Table 1. Presented data are the results of the following mRNA species: albumin, asialoglycoprotein receptor 1 (ASGR1), ASGR2, α1-antitrypsin, transferrin, apolipoprotein, cytochrome P450 2C9 (CYP2C9), CYP3A4, hepatocyte nuclear factor 1α (HNF-1α), HNF-1β, and HNF-3β. (B) Representative genes that were differentially expressed between HuH-7-derived cell lines and Li23-derived cell lines. Total RNAs prepared from HuH-7-derived cells (HuH-7, Oc, and OAc) and Li23-derived cells (Li23, OL8c, OL11c, OL8, and OL11) were subjected to RT-PCR using the primer sets listed in Supplementary Table S1. Lane M, 100 bp DNA ladder; NC, no RNA. The data presented are the results of the following mRNA species: cancer testis antigen (NY-ESO-1), melanoma-specific antigen family A (MAGEA), insulin-like growth factor binding protein 2 (IGFBP2), β-defensin-1, transmembrane 4 superfamily member 3 (TM4SF3), UDP glycosyltransferase 2 family polypeptide B4 (UGT2B4), galectin 3, and arylacetamide deacetylase (AADAC). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control. (C) Expression levels of CD81, SR-BI, Claudin-1, and Occludin between HuH-7- and Li23-derived cells. RNA preparation and RT-PCR were performed as described in (B).

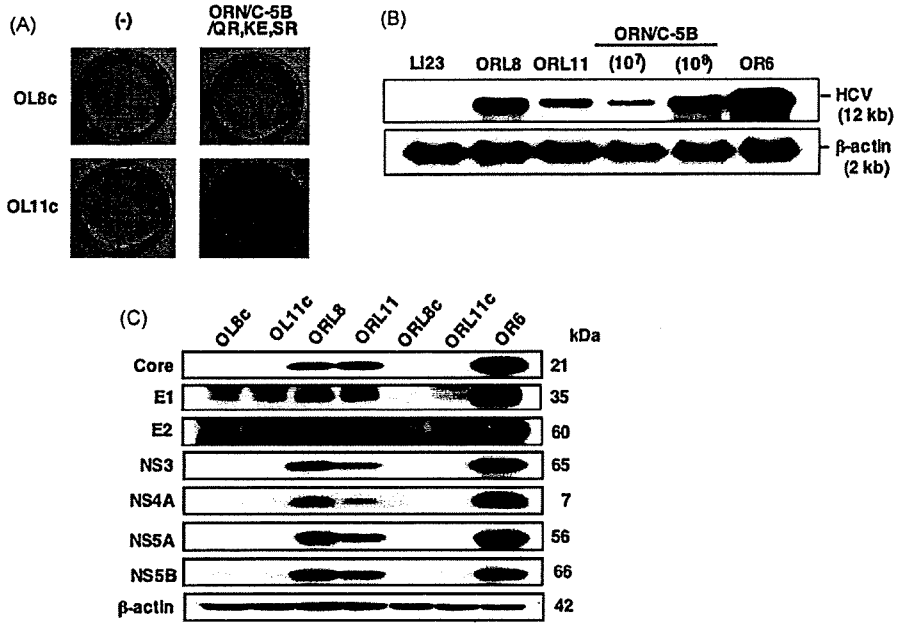

Fig. 4. Establishment of OL8- and OL11-derived cell lines harboring replicative genome-length HCV RNA encoding renilla luciferase. (A) G418-resistant colonies from OL8c or OL11c cells transfected with genome-length HCV RNA (ORN/C-5B/QR,KE,SR) encoding renilla luciferase gene. The panels show G418-resistant colonies that were stained as described in Fig. 1A. (B) Northern blot analysis of total RNA prepared from genome-length HCV RNA replicating ORL8 and ORL11 cells. Synthetic RNA, given number of synthetic ORN/C-5B RNA; Li23, negative control. HuH-7-derived OR6 cells replicating genome-length HCV RNA encoding renilla luciferase gene (ORN/C-5B/KE,SR) served as positive control. (C) Western blot analysis of ORL8 and ORL11 cells for HCV proteins, core, E1, E2, NS3, NS4A, NS5A, and NS5B. OL8c, OL11c, ORL8c, and ORL11c, negative controls; OR6, positive control.

3.3. *Development of new luciferase reporter assay systems that facilitate the quantitative monitoring of HCV RNA replication*

Since the reporter assay system using HuH-7-derived OR6 cells, which robustly replicates genome-length HCV RNA encoding renilla luciferase, is potentially useful for the quantitative evaluation of anti-HCV activity (Ikeda et al., 2005, 2006; Ikeda and Kato, 2007), we have tried to develop an Li23-derived assay system corresponding to the OR6 assay system. A genome-length HCV RNA encoding renilla luciferase (ORN/C-5B/QR,KE,SR) (Supplemental Fig. 1) was transfected into OL8c or OL11c cells. Following G418 selection, several OL8c colonies and several hundred OL11c colonies were obtained from the cells transfected with ORN/C-5B/QR,KE,SR (Fig. 4A). Regarding ORN/C-5B/QR,KE,SR, 9 OL8c-derived clones and 16 OL11c-derived clones were successfully proliferated as cell lines. Each clone possessing the highest

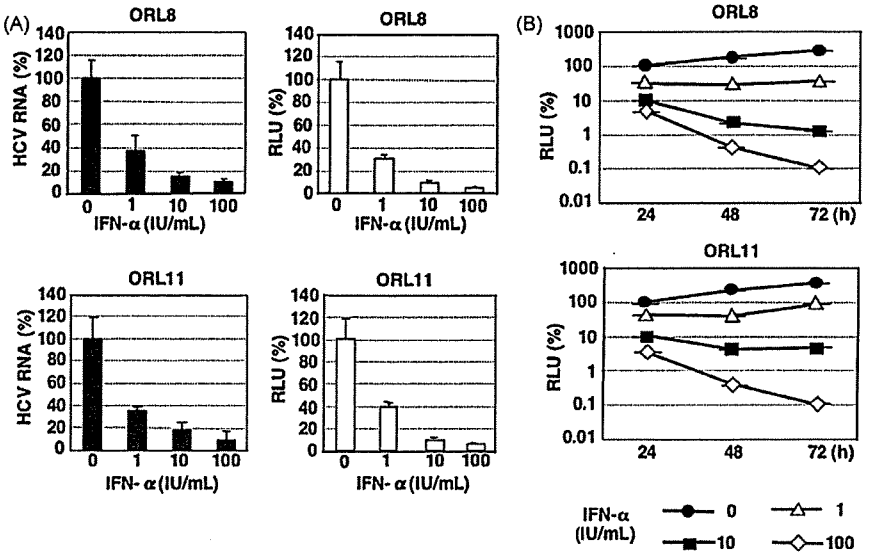

Fig. 5. ORL8 and ORL11 reporter assay system to monitor genome-length HCV RNA replication. (A) Renilla luciferase activity is correlated with HCV RNA level. The ORL8 (upper panels) and ORL11 (lower panels) cells were treated with IFN-α (0, 1, 10, and 100 IU/ml) for 24 h, and then luciferase reporter assay (right panels) and quantitative RT-PCR (left panels) were performed. The relative luciferase activity (RLU) (%) or HCV RNA (%) calculated at each point, when the level of luciferase activity or HCV RNA in non-treated cells was assigned to be 100%, is presented here. (B) IFN-α sensitivity of HCV RNA replication in ORL8 and ORL11 cells. The ORL8 (upper panel) and ORL11 (lower panel) cells were treated with IFN-α (0, 1, 10, and 100 IU/ml); the luciferase assay was performed at 24, 48, and 72 h after the treatment. The RLU (%) calculated at each point, when the luciferase activity of non-treated cells at 24 h was assigned to be 100%, is presented here. The experiments were performed in at least triplicate.

titer of HCV RNA was selected by quantitative RT-PCR and was thereafter referred to as ORL8 and ORL11 (data not shown). We demonstrated that the HCV RNA sequence was not integrated into the genomic DNA in ORL8 or ORL11 cells (data not shown). Northern and Western blot analyses showed that ORL8 and ORL11 cells expressed sufficient levels of HCV RNA and proteins for the quantitative monitoring of HCV RNA replication, although these levels were somewhat lower than those in OR6 cells (Fig. 4B and C). We performed sequence analysis of HCV RNAs derived from ORL8 and ORL11 cells, but no additional mutations were detected commonly among the three independent clones sequenced (data not shown). We demonstrated good correlations between the levels of luciferase activity and HCV RNA in ORL8 and ORL11 cells (Fig. 5A), as we previously demonstrated in OR6 cells treated with IFN-α for 24 h (Ikeda et al., 2005). Time course assays (24, 48, and 72 h) on IFN-α treatment demonstrated that the luciferase activity decreased in a dose- and time-dependent manner, and revealed that the luciferase activity had decreased to less than 0.1% at 72 h after treatment with 100 IU/ml IFN-α (Fig. 5B).

