CE-TOFMS. The values of each measurement are shown at left. The right graph shows means with SD of the data at left. Open bar; Huh-7 cells, gray bar; JFH-1/4-5 cells.
(TIF)

Figure S2 Cytoplasmic ATP levels in HCV replicon cells and IFN-treated cells. (Left) The HCV replicon cells JFH-1/4-1, JFH-1/4-5 (genotype 2a) and NK5.1/0-9 (genotype 1b), and parental Huh-7 cells were cultured for 72 h in the absence or presence of 1,000 IU/ml IFN-alpha. Forty-eight hours after transfection with AT1.03, the Venus/CFP emission ratio of each cell was calculated from fluorescent images acquired with the confocal microscope FV1000. All data are presented as means and SD for at least 10 independent cells. (Right) HCV RNA titers in cells corresponding to the left panel were determined using real-time quantitative RT-PCR. Data are presented as means and SD for three independent samples. NTD indicates not detected.
(TIF)

Figure S3 Increase in ATP-enriched dot-like structures in cells replicating SGR-ATeam. Huh-7 cells were transfected with SGR-AT1.03, and analyzed in the same way as described in the legends for Figures 5A and 5B. The lower four panels are five-fold magnifications of the boxed areas in independent cells. Scale bars, 40 μm.
(TIF)

Figure S4 Visualization of the ATP level in cells expressing replication-defective HCV polyprotein. (A) A schematic representation of the NS3-NS5B-AT1.03 plasmid is shown. The HCV polyprotein is indicated by the open boxes. The ATeam gene was inserted into the same site as that for NS5A-ATeam and SGR-ATeam insertion as indicated in the legend for Figure 4A. CAG, CAG promoter. (B) Cells transfected with constructs encoding NS5A, NS5A-AT1.03, NS3-NS5B-AT1.03, SGR or SGR-AT1.03 were analyzed by immunoblotting with anti-NS5A, anti-NS5B or anti-beta-actin antibodies. (C) Huh-7 cells were transfected with NS3-NS5B-AT1.03, and analyzed in the same way as described in the legends for Figures 5A and 5B. The upper panel (Fluorescence) demonstrates signal intensity from a spectral channel with maximum intensity and represents the expression pattern of NS5A-ATeam processed from NS3-NS5B-AT1.03. The lower panels (Venus/CFP ratio) indicate the FRET ratio and a five-fold magnification of the boxed area. Scale bar, 20 μm.
(TIF)

Figure S5 Relationship between ATP-enriched dot-like structures and mitochondria. Huh-7 cells replicating SGR-AT1.03 (right panels) and parental cells (left panel) were analyzed. Active mitochondria were labeled with MitoTracker Red CMXRos in living cells, and were analyzed in the same way as described in the legends for Figures 5A and 5B, using a reference for the MitoTracker spectrum. The lowest panels of SGR-ATeam cells indicate five-fold magnifications of the boxed areas. Scale bars, 20 μm.
(TIF)

Figure S6 Inhibitory effect of PSI-6130 on HCV RNA replication. (A) Replication levels of SGR/luc-AT1.03 RNA in transfected cells were determined by luciferase assay 3 days after treatment with PSI-6130 at the indicated concentrations (μM). The values shown were normalized for transfection efficiency with luciferase activity determined 24 h post-transfection. All data are presented as means and SD for three independent samples. (B) Cell viability was assessed using the MTT assay.
(TIF)

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Author Contributions

Conceived and designed the experiments: T. Ando, H. Imamura, T. Wakita, T. Suzuki. Performed the experiments: T. Ando, H. Aizaki. Analyzed the data: T. Ando, H. Imamura, T. Watanabe, T. Wakita, T. Suzuki. Contributed reagents/materials/analysis tools: H. Imamura, R. Suzuki, H. Aizaki. Wrote the paper: T. Ando, T. Suzuki.