3.4. ORL8 and ORL11 assay systems are frequently more sensitive than the OR6 assay system

Using ORL8 and ORL11 assay systems, we evaluated the anti-HCV activities of representative reagents identified by HuH-7-derived assay systems (Ikeda and Kato, 2007; Moriishi and Matsuura, 2007). For the sake of comparison, we also evaluated these activities using the OR6 assay system along with the same culture medium that we used for the ORL8 and ORL11 assays, since we had already confirmed that HCV RNA in OR6 cells was efficiently replicated using this culture medium (data not shown). First, we measured the 50% effective concentration (EC_{50}) of IFN-α against HCV RNA replication. The EC_{50} values of IFN-α in ORL8, ORL11, and OR6 assays were assigned as 0.13, 0.30, and 0.40 IU/ml, respectively, without suppression of cell growth (Fig. 6A). Regarding IFN-β, IFN-γ, and cyclosporine A also, the ORL8 and ORL11 assays were each more sensitive than the OR6 assay (Fig. 6B). It is noteworthy that the EC_{50} values of fluvastatin and simvastatin in the ORL8 and ORL11 assays were fairly lower than those in the OR6 assay (Fig. 6B). In contrast, we observed that the OR6 assay for geldanamycin was slightly more sensitive than the ORL8 or ORL11 assay (Fig. 6B). When the number of cells without treatment was compared to that of cells with treatment, no significant decrease in cell number was observed following treatment with anti-HCV reagents used in Fig. 6B (data not shown). Co-treatment of IFN-α and fluvastatin also demonstrated that the ORL8 and ORL11 assays were much more sensitive than the OR6 assay (Fig. 6C), indicating that these two systems are powerful biosensors of RNA viral replication.

3.5. Persistent reproduction of HCV life cycle in Li23-derived cells

A most interesting point is whether or not infectious HCV is produced in Li23-derived cell lines and thus enables robust HCV RNA replication. To clarify this point, we used HCV-JFH1 (genotype 2a), the only infectious HCV molecular clone identified in a cell culture to date (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al.,

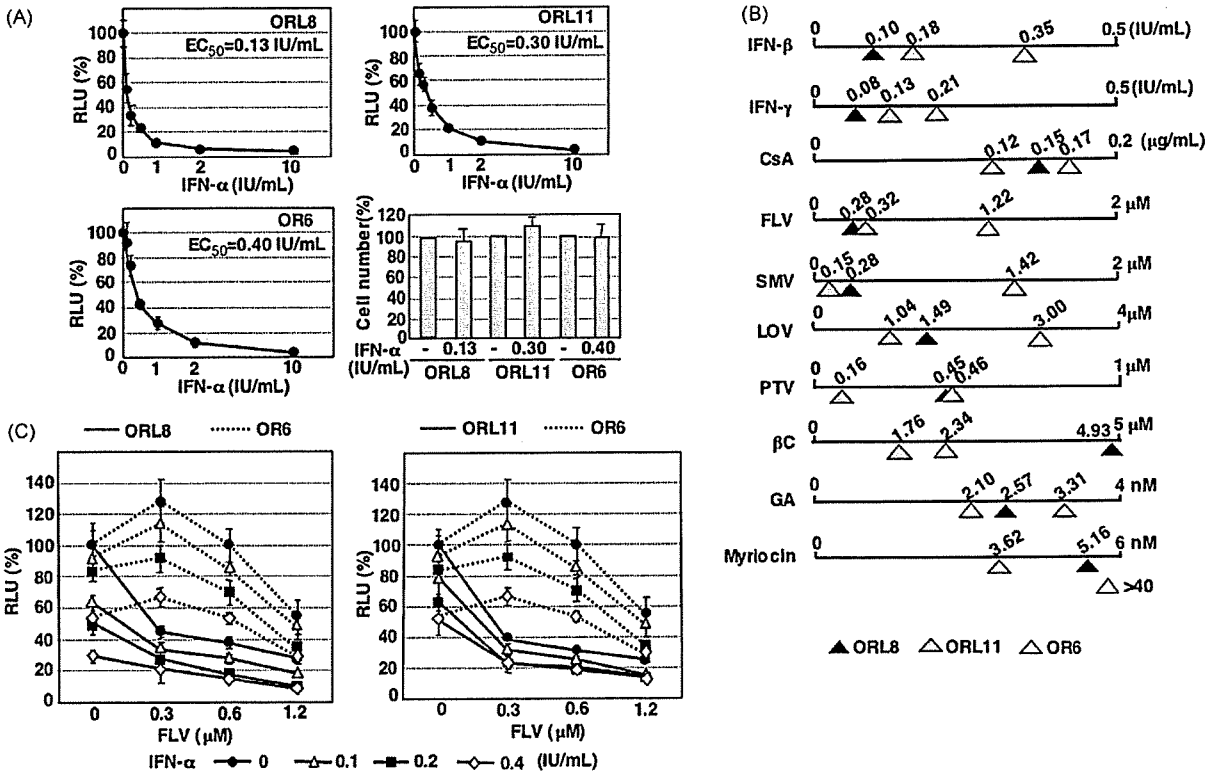

Fig. 6. The diverse effects of anti-HCV reagents in ORL8, ORL11, and OR6 assay systems. (A) IFN-α sensitivities on genome-length HCV RNA replication in ORL8, ORL11, and OR6 assay systems. The ORL8, ORL11, and OR6 cells were treated with IFN-α (0, 0.06, 0.13, 0.25, 0.5, 1, 2, and 10 IU/ml) for 72 h, and then luciferase assay was performed as described in Fig. 5A. ORL8, ORL11, and OR6 cells were cultured in the absence or presence of IFN-α at each 50% effective concentration (EC_{50}) for 72 h, and then the cells were counted as described in Section 2. (B) Diverse EC_{50} values of anti-HCV reagents on genome-length HCV RNA replication in ORL8, ORL11, and OR6 cells. ORL8, ORL11, and OR6 cells were treated with several different concentrations of IFN-β, IFN-γ, CsA, FLV, simvastatin (SMV), lovastatin (LOV), pitavastatin (PTV), β-carotene (βC), geldanamycin (GA), or myriocin for 72 h, after which luciferase assay was performed as described in Fig. 5A. EC_{50} values were calculated from the data of each triplicate assay. (C) ORL8 and ORL11 assay systems are more sensitive than the OR6 assay system in the combination analysis of IFN-α and FLV. ORL8, ORL11, and OR6 cells were treated with a combination of IFN-α (0, 0.1, 0.2, and 0.4 IU/ml) and FLV (0, 0.3, 0.6, and 1.2 μM) for 72 h, after which a luciferase assay was performed as described in Fig. 5A.