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Trans-complemented hepatitis C virus particles as a versatile tool for study of virus assembly and infection

Ryosuke Suzuki [a,*], Kenji Saito [a], Takanobu Kato [a], Masayuki Shirakura [b], Daisuke Akazawa [a], Koji Ishii [a], Hideki Aizaki [a], Yumi Kanegae [c], Yoshiharu Matsuura [d], Izumu Saito [c], Takaji Wakita [a], Tetsuro Suzuki [e,**]

[a] Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
[b] Influenza Virus Research Center, National Institute of Infectious Diseases, Tokyo 208-0011, Japan
[c] Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan
[d] Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan
[e] Department of Infectious Diseases, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu, Shizuoka 431-3192, Japan

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ABSTRACT

In this study, we compared the entry processes of *trans*-complemented hepatitis C virus particles (HCVtcp), cell culture-produced HCV (HCVcc) and HCV pseudoparticles (HCVpp). Anti-CD81 antibody reduced the entry of HCVtcp and HCVcc to almost background levels, and that of HCVpp by approximately 50%. Apolipoprotein E-dependent infection was observed with HCVtcp and HCVcc, but not with HCVpp, suggesting that the HCVtcp system is more relevant as a model of HCV infection than HCVpp. We improved the productivity of HCVtcp by introducing adapted mutations and by deleting sequences not required for replication from the subgenomic replicon construct. Furthermore, blind passage of the HCVtcp in packaging cells resulted in a novel mutation in the NS3 region, N1586D, which contributed to assembly of infectious virus. These results demonstrate that our plasmid-based system for efficient production of HCVtcp is beneficial for studying HCV life cycles, particularly in viral assembly and infection.

Introduction

Over 170 million people worldwide are chronically infected with hepatitis C virus (HCV), and are at risk of developing chronic liver diseases (Hoofnagle, 2002). HCV is an enveloped virus of the family *Flaviviridae*, and its genome is a positive-strand RNA consisting of the 5′-untranslated region (UTR), an open reading frame encoding viral proteins (core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) and the 3′-UTR (Suzuki et al., 2007).

Host–virus interactions are required during the initial steps of viral infection. It was previously reported that CD81 (Bartosch et al., 2003a, b; McKeating et al., 2004; Pileri et al., 1998), scavenger receptor class B type I (Bartosch et al., 2003a, b; Scarselli et al., 2002), claudin-1 (Evans et al., 2007; Liu et al., 2009) and occludin (Benedicto et al., 2009; Evans et al., 2007; Liu et al., 2009; Ploss et al., 2009) are critical molecules for HCV entry into cells. CD81 interacts with HCV E2 via a second extracellular loop (Bartosch et al., 2003a, b; Hsu et al., 2003) and its role in the internalization process was confirmed (Cormier et al., 2004; Flint et al., 2006). It has also been shown that infectious HCV particles produced in cell cultures (HCVcc) exist as apolipoprotein E (ApoE)-enriched lipoprotein particles (Chang et al., 2007) and that ApoE is important for HCV infectivity (Owen et al., 2009).

Investigation of HCV had been hampered by difficulties in amplifying the virus in vitro before development of robust cell culture systems based on JFH-1 isolates (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). Retrovirus-based HCV pseudoparticles (HCVpp), in which cell entry is dependent on HCV glycoproteins, have been used to study virus entry (Bartosch et al., 2003a; Hsu et al., 2003). Vesicular stomatitis virus (VSV)-based pseudotypic viruses bearing HCV E1 and E2 and replication-competent recombinant VSV encoding HCV envelopes have also been available as surrogate models for studies of HCV infection (Mazumdar et al., 2011; Tani et al., 2007).

It was recently shown that HCV subgenomic replicons can be packaged when structural proteins are supplied in *trans* (Adair et al., 2009; Ishii et al., 2008; Masaki et al., 2010; Steinmann et al., 2008). These *trans*-complemented HCV particles (HCVtcp) are infectious, but support only single-round infection and are unable to spread. Establishment of flexible systems to efficiently produce HCVtcp should contribute to studying HCV assembly, in particular encapsidation of the viral genome, and entry to cells with less stringent biosafety and biosecurity measures. Although single-round infection can be achieved by using the HCVcc system with receptor knock-out

* Corresponding author. Fax: +81 3 5285 1161.
** Corresponding author. Fax: +81 53 435 2338.
E-mail addresses: ryosuke@nih.go.jp (R. Suzuki), tesuzuki@hama-med.ac.jp (T. Suzuki).