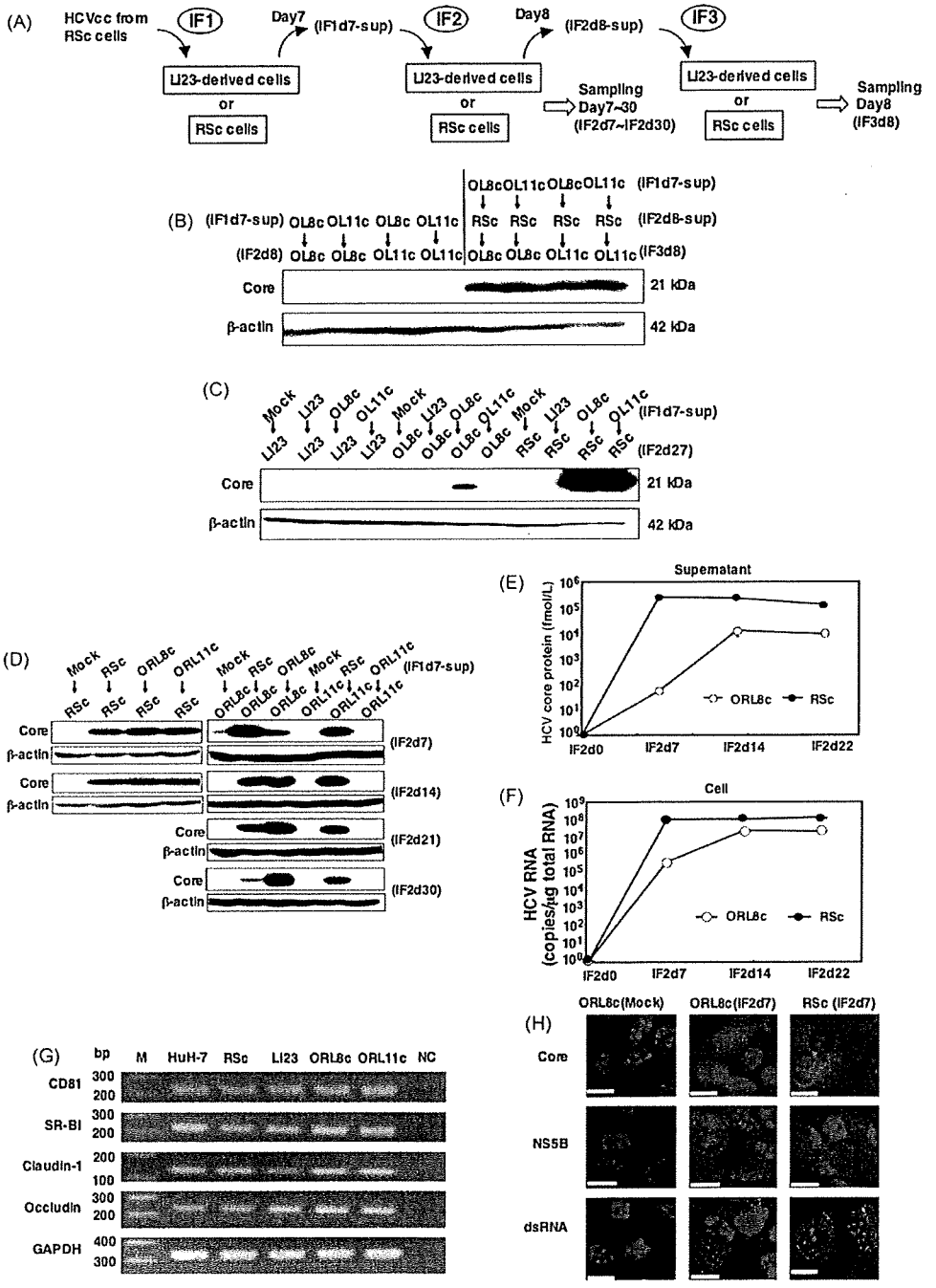

Fig. 7. Reproduction of HCV life cycle in OL8c and ORL8c cells. (A) Flow chart of experiments on HCVcc infection. A time schedule for HCVcc infection and sampling is shown. IF1d7-sup refers to the supernatant of the first-infected cells at 7 days p.i. IF2d7 refers to the secondly infected cells at 7 days p.i. (B) Production of infectious HCV from HCVcc-infected OL8c and OL11c cells. OL8c or OL11c cells were infected with HCVcc produced from RSc cells, and each supernatant at 7 days p.i. (IF1d7-sup) was used for inoculation to OL8c, OL11c, or RSc cells. Western blot analysis was performed to detect HCV core protein in OL8c or OL11c cells at 8 days p.i. (IF2d8). The supernatant (IF2d8-sup) from RSc cells at 8 days p.i. was used for further inoculation to OL8c or OL11c cells. Western blot analysis was performed for the detection of HCV core protein in OL8c or OL11c cells at 8 days p.i. (IF3d8). (C) HCVcc produced from OL8c cells is infectious to OL8c cells. The supernatants (IF1d7-sup) described in (B) were used for inoculation to OL8c and RSc cells. Li23 cells were used as negative controls. Western blot analysis was performed to detect HCV core protein in OL8c, RSc, or Li23 cells at 27 days p.i. (IF2d27). (D) Persistent production of infectious HCVcc from ORL8c cells. ORL8c or ORL11c cells were infected with HCVcc produced from RSc cells, and each supernatant at 7 days p.i. (IF1d7-sup) was used for inoculation to ORL8c or ORL11c cells. RSc cells were used as positive controls. Western blot analysis was performed to detect HCV core protein in ORL8c or ORL11c cells at 7, 14, 21, and 30 days p.i. (IF2). HCV core protein in RSc cells at 7 and 14 days p.i. was also detected by Western blot analysis. (E) Secretion of HCV core protein in culture supernatant. The culture supernatants (IF2d7-, IF2d14-, and IF2d22-sup) of ORL8c or RSc cells were used to determine the levels of core protein by enzyme-linked immunosorbent assay. Experiments were performed in triplicate. (F) Levels of HCV RNA in HCVcc-infected cells. The levels of intracellular HCV RNAs of ORL8c or RSc cells (IF2d7, IF2d14, and IF2d22) were determined by quantitative RT-PCR. Experiments were performed in triplicate. (G) Expression levels of CD81, SR-BI, Claudin-1, and Occludin among RSc, ORL8c, and ORL11c cells. RNA preparation and RT-PCR were performed as described in Fig. 3B. (H) Immunofluorescence analysis of HCVcc-infected cells. ORL8c or RSc cells (IF2d7) were processed and stained with anti-core, anti-NS5B, and anti-dsRNA antibodies and Cy2-conjugated secondary antibody. Mock-infected ORL8c cells served as negative controls. Bar, 20 μm.

2005), and various Li23-derived cell lines obtained in this study (Supplemental Table 2). A flow chart of the expression procedure is shown in Fig. 7A.

Since we detected the transient expression of HCV-JFH1 RNA in OL8c and OL11c cells (Supplemental Fig. 4A), we examined the susceptibility of OL1c, OL2c, OL3c, OL4c, OL8c, OL11c, or OL14c cells to cell culture-generated HCV-JFH1 (HCVcc) produced from HuH-7-derived RSc cells that HCVcc could infect and efficiently replicate (Ariumi et al., 2007, 2008; Kuroki et al., 2009). At 16 days post-infection (p.i.), the core protein was detected in OL2c, OL3c, OL8c, OL11c, and OL14c cells, but not in OL1c and OL4c cells (Supplemental Fig. 4B), indicating that most OLc series cells exhibit good susceptibility to HCVcc. In this context, the supernatant (IF1d7-sup) of HCVcc-infected OL8c or OL11c cells at 7 days p.i. was inoculated to naïve OL8c or OL11c cells; however, we failed to detect the core protein in the cells (IF2d8) at 8 days p.i. (Fig. 7B). However, when the supernatant (IF2d8-sup) of IF1d7-sup-inoculated RSc cells at 8 days p.i. was inoculated to naïve OL8c or OL11c cells, core expression was strongly detected in either case at 8 days p.i. (IF3d8) (Fig. 7B). This suggested that small amount of infectious HCV was produced from OL8c or OL11c cells. Accordingly, the expression of core protein was detected in IF1d7-sup-inoculated OL8c cells, but not in OL11c cells, at 27 days p.i. (IF2d27) (Fig. 7C), indicating that OL8c-derived HCVcc may infect and replicate in naïve OL8c cells. This finding leads to the assumption that ORL8c or ORL11c cells are better than OL8c cells, because ORL8c and ORL11c cells are derived from luciferase reporter full-length HCV RNA-replicating cells (ORL8 and ORL11), and because they each have a more permissible environment for HCV RNA replication. To test this hypothesis, RSc-derived HCVcc was commonly used in order to avoid the issue of uncertain efficiency of RNA transfection to RSc, ORL8c, or ORL11c cells. RSc-derived HCVcc was inoculated to naïve RSc, ORL8c, or ORL11c cells; and RSc, ORL8c, or ORL11c-derived IF1d7-sup was further inoculated to naïve RSc, ORL8c, or ORL11c cells. Expectedly, core expression in the ORL8c cells inoculated with ORL8c-derived IF1d7-sup was strongly detected until at least 30 days p.i. (IF2d30) (Fig. 7D). The level of core protein in the ORL8c cells was equivalent to that in the RSc cells inoculated with RSc, ORL8c, or ORL11c-derived IF1d7-sup (Fig. 7D). Regarding the NS5B expression, similar results were obtained (data not shown). These results suggest that HCV production in ORL8c cells is comparable to that in RSc cells. In contrast, core protein was not detected in ORL11c cells inoculated with ORL11c-derived IF1d7-sup (Fig. 7D). The core protein released into the culture supernatants (IF2d7-, IF2d14-, and IF2d22-sup) of HCVcc-infected ORL8c cells was persistently detected, although at somewhat lower levels than in the RSc cells (Fig. 7E). The level of intracellular HCV RNA in the ORL8c cells was $>10^7$ copies/μg total RNA at IF2d14; this is also somewhat lower than in the RSc cells (Fig. 7F). However, RT-PCR analysis revealed that the expression levels of HCV entry factors (CD81, SR-BI, Claudin-1, and Occuldin) were comparable among HuH-7, RSc, Li23, ORL8c, and ORL11c cells (Fig. 7G). Immunofluorescence analysis showed that the staining levels of dsRNA and HCV proteins were also comparable between HCVcc-infected ORL8c and RSc cells (IF2d7) (Fig. 7H). Colocalization of lipid droplet and HCV core protein was also observed in HCVcc-infected ORL8c and RSc cells (Supplemental Fig. 5), as previously reported (Miyanari et al., 2007). In summary, we demonstrated that ORL8c cells persistently supported the HCV life cycle.

4. Discussion

In this study, we found that human hepatoma Li23-derived cells possess the environments needed for robust genome-length HCV RNA replication and persistent production of infectious HCV. Using Li23-derived cell lines, we developed subgenomic and genome-length HCV RNA replication systems, drug assay systems, and a persistent HCV production system, which correspond to the counterparts of those using HuH-7-derived cell lines (Supplemental Table 2). It is noteworthy that the ORL8c cells cured from ORL8 cells, which were selected by the indicator of HCV RNA replication, showed good potential for producing HCV-JFH1. This finding suggests that the host factors required for robust HCV RNA replication – rather than those for HCV infection or reformation – are key determinants for reproducing the HCV life cycle in cell culture. In fact, we observed similar expression levels of the HCV entry factors between Li23- and HuH-7-derived cells. Therefore, such host factors might be commonly expressed in both ORL8c and RSc cells (Ariumi et al., 2007, 2008; Kuroki et al., 2009).

Our microarray analysis clearly demonstrated that OL8 and OL11 cell lines established in this study were not of HuH-7 cell origin, and revealed that Li23-derived cells possessed rather different expression profiles from those in HuH-7-derived cells (Fig. 3B), although similar liver-specific gene expression profiles were observed in both cell lineages (Fig. 3A). In addition, this analysis revealed that at least OL8 and OL11 cells possessed characteristic expression profiles of the parental Li23 cells, as Oc and OAc cells also showed the HuH-7-type expression profile. Therefore, further comparative studies on the mechanism(s) of HCV proliferation using Li23- and HuH-7-derived cell lines (e.g. ORL8c vs. RSc) may identify new host factor(s) required for efficient HCV proliferation.

A specific combination of adaptive mutations (Q1112R, K1609E, and S2200R) (Abe et al., 2007) is also a key determinant with which to find the Li23 cell line. Until the finding of such a combination of adaptive mutations, we had failed to establish any non-HuH-7-derived cells harboring the HCV replicon. Although it remains unclear what mechanism underlies these adaptive mutations that enhance HCV RNA replication, these mutations might be useful for the development HCV RNA replication systems of various HCV strains.

ORL8 and ORL11 assay systems might become important tools for evaluating or screening anti-HCV reagents, because these assay systems were frequently more sensitive to anti-HCV reagents than the HuH-7-derived OR6 assay system. However, the fact that the ORL8 and ORL11 assays were each more sensitive than the OR6 assay may be due to the fact that OR6 has a higher level of HCV RNA replication than ORL8 and ORL11 cells. Recently, we developed HCV replicon reporter assay systems using four genotype 1b HCV strains (1B-4, KAH5, O, and 1B-5), and found diverse sensitivities against various anti-HCV reagents among the replicons (Nishimura et al., 2009). In that study, we demonstrated that the sensitivities to anti-HCV reagents were not dependent on the replication levels of HCV RNA, and suggested that factor(s) other than the HCV RNA level are involved in conferring sensitivities to anti-HCV reagents including IFN-α (Nishimura et al., 2009). Therefore, the practical use of HuH-7- and Li23-derived assay systems would be very effective for accurately evaluating anti-HCV activity.

Finally, the most important feature of this report is that we were able to persistently produce infectious HCVcc using ORL8c cells. ORL8c-produced HCVcc would be very useful not only for verification of data obtained from HuH-7-derived cells but also for obtaining a variety of new information about the HCV life cycle.

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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.virusres.2009.08.006.

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ORIGINAL ARTICLE

HCV genotype 1b chimeric replicon with NS5B of JFH-1 exhibited resistance to cyclosporine A

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Abstract Cyclosporine A (CsA) is a well-characterized anti-HCV reagent. Recently it was reported that the genotype 2a JFH-1 strain was more resistant than genotype 1 HCV strains to CsA in a cell culture system. However, the JFH-1 responsible region for the resistance to CsA remains unclear. It was also demonstrated that in genotype 1b HCVs, NS5B interacts with cyclophilin (CyP). To clarify whether or not NS5B of JFH-1 is significant for CsA resistance, we developed a chimeric replicon with NS5B of JFH-1 in the genotype 1b backbone. The chimeric replicon was more resistant to CsA than the parental genotype 1b replicon. Furthermore, reduction of CyPA had a greater effect on HCV RNA replication and sensitivity to CsA than reduction of CyPB. Here, we demonstrated that NS5B of JFH-1 contributed to this strain's CsA-resistant phenotype. NS5B and CyPA are significant for determining HCV's sensitivity to CsA.

Introduction

The combination of a pegylated interferon (IFN) with ribavirin (RBV) is the current standard therapy for chronic hepatitis C and yields a sustained virological response (SVR) rate of about 55% [6]. This means that about 45% of patients with chronic hepatitis C are still threatened by the progress of the disease to cirrhosis and hepatocellular carcinoma. To find a more effective therapy, several anti-HCV reagents have been reported using HCV replicon systems [11, 14]. Especially, cyclosporine A (CsA), which is widely used as an immunosuppressive reagent, and its derivatives, which lack immunosuppressive activity, possess anti-HCV activity [8, 18, 19]. These reagents will help to improve the SVR rate.

Cyclophilins (CyPs), CsA ligands, are a family of cellular enzymes possessing peptidyl-prolyl isomerase activity. CyP family members play significant roles in numerous cellular processes, including transcriptional regulation, immune response, protein secretion and mitochondrial function [7]. CsA possesses three major cellular targets: CyP, the calcineurin-nuclear factor of activated T-cells pathway and P-glycoprotein [7]. The mechanism of anti-HCV activity of CsA is through disassociation between CyP and HCV nonstructural protein 5B (NS5B), an RNA-dependent RNA polymerase [20]. Fernandes et al. [5] also reported that NS5A was significant in the sensitivity of HCV to CsA. However, the role of CyPs as a cellular target of CsA in HCV RNA replication remains controversial [17, 20, 21]. While genotype 1a and 1b HCV strains were highly sensitive to CsA, a genotype 2a strain, JFH-1, was less sensitive to CsA [12, 21]. Moreover, in genotype 1b HCV, interaction between CyPB and HCV NS5B is required for robust HCV RNA replication [10].

To investigate whether or not NS5B of JFH-1 is an important factor for determining sensitivity to CsA, we engineered a 1b/2a chimeric HCV subgenomic replicon derived from genotype 1b HCV-O RNA, in which NS5B and a 3′-untranslated region (UTR) were replaced with

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those of HCV JFH-1 RNA. This replicon system enables to investigate the direct effect of NS5B on the CyPs.

We report here that NS5B of JFH-1 contributes to the CsA-resistant phenotype of this strain. Furthermore, CyPA but not CyPB is essential for HCV RNA replication in 1b and 1b/2a chimeric replicon-harboring cells. Finally, supplementation with vitamin E (VE) negates the anti-HCV activity of CsA in the presence or absence of CyPs. These results contribute to our understanding of the mechanism(s) that mediate the efficacy of CsA's anti-HCV activity.

Materials and methods

Cell culture

293FT cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum. The HuH-7-derived OR6c cells were cultured as previously described [10]. The cells harboring the subgenomic replicon were maintained in the culture medium containing G418 (0.3 mg/ml; Promega, Madison, WI).

Reagents

IFN-α, IFN-γ, and VE were purchased from Sigma-Aldrich (St. Louis, MO). CsA was purchased from Calbiochem (San Diego, CA). Pitavastatin (PTV) was purchased from KOWA Co., Ltd. (Tokyo, Japan). Mizoribine (MZB) and RBV were kindly provided by Asahi Kasei Pharma (Tokyo, Japan) and Yamasa Corporation (Choshi, Japan), respectively.

Plasmid construction

The plasmid of pORN/3-5B/QR,KE,RS/5B(J) was based on pORN/3-5B/QR,KE,RS [1] and was constructed by replacing the NS5B coding region and 3′UTR with the corresponding JFH-1 sequence. The NS5A/NS5B junction was set after amino acid 2419 of HCV-O and generated by polymerase chain reaction (PCR). The sequence numbering for coding and non-coding regions was based on a sequence from GenBank: HCV-O (accession no. **AB191333**) and JFH-1 (accession no. **AB047639**). Retroviral vector pCX4bsr [2] was used as an expression vector. To obtain full-length CyPA and CyPB cDNAs, reverse transcription (RT)-PCR with KOD-plus DNA polymerase (Toyobo, Osaka, Japan) was performed as previously described [4]. The pCX4bsr/Myc-CyPA and pCX4bsr/Myc-CyPB plasmids expressing Myc-tagged CyPA and CyPB, respectively, were obtained by inserting the PCR products of full-length CyPA and CyPB into the MluI-NotI sites of the pCX4bsr/Myc vector. Expression plasmids for HA-tagged NS5B (HCV-O and JFH-1) were generated by insertion of PCR fragments encoding each HCV protein into the MluI-NotI sites of the pCX4bsr/HA vector. The sequences of all constructed plasmids were confirmed by the sequencing analysis as described previously [1].

RNA synthesis, RNA transfection, and selection of G418 cells

Plasmid DNAs were linearized by XbaI and used for the RNA synthesis with the T7 MEGAScript kit (Ambion, Austin, TX). In vitro transcribed RNA was transfected into OR6c cells by electroporation [9]. The transfected cells were selected in culture medium containing G418 (0.3 mg/ml) for 3 weeks.

RNA interference, lentiviral vector construction

A detailed description of methods of RNA interference and lentiviral vector construction is available in Supplementary Materials.

Western blot analysis

Western blot analysis was performed as described previously [1]. The antibodies used in this study were those against NS3 (Novocatra Laboratories, UK), NS5A (a generous gift from Dr. A Takamizawa, Research Foundation for Microbial Diseases, Osaka University), NS5B (1b, a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science), NS5B (1b/2a) [15], CyPA (BIOMOL, Plymouth Meeting, PA), CyPB (Affinity BioReagents, Rockford, IL), and β-actin (AC-15; Sigma).

Immunoprecipitation

Immunoprecipitation was performed as described previously [10]. Briefly, pre-cleared cell lysates were incubated with an anti-Myc antibody (PL14; MBL, Nagoya, Japan). Immunocomplexes were recovered by adsorption to protein G-Sepharose resin (GE Healthcare Bioscience, Uppsala, Sweden). After three washes with lysis buffer, the immunoprecipitates were analyzed by immunoblot analysis using anti-Myc and anti-HA (3F10; Roche, Mannheim, Germany) antibodies.

Evaluation of sensitivity of anti-HCV reagents

The cells were plated onto 24-well plates ($1.5–2 \times 10^4$ cells/well). After 24 h culture, the culture medium was replaced with anti-HCV reagent containing medium. After 72 h additional culture, the cells were washed with

phosphate-buffered saline once, harvested with Renilla lysis reagents (Promega), and subjected to Renilla luciferase (RL) assay according to the manufacturer's protocol.

Statistical analysis

The luciferase activities were statistically compared between the various treatment groups using Student's *t* test. *P* values of less than 0.05 were considered statistically significant.

Results

The 1b/2a chimeric replicon is less sensitive to CsA than the 1b replicon

To investigate the mechanism(s) underlying CsA's anti-HCV activity, we engineered a 1b/2a chimeric HCV subgenomic replicon derived from genotype 1b HCV-O RNA, in which the NS5B and 3′UTR regions were replaced with those of HCV JFH-1 RNA (Fig. 1a). These RNAs were transfected into OR6c cells. After 3 weeks' selection of G418, we successfully obtained a 1b replicon or 1b/2a chimeric replicon-harboring cells as polyclones (see Supplementary Material). The colony forming efficiencies of the 1b replicon and the 1b/2a chimeric replicon were 5150 ± 361 and 62 ± 10 colonies/μg RNA, respectively. Sequence analysis of HCV RNA in 1b replicon or 1b/2a chimeric replicon-harboring cells revealed that there was no conserved amino acid substitution (data not shown). The RT-quantitative PCR analysis revealed that intracellular HCV RNA copies were $3.8 \pm 0.1 \times 10^7$ and $1.1 \pm 0.1 \times 10^7$ copies/μg total RNA in 1b replicon and 1b/2a chimeric replicon-harboring cells, respectively.