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cells, the single-round HCVcc system is not suitable for studying virus entry. We previously described plasmid-based production of HCVcc and HCVtcp (Masaki et al., 2010). Here, we demonstrated that HCVtcp production can be enhanced by introducing the previously reported cell-culture adaptive mutations and by deleting sequences not essential for replication in the subgenomic replicon construct. By providing genotype 1b-derived core-to-p7 in addition to intragenotypic viral proteins, chimeric HCVtcp were generated. Furthermore, blind passage of HCVtcp in the packaging cells resulted in the identification of a novel cell culture-adaptive mutation in NS3 that enables us to establish the efficient production of HCVtcp with structural proteins from various strains. Taken together, our system for producing single-cycle infectious HCV particles should be useful in the study of entry and assembly steps of the HCV life cycles. This technology may also have potential to be the basis for the safer vaccine development.

Results

Enhancement of HCVtcp production by adaptive mutations in E2, p7 and NS2 and by deleting sequences not essential for replication from replicon construct

In our HCVtcp system, the RNA polymerase I (Pol I)-driven replicon plasmid, which carries a dicistronic subgenomic luciferase reporter replicon of JFH-1 strain with a Pol I promoter and terminator (pHH/SGR-Luc), as well as a plasmid containing core-NS2 cDNA under the CAG promoter (pCAGC-NS2) were used (Masaki et al., 2010). In an effort to improve the yield of HCVtcp production, cell culture-adaptive mutations in E2 (N417S), p7 (N765D) and NS2 (Q1012R) which were previously selected from serial passage of HCVcc (Russell et al., 2008) were introduced into the core-NS2 expression plasmid (Fig. 1A) (residues are numbered

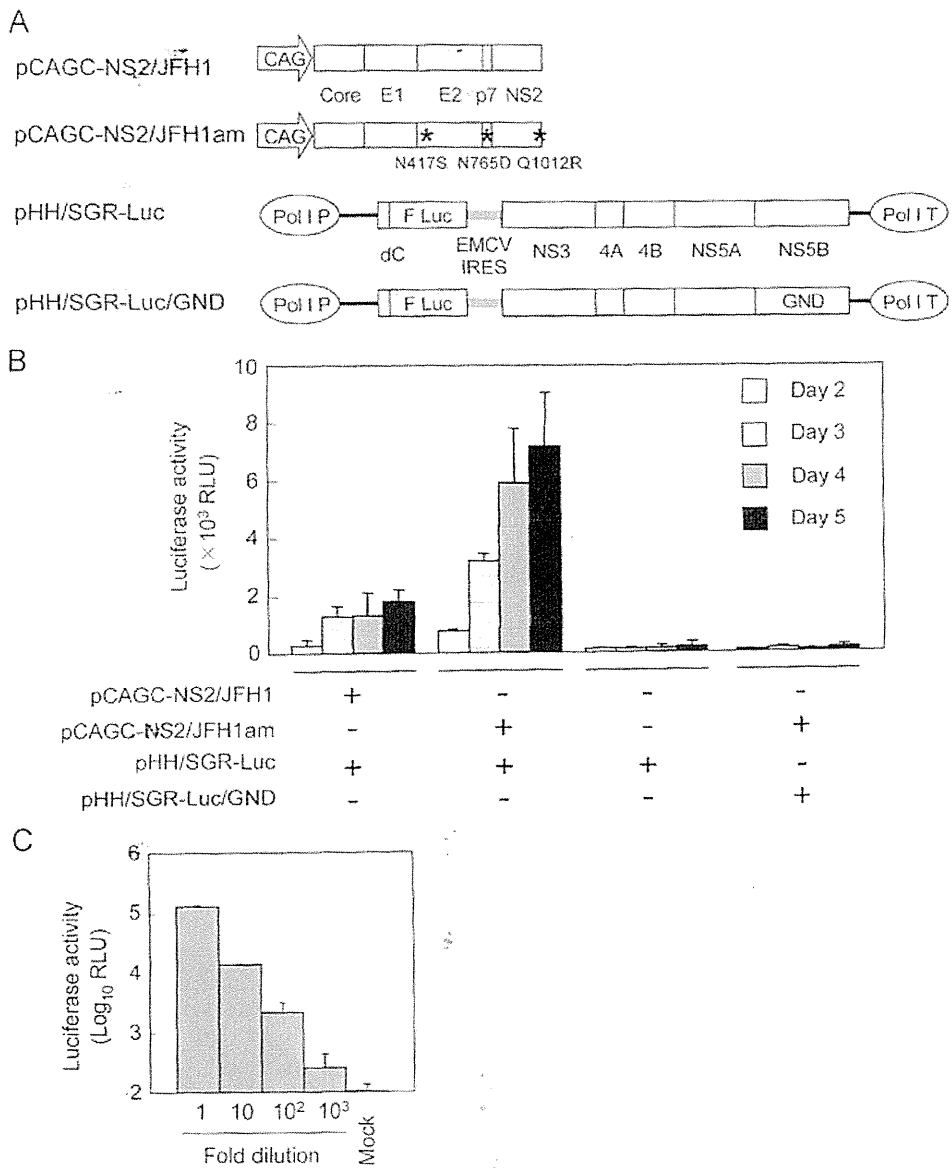

Fig. 1. HCVtcp production by two-plasmid transfection. (A) Schematic representation of plasmids is shown. HCV polyproteins derived from JFH-1 are indicated by white boxes. HCV UTRs are indicated by bold lines. The internal ribosomal entry site from encephalomyocarditis virus (EMCV IRES) is denoted as gray lines. Adaptive mutations are indicated as asterisks. F Luc: firefly luciferase gene; CAG: CAG promoter; Pol I P: RNA polymerase I promoter; Pol I T: RNA polymerase I terminator; GND: replication-deficient GND mutation. (B) Luciferase activity in Huh7.5.1 cells inoculated with supernatant from cells transfected with indicated plasmids at the indicated time points. Data are averages of triplicate values with error bars showing standard deviations. (C) Luciferase activity in cells inoculated with serially diluted HCVtcp.

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according to positions within the JFH-1 polyprotein). Supernatants of cells transfected with plasmids (Fig. 1A) were collected and were used to infect Huh7.5.1 cells, which were analyzed by luciferase assay. Introduction of adaptive mutations (pCAGC-NS2/JFH1am) resulted in more than 4-fold higher production of HCVtcp at 5 day post-transfection, as compared to wild-type (WT) (pCAGC-NS2/JFH1) (Fig. 1B), indicating that the adaptive mutations contribute to enhancing HCVtcp production. To confirm that luciferase activity levels in HCVtcp-infected cells are correlated with the number of infectious particles, Huh7.5.1 cells were inoculated with serial dilutions of HCVtcp. Luciferase activity was well correlated with viral load (Fig. 1C), indicating that luciferase assay in HCVtcp-infected cells can be used to quantify HCV infection.

In order to further explore the efficient production of HCVtcp, we generated replicon constructs that lack the luciferase gene or include the partial coding sequences for structural proteins instead of reporter (Fig. 2A). Replication of each replicon in plasmid-transfected cells was then assessed by Western blotting (Fig. 2B). Among the constructs tested, NS5B levels were lowest in cells expressing pHH/SGR-Luc. NS5B levels in cells replicating other replicons appeared to be comparable. Cells were infected with supernatants of cells transfected with each replicon plasmid, along with pCAGC-NS2/JFH1am, followed by infectious unit assay (Fig. 2C). The highest production of HCVtcp was obtained from cells transfected with pHH/SGR, where the luciferase sequence was deleted from pHH/SGR-Luc, thus suggesting that deletion of the sequence not essential for RNA replication in the replicon may contribute to enhancing HCVtcp production.