Next, we examined the sensitivity of the 1b replicon and 1b/2a chimeric replicon to anti-HCV reagents (Fig. 1b). HCV RNA replication was monitored through reporter

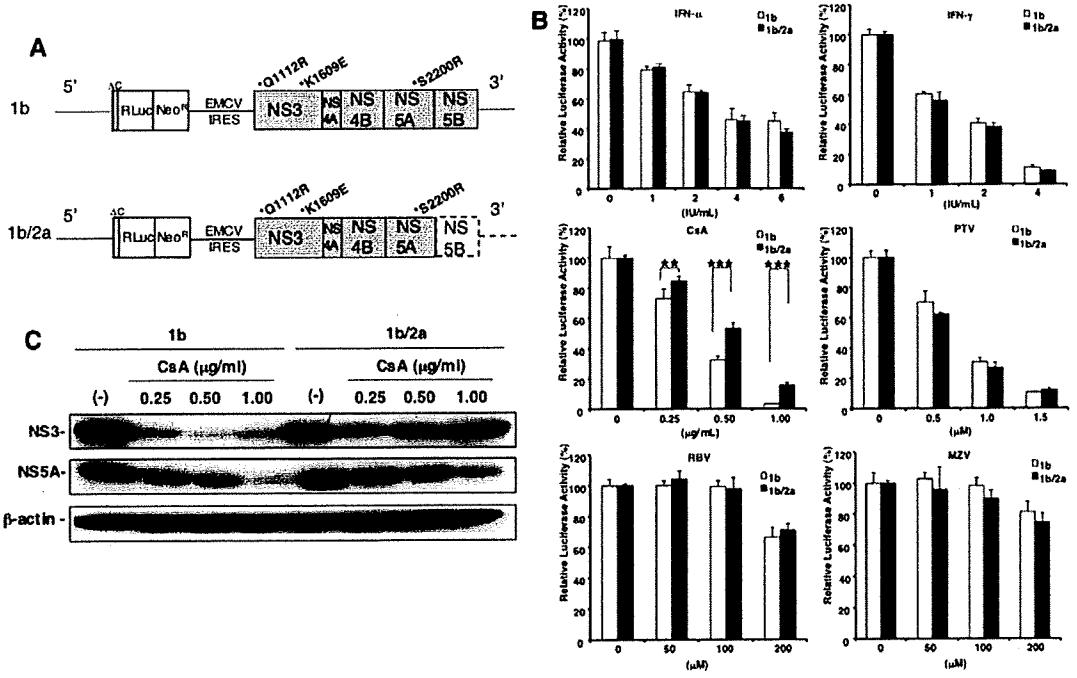

Fig. 1 1b/2a chimeric replicon-harboring cells are less sensitive to CsA. **a** Gene organization of subgenomic RNA. RLuc and DC indicate the RL gene and the 12 N-terminal amino acid residues of the core protein as a part of internal ribosomal entry site (IRES), respectively. The positions of adaptive mutations are indicated by *asterisks*. *Shaded boxes*, *dotted open boxes*, *thin lines*, *dotted lines*, *sick lines*, and *open boxes* indicate open reading frame (ORF) derived from HCV-O strain, ORF derived from JFH-1 strain, UTR of HCV-O strain, UTR of JFH-1 strain, encephalomyocarditis virus IRES, and fusion protein RL with neomycin phosphotransferase (Neo^R), respectively. **b** Effects of various anti-HCV reagents on HCV RNA replication in the 1b replicon (*open columns*) and in the 1b/2a chimeric replicon (*closed columns*) harboring cells. The cells were treated with IFN-α, IFN-γ, CsA, PTV, RBV, and MZB, respectively. After 72 h of treatment, the RL assay was performed according to the manufacturer's protocol. The relative luciferase activity is calculated when the RL activity of untreated cells was assigned as 100% (** $P < 0.01$; *** $P < 0.001$). **c** Western blot analysis of HCV proteins. The 1b replicon or 1b/2a chimeric replicon-harboring cells were treated with CsA for 72 h. After treatment, the cell lysates were subjected to Western blot analysis. The production of NS3 and NS5A was analyzed using anti-NS3 and anti-NS5A antibodies, respectively. β-Actin was used as a control for the amount of protein loaded per lane

activity encoded by replicon RNAs in stable cell lines harboring these autonomously-replicating RNAs. The results revealed that the 1b/2a chimeric replicon was less sensitive to CsA than the 1b replicon. However, there were no differences in sensitivity to other anti-HCV reagents (IFN-α, IFN-γ, PTV, RBV, and MZB) between the 1b replicon and 1b/2a chimeric replicon (Fig. 1b). We also tested the expression levels of HCV proteins (NS3 and NS5A) in CsA-treated replicon-harboring cells (Fig. 1c). CsA decreased HCV protein expression levels in the 1b eplicon-harboring cells in a dose-dependent manner. On the other hand, in the 1b/2a chimeric replicon-harboring cells those levels were not changed at the higher concentration of CsA treatment. These results suggest that NS5B of JFH-1 decreased the sensitivity to CsA in 1b/2a chimeric replicon-harboring cells.

CyPA is essential for HCV RNA replication

It has been reported that CyPs are responsible for CsA's anti-HCV activity [17, 20, 21]. Therefore, we next examined the role of CyPs in HCV RNA replication and the anti-HCV activity of CsA using short-hairpin RNA (shRNA) against CyPA or CyPB. The silencing of CyPA or CyPB by shRNA was confirmed by Western blot analysis (Fig. 2a). The silencing of CyPA significantly suppressed HCV protein expression in 1b replicon-harboring cells and in 1b/2a chimeric replicon-harboring cells. The silencing of CyPB didn't suppress HCV protein expression in the former replicon-harboring cells and slightly suppressed in the latter replicon-harboring cells. We also demonstrated that the HCV RNA levels in these cells correlated with the results of Western blot analysis (data not shown). These results suggest that CyPA is essential for replication of both 1b and 1b/2a chimeric replicon. On the other hand, CyPB might partially affect HCV RNA replication of the 1b/2a chimeric replicon.

We next evaluated the sensitivity to anti-HCV reagents (CsA and IFN-α) between control cells and CyPs-knockdown cells. The results revealed that the sensitivity to CsA was drastically enhanced in both CyPA-knockdown 1b replicon-harboring cells and 1b/2a replicon-harboring cells (Fig. 2b), while the sensitivity to IFN-α was not dramatically changed in replicon-harboring either cells (see Supplementary Material). The silencing of CyPB slightly improved the sensitivity to CsA in the 1b/2a chimeric

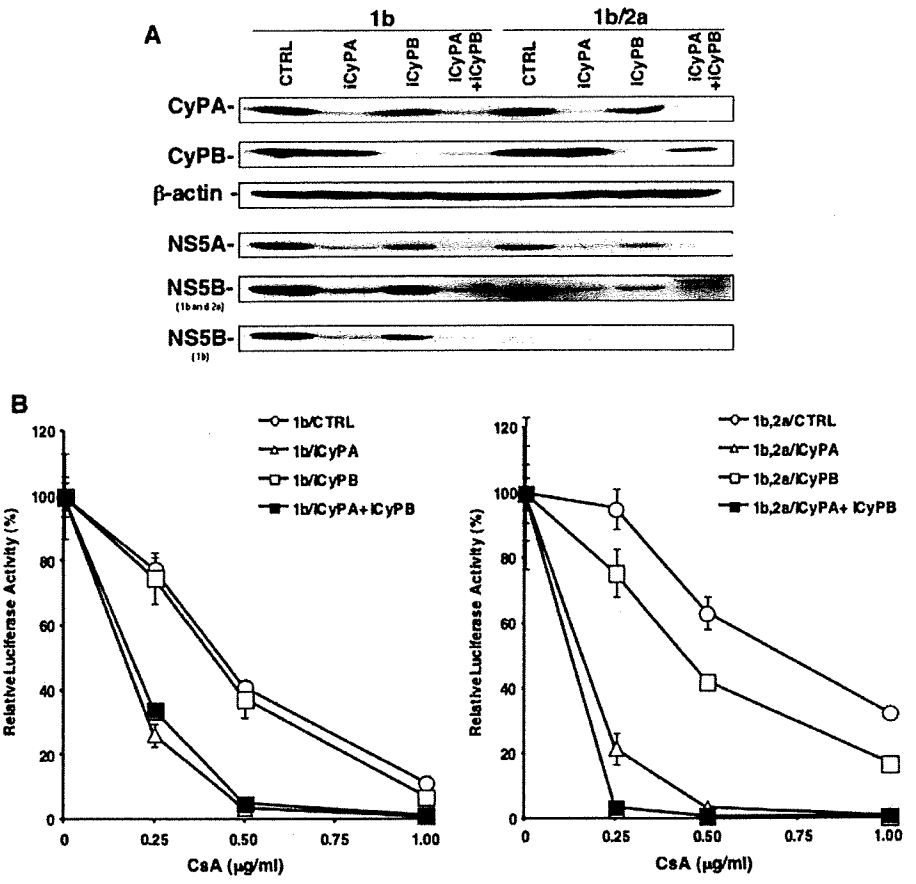

Fig. 2 CyPA is essential for HCV RNA replication and modulates the anti-HCV activity of CsA. **a** Western blot analysis of HCV proteins and CyPs. The 1b replicon and 1b/2a chimeric replicon-harboring cells were transduced with the indicated shRNA for 1 week. The cell lysates were subjected to Western blot analysis. CTRL indicates the control cells transfected with the empty vector. **b** Effects of CyPs on CsA's anti-HCV activity in the 1b replicon- (*left panel*) and 1b/2a chimeric replicon- (*right panel*) harboring cells. After 72 h treatment with CsA, the RL assay was performed. The relative luciferase activity was calculated as described in Fig. 1b. The cells transfected with CTRL, CyPA, CyPB, and both CyPA and CyPB shRNA indicate *open circles*, *open triangles*, *open squares*, and *closed squares*, respectively

replicon-harboring cells but made no improvement in the 1b replicon-harboring cells. Moreover, the silencing of CyPA and CyPB additively enhanced the sensitivity to CsA in the 1b/2a chimeric replicon-harboring cells but not in the 1b replicon-harboring cells. These results suggest that the major cellular determining factor in HCV RNA replication is CyPA rather than CyPB in the 1b/2a chimeric replicon-harboring cells.

HCV NS5B interacts more strongly with CyPB than with CyPA

Although it has been reported that the interaction between CyPs and NS5B is important for HCV RNA replication using glutathione S-transferase pull-down assay [20, 21], the binding activity of NS5B to CyPs in physiological conditions remains unclear. To evaluate the interaction between CyPs and NS5B, we performed an immunoprecipitation assay using 293FT cells transfected with the expression vectors of CyPs (CyPA or CyPB) and NS5B (HCV-O or JFH-1 strain). The obtained results revealed that both NS5Bs interacted more strongly with CyPB than with CyPA. Furthermore, NS5B of HCV-O interacted more strongly with CyPs than did NS5B of JFH-1 (Fig. 3). Since CyPA expression is important for robust HCV RNA replication, these results suggest that the interaction between CyPA and NS5B might not be important for HCV RNA replication or for the anti-HCV activity of CsA.

VE completely negates CsA's anti-HCV activity in the presence or absence of CyPs

We previously reported that VE supplementation negated CsA's anti-HCV activity [22]. To rule out the possibility that VE negates CsA's anti-HCV activity in only CyP-expressing cells, we examined whether or not VE could negate CsA's anti-HCV activity in the presence or absence of CyPs (Fig. 4). Surprisingly, VE negated CsA's anti-HCV activity in the presence or absence of CyPs. It is noteworthy that VE negated this activity more efficiently in CyPA knockdown cells than in the control or CyPB knockdown cells.

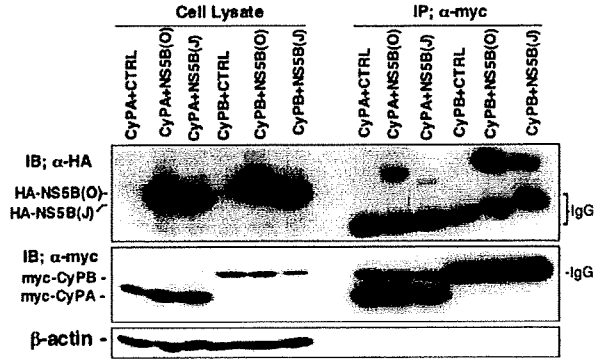

Fig. 3 CyPs interact with HCV NS5B. 293FT cells were cotransfected with plasmids expressing Myc-tagged CyP and HA-tagged HCV NS5B for 48 h. The cells were lysed and subjected to immunoprecipitation with monoclonal Myc-antibody, followed by immunoblot analysis with either anti-HA (*top*) or anti-Myc (*bottom*) antibodies. CTRL indicates empty vector

Discussion

Since it was first reported that CsA possesses anti-HCV activity, several groups have found that CsA suppresses HCV RNA replication using HCV replicon-harboring cells. In addition, the genotype 1a and 1b replicons possess high sensitivity to CsA [17, 19, 21], but the replicon of genotype 2a, JFH-1 strain, is less sensitive to CsA [12]. However, the mechanism of CsA resistance remains unclear.

Recently, Murayama et al. [16] reported that NS3 helicase and NS5B of JFH-1 were essential for robust replication using intragenotypic 2a replicon with J6 backbone. In contrast, Binder et al. [3] demonstrated that NS3 helicase from JFH-1 reduced replication efficiency of 1b/2a chimeric replicon with NS5B from JFH-1 in genotype 1b Con1 backbone. These results suggest that the effect of co-substitution of NS3 helicase with NS5B on HCV RNA replication is different between genotype 1b and 2a backbones.

In this study, we clearly demonstrated that the viral determining factor of sensitivity to CsA is NS5B, by using 1b/2a chimeric replicon-harboring cells. The homology of NS5B region between HCV-O and JFH-1 is 75% in amino acids. Fernandes et al. [5] reported amino acid change from serine to glycine at position 556 of NS5B in CsA resistant 1b replicon. Interestingly, amino acid at this position in HCV-O and JFH-1 are serine and glycine, respectively. The results indicate that the difference in amino acid sequences in NS5B between the HCV-O and JFH-1 strains contributes to the sensitivity to CsA.

Moreover, we further demonstrated that the major cellular determining factor for HCV RNA replication is CyPA rather than CyPB. CyPB is partially involved in only 1b/2a chimeric replicon RNA replication. These results suggest that decreased endogenous expression of CyPA by shRNA contributes to suppression of HCV RNA replication. Furthermore, the knockdown of CyPA enhances CsA's anti-HCV activity. Since the silencing of CyPB slightly enhanced the sensitivity to CsA in only the 1b/2a chimeric replicon-harboring cells, the expression level of CyPB might contribute to the suppression of HCV RNA replication in the case of genotype 2a, JFH-1 strain.

It has been reported that CyPB binds to NS5B and regulates its activity [20]. We also demonstrated that NS5B

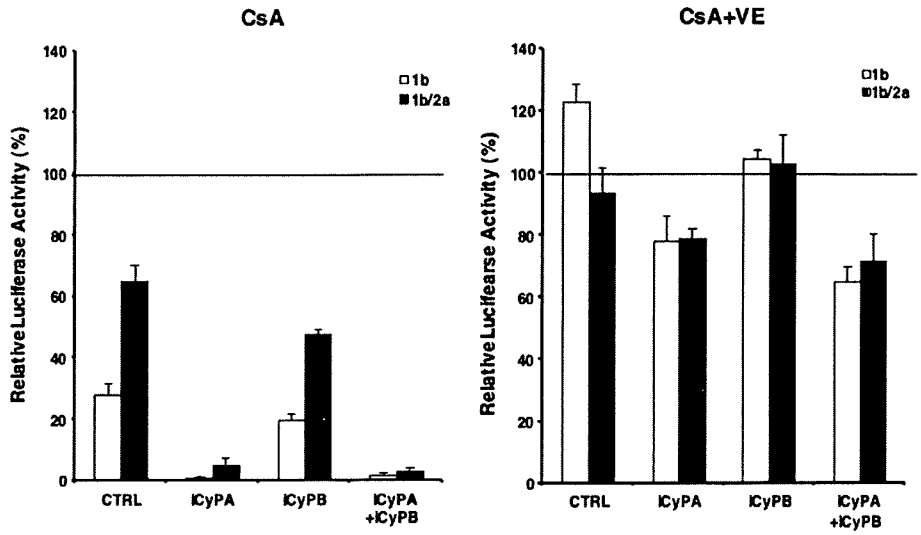

Fig. 4 The effect of CsA (0.5 μg/ml) in combination with VE (10 μM) on HCV RNA replication in the 1b replicon- (*open columns*) and the 1b/2a chimeric replicon- (*closed columns*) harboring cells. After 72 h treatment, the RL assay was performed according to the manufacturer's protocol. The relative luciferase activity was calculated as described in Fig. 1b

bound to CyPB. However, our results revealed that NS5B more strongly interacted with CyPB than with CyPA. The difference in binding activity between CyPA and CyPB may be caused by subcellular localization. It has been reported that CyPA and CyPB are localized in cytoplasm and endoplasmic reticulum (ER), respectively [7]. On the other hand, NS5B localizes with ER membranes [13]. Our data, showing that NS5B interacted more strongly with CyPB than with CyPA, might be attributable to the difference in subcellular localization between cytoplasm and ER. We also demonstrated that NS5B of HCV-O interacted more strongly with CyPs than did NS5B of JFH-1. Moreover, the expression of CyPA plays a major role in robust HCV RNA replication. On the other hand, CyPB has little impact on HCV RNA replication. Taken together, these results suggest that the interaction between CyPA and NS5B might partially affect HCV RNA replication and the anti-HCV activity of CsA.

It is noteworthy that VE can negate CsA's anti-HCV activity in the presence or absence of CyPs. We also examined whether or not the combination treatment of CsA and other antioxidants (vitamin C, sodium selenate, and coenzyme Q10) could negate CsA's anti-HCV activity. Among these antioxidants, only VE negated CsA's anti-HCV activity (data not shown). Understanding VE's involvement in CsA's anti-HCV activity may help us identify factors other than the interaction between CyPA and NS5B.

CsA derivatives that affect only CyPA and that also lack immunosuppressive function will have advantages over CsA. A combination therapy of CsA or CsA derivatives with VE should be avoided so as not to affect CsA's anti-HCV activity clinically. In conclusion, we have demonstrated that NS5B of JFH-1 contributed to the CsA-resistant phenotype of this strain using 1b/2a chimeric replicon-harboring cells and CyPA is a major cellular determining factor in HCV RNA replication.

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Case Report

Early emergence of entecavir-resistant hepatitis B virus in a patient with hepatitis B virus/human immunodeficiency virus coinfection

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The efficacy of entecavir for patients with hepatitis B virus/human immunodeficiency virus coinfection has not been fully elucidated. Here we examined a patient coinfected with both viruses in whom entecavir-resistant hepatitis B virus appeared. The 60-year-old Japanese male with the coinfection received antiretroviral therapy including lamivudine. The therapy initially suppressed replication of both viruses, followed by reactivation of the hepatitis B virus alone by 2 years of therapy. He subsequently received entecavir therapy in addition to the antiretroviral regimen. After entecavir administration, the hepatitis B virus DNA level was slightly reduced, but then increased after 6 months of entecavir therapy. In the sequencing analysis of hepatitis B virus, no drug resistance-associated amino acid substitutions were observed in the reverse transcriptase (rt) domain before antiretroviral therapy. The lamivudine-resistant amino acid substitutions at rt173, rt180 and rt204 were detected before entecavir administration, and further the entecavir-resistant rt202 substitution was observed after 6 months of entecavir therapy. The full-length hepatitis B sequences showed that the viral strain derived from the patient belonged to genotype H. In summary, this report describes a patient with hepatitis B virus/human immunodeficiency virus coinfection who received entecavir therapy in addition to an antiretroviral regimen and showed the early emergence of entecavir-resistance hepatitis B virus. In entecavir therapy for patients infected with both viruses, great care should be taken with respect to the emergence of entecavir-resistant hepatitis B virus, especially in patients with pre-existing lamivudine-resistant virus.

Key words: coinfection, drug-resistant hepatitis B virus, entecavir, hepatitis B virus, human immunodeficiency virus, lamivudine

INTRODUCTION

CHRONIC CARRIERS OF hepatitis B virus (HBV) number more than 350 million worldwide.[1] Chronic HBV infection is seen in approximately 10% of human immunodeficiency virus (HIV)-infected patients,[2] and coinfection with HBV and HIV is a serious health problem due to the shared mode of transmission. Since the prognosis of HIV-infected patients can be dramatically improved by highly active antiretroviral therapy (HAART), one of the major causes of mortality in HIV-infected patients is chronic liver disease due to HBV infection.[3]

Lamivudine (LAM, also abbreviated to 3TC), one of the antiretroviral drugs, has also been used for the reduction of HBV replication and improvement of HBV-related liver diseases.[4,5] However, the anti-HBV effect of LAM is hampered by the emergence of LAM-resistant mutant virus in cases of HBV monoinfection and HBV/

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HIV coinfection.[6,7] The LAM-resistant HBV strain is based on point mutation occurring within the reverse transcriptase (rt) domain of the polymerase gene. A methionine-to-valine/isoleucine amino acid substitution at rt204 (rtM204V/I) is known to confer LAM resistance.[8,9] A leucine-to-methionine substitution at rt180 (rtL180M) and a valine-to-leucine substitution at rt173 (rtV173L) have also been shown to appear in association with LAM resistance.[8,10,11] The emergence rate of LAM-resistant virus in patients coinfected with HBV and HIV has been reported to be approximately 50% after 2 years of therapy.[9]

Recently, entecavir (ETV) has been reported to be superior to LAM for the suppression of viral replication and disease activity in patients with HBV monoinfection who had not received previous treatment with other anti-HBV drugs (naïve patients).[12,13] ETV has also been shown to be effective in HBV-infected patients who had been treated with LAM and showed LAM resistance.[14] It has been demonstrated that ETV resistance occurs based with amino acid substitution(s) at rt184, rt202 and/or rt250, together with the LAM-resistant rtM204V/I and rtL180M substitutions.[15] The emergence rate of ETV-resistant virus after 3 years of therapy has been reported to be less than 1% in naïve patients and 15% in LAM-resistant patients with chronic HBV monoinfection.[16] However, the anti-HBV efficacy of ETV for HBV/HIV coinfection has not been fully clarified.

In this study, we examined a patient with concomitant HBV/HIV infection who underwent HAART including LAM, and showed the appearance of LAM-resistant HBV. Subsequent ETV administration did not lead to an adequate reduction of the HBV replicative level, followed by the early emergence of the ETV-resistant virus. We investigated the serial change in the drug resistance-associated mutation status within the rt domain of the HBV polymerase gene, as well as full-length nucleotide sequences of the ETV-resistant HBV strain derived from the patient.

CASE REPORT

Patient and serum sampling

A 60-YEAR-OLD JAPANESE heterosexual male first visited to the National Hospital Organization Osaka National Hospital in December 2001 due to a positive result from an HIV antibody (anti-HIV) test in voluntary HIV screening. From his anamnestic record, he had been admitted with type B acute hepatitis to another hospital 3 years earlier. Anti-HIV had been negative at that time. On his first visit, the anti-HIV positivity was confirmed by Western blot analysis. Antibodies to HIV-1 proteins, gp160, gp110/120, p68, p52, gp41, p40 and p34 were positive. As for antibodies to HIV-2 proteins, only an antibody to p68 was positive. According to these, he was judged to be infected with HIV-1. The HIV-RNA level was $10^{4.3}$ copies/mL, and the CD4+ T cell counts were 275/mm^3 (normal range, >300/mm^3). He tested positive for hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg), and negative for antibody to HBsAg (anti-HBs) and antibody to HBeAg (anti-HBe). The HBV-DNA level was $>10^{7.6}$ copies/mL, and the alanine aminotransferase (ALT) level was 106 IU/L. The patient was free of HIV-related symptoms and had no opportunistic infectious diseases. HAART with LAM (300 mg/day), zidovudine (AZT) (600 mg/day) and efavirenz (EFV) (600 mg/day) was started in April 2002. AZT and EFV were then substituted for sanilvudine (d4T) (60 mg/day) and avacavir (ABC) (600 mg/day) in July 2002 because of anemia and dizziness. By July 2002, HIV-RNA decreased to below the detection limit ($<10^{1.7}$ copies/mL), whereas the CD4+ T cell counts tended to rise up to >500/mm^3. In August 2006, fosamprenavir (FPV) (2400 mg/day) was commenced in place of d4T due to peripheral nerve palsy. Suppression of HIV-RNA below the detection limit continued at the end of follow-up, irrespective of repeated alterations in the therapeutic regimen of HAART. As for HBV status, HBV-DNA declined to $10^{3.9}$ copies/mL in April 2003 but increased again to $>10^{7.6}$ copies/mL in May 2005. To control HBV replication, ETV (0.5 mg/day) was added in October 2006. After the ETV administration, HBV-DNA slightly decreased from $>10^{7.6}$ to $10^{6.2}$ copies/mL in January 2007 but rose to $10^{7.2}$ copies/mL 3 months later. ALT remained abnormal and HBeAg continued to be positive throughout the follow-up period. The clinical course of the patient is summarized in Figure 1a.

For the nucleotide sequencing of HBV-DNA, the serum samples were obtained in December 2001 (before HAART), August 2006 (before ETV administration), and April 2007 (after 6 months of ETV therapy). These serum sampling points were designated as P1, P2 and P3 (see Fig. 1a). Serum samples were stored at −80°C until use. Informed consent was obtained from the patient.

Virus markers and nucleotide sequencing

HBsAg, anti-HBs, HBeAg, anti-HBe and anti-HIV were tested by chemiluminescent immunoassay. A

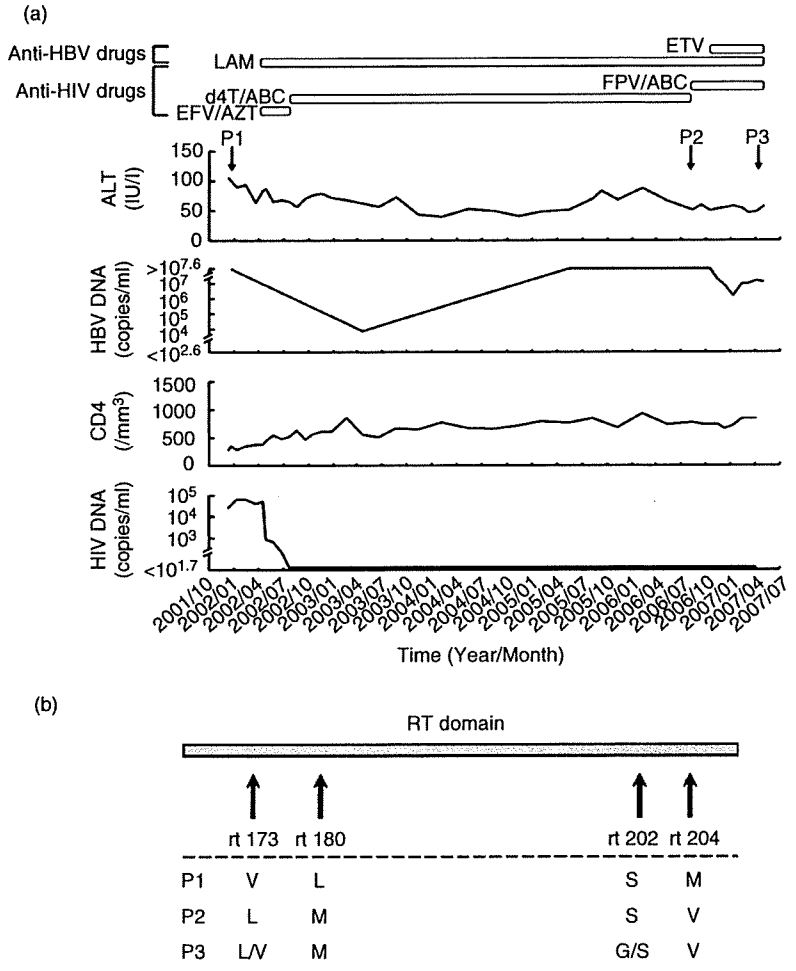

Figure 1 (a) Patient clinical course and serum sampling points. P1, P2 and P3 are the points at which serum samples were obtained. P1 was taken in December 2001 (before HAART), P2 in August 2006 (before ETV administration) and P3 in April 2007 (after 6 months of ETV therapy). ABC, avacavir; ALT, alanine aminotransferase; AZT, zidovudine; d4T, sanilvudine; EFV, efavirenz; ETV, entecavir; FPV, fosamprenavir; HBV, hepatitis B virus; HIV, human immunodeficiency virus; LAM, lamivudine. (b) Serial change in the status of drug resistance-associated amino acid substitutions.

confirmatory anti-HIV-1/2 testing was carried out by Western blot analysis. Serum HBV-DNA was detected by means of a PCR assay (Amplicor HB monitor; Roche Diagnostics, Basel, Switzerland) with a lower detection limit of $10^{2.6}$ (=400) copies/mL. Plasma HIV-RNA was quantified by a PCR assay (Amplicor HIV-1 monitor; Roche) whose lower detection limit was $10^{1.7}$ (=50) copies/mL.

The nucleotide sequences of HBV-DNA were determined by a method based on nested PCR and direct sequencing, as described elsewhere.[17] In this study, primers BF5-2 (5′-TCC TCA GGC CAT GCA GTG GA-3′, nt 3201-20) and BR8 (5′-TTG CGT CAG CAA ACA CTT GG-3′, nt 1195-76) were also used. Nucleotide sequences of the entire rt domain in the polymerase gene were examined in HBV strains derived from the P1 and P2 serum samples (GenBank accession nos. AB353765 and AB353766), whereas the full-length HBV-DNA was determined in the strain derived from the P3 serum sample (GenBank accession no. AB353764). The full-length HBV strain obtained in this study (designated as HBDI03), the seven representative HBV strains of genotypes A–G and the eight previously isolated HBV strains of genotype H were aligned, and the phylogenetic tree was constructed. These analyses were done at the homepage of the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp).

Results of sequencing analysis of HBV

The serial change in the nucleotide sequences in the rt domain of the HBV polymerase gene was first examined

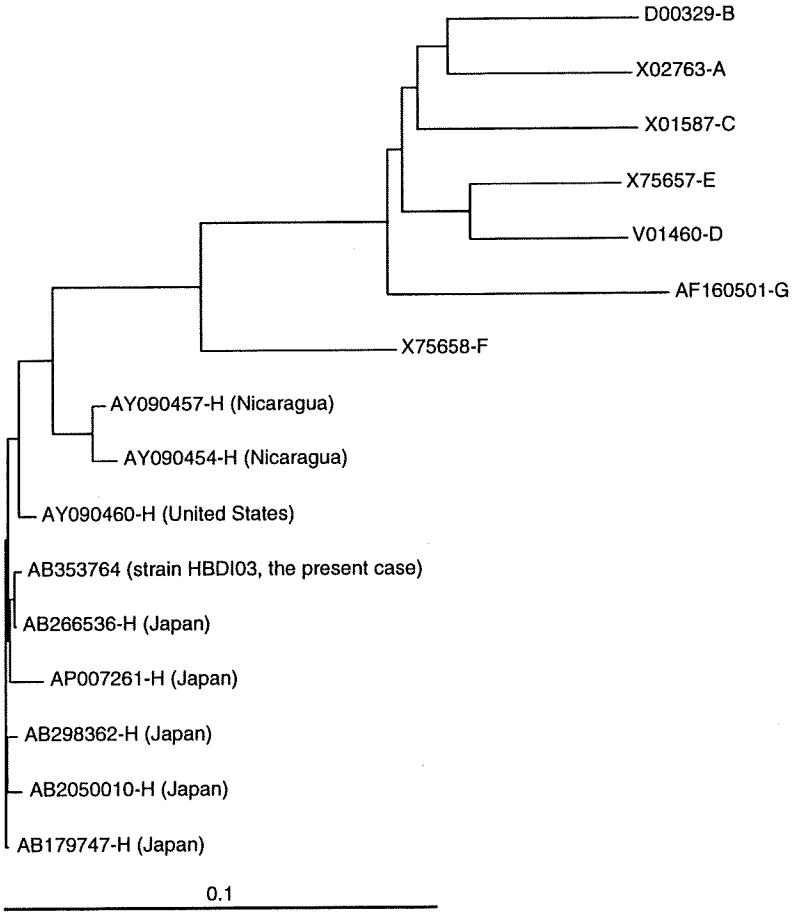

Figure 2 Phylogenetic tree analysis including the HBV strain HBDI03 obtained in this study, the seven representative HBV strains of genotypes A–G, and the eight previously isolated HBV strains of genotype H.

using serum samples obtained at P1–P3 (Fig. 1b). At point P1, no drug resistance-associated mutations were found in the rt domain, but three LAM resistance-associated substitutions, rtM204V, rtL180M and rtV173L, emerged at point P2. A serine-to-glycine substitution at rt202 (rtS202G), which has been shown to be one of the ETV resistance-associated substitutions,[15] was further observed at point P3, although rtS202G and rtV173L substitutions occurred incompletely. No other amino acid substitutions were seen in the rt domain of the HBV polymerase gene from point P1 to P3. Thus, in the patient with HBV/HIV coinfection, the emergence of the drug resistance-associated amino acid substitutions revealed a close relationship with the poor anti-HBV efficacy of LAM and ETV.

Next, the full-length nucleotide sequences of HBV were determined from the P3 serum sample of the patient with HBV/HIV coinfection showing ETV resistance. The full-length HBV strain HBDI03 comprised a total of 3215 nucleotide lengths. The phylogenetic tree was depicted using the HBV strain HBDI03, the seven representative HBV strains of genotypes A–G and the eight previously identified genotype H HBV strains. As shown in Figure 2, the HBV strain HBDI03 obtained in this study was classified as genotype H. When the nucleotide sequences of the strain HBDI03 were compared with the eight reported genotype H HBV strains, the strain HBDI03 showed a 97.2–99.8% identity with these strains. The unique amino acid substitutions in the strain HBDI03 were further investigated in comparison with these eight genotype H HBV strains. As shown in Table 1, four drug resistance-associated substitutions within the rt domain were observed, as described above. The two amino acid substitutions in the S gene were also caused by the same mutations of the drug resistance-associated rtV173L and rtM204V

Table 1 The unique amino acid substitutions in strain HBDI03 in comparison with eight previously isolated genotype H hepatitis B virus strains

Amino acid position	Consensus residue of genotype H	Residue unique to strain HBDI03
Polymerase		
519 (rt173)	V	L/V
526 (rt180)	L	M
548 (rt202)	S	G/S
550 (rt204)	M	V
Surface		
164	E	D/E
195	I	M
X		
32	W	G

Consensus residues of genotype H were from the eight reported hepatitis B virus (HBV) strains (GenBank accession nos. AY090454, AY090457, AY090460, AP007261, AB179747, AB205010, AB266536 and AB298362).

changes. As for the remaining one amino acid substitution in the X gene, the substituted glycine residue observed in the HBDI03 strain was a common one in the representative HBV strains of genotypes A–G at the corresponding codon position. Taken together, the HBDI03 strain did not appear to have any distinctive features other than the presence of the drug-associated amino acid substitutions.

DISCUSSION

RECENTLY, ETV HAS been widely accepted as an effective drug for the treatment of HBV monoinfection because of its stronger inhibitory effect on HBV replication and lower emergence rate of drug-resistant mutant virus compared to LAM.[12–14] ETV-resistant HBV has been demonstrated to be established by amino acid substitution(s) at rt184, rt202 and/or rt250, in addition to the LAM-resistant rtM204V/I and rtL180M substitutions.[15] The emergence rate of ETV-resistant virus has been reported to be higher in LAM-resistant patients than in naïve patients.[16] There has so far been little evidence concerning the anti-HBV efficacy of ETV for patients with HBV/HIV coinfection. In particular, LAM-resistant HBV has been shown to emerge frequently in patients with HBV/HIV coinfection who received LAM therapy as a component of HAART.[7] The therapeutic efficacy of ETV on LAM-resistant HBV should be assessed in patients with HBV/HIV coinfection. In this study, we examined a patient with HBV/HIV coinfection who had LAM-resistant HBV induced by HAART including LAM, and underwent subsequent ETV therapy. The patient showed a rather weak suppressive effect of ETV on HBV replication, followed by the emergence of ETV-resistant HBV in the early phase of therapy.

In the sequence analysis of the HBV genome, no drug-resistant HBV mutations were detected before HAART, but continuous LAM administration induced the LAM-resistant mutant HBV with rtM204V, rtL180M and rtV173L amino acid substitutions. Subsequent ETV therapy resulted in the emergence of an ETV-resistant virus possessing the rtS202G substitution in addition to the three LAM resistance-associated substitutions after no more than 6 months of ETV therapy, although the rtS202G and rtV173L substitutions were incomplete. In LAM-resistant patients with HBV monoinfection, the emergence rate of the ETV-resistant mutation has been reported to be merely 15% after 3 years of therapy.[16] In comparison with this, ETV-resistant HBV appeared in an extremely early phase of therapy in our patient with HBV/HIV coinfection. According to this, ETV resistance is speculated to be established earlier in patients with HBV/HIV coinfection than in those with HBV monoinfection, although concomitant HIV infection has not thus far been suggested to result in a higher incidence of the drug-resistant HBV strain in the treatment with other anti-HBV drugs in chronic HBV infection. The latent immune deficiency caused by HIV infection might prevent HBV eradication through a host immune response, resulting in poor anti-HBV efficacy of ETV. Alternatively, simultaneous usage of multiple antiretroviral drugs might in some way contribute to the emergence of ETV-resistant HBV.

Very recently, it has been shown that ETV possesses modest anti-HIV activity both *in vitro* and *in vivo* and can induce the drug-resistant mutant HIV strain in patients with HBV/HIV coinfection.[18] This suggests that ETV may not be appropriate for the treatment of patients with HBV/HIV coinfection in whom HAART is not needed. On the other hand, ETV is considered to be beneficial for patients with HBV/HIV coinfection undergoing a stable continuation of HAART. In particular, the therapeutic efficacy of ETV may be more promising in patients without LAM-resistant HBV than in those with it. Although the present case of the patient under discussion, who already displayed LAM-resistant HBV due to the preceding HAART, did not support the usefulness of ETV therapy because of the early emergence of ETV-resistant HBV, further studies with a large number of