Production of chimeric HCVtcp by providing heterologous core-p7

In order to elucidate whether *trans*-encapsidation of JFH-1 replicon can be achieved by providing core-p7 from other HCV strains, core-NS2 plasmids were constructed (Fig. 3A). In these plasmids, core through the N-terminal 33 aa of NS2, which contains transmembrane domain 1 of NS2, was derived from either H77c (genotype 1a), THpa (genotype 1b), Con1 (genotype 1b) or J6 (genotype 2a) strain. Residual NS2 was derived from JFH-1, as described previously (Pietschmann et al., 2006). HCVtcp was efficiently produced by core-p7 of J6 and THpa strains, but its production was less efficient in the case of Con1 strain. *Trans*-packaging was not detectable when core-p7 of H77c strain was used (Fig. 3C). Among HCV strains tested, difference in luciferase activity levels in HCVtcp-infected cells (Fig. 3C) were in agreement with that in the viral RNA levels in the culture supernatants of the transfected cells (Fig. 3B). Although the efficacy of *trans*-complementation was variable among strains, chimeric HCVtcp can be generated by providing genotype 1b-derived core-p7 in addition to intragenotypic viral proteins, and was used in subsequent studies.

ApoE- and CD81-dependent infection by HCVtcp

There is accumulating evidence that apolipoproteins, particularly ApoE, contribute to HCV production and infectivity (Chang et al., 2007; Owen et al., 2009). To determine whether ApoE is involved in infection of target cells by HCVtcp, we infected cells in the presence of increasing concentrations of anti-ApoE antibody.

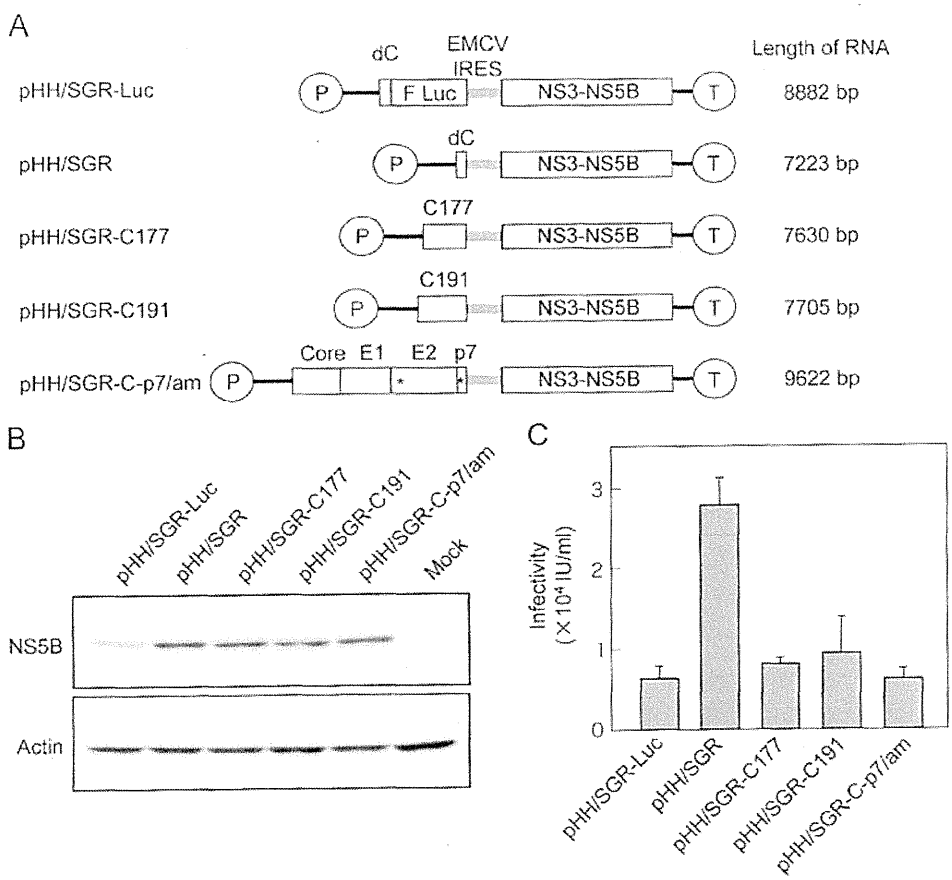

Fig. 2. Production of HCVtcp with different replicon constructs. (A) Schematic representation of plasmids used for production of HCVtcp. Deduced length of transcribed RNA from each construct is shown on the right. HCV polyproteins from JFH-1 strain are indicated by open boxes. HCV UTRs are indicated by bold lines. The EMCV IRES is denoted by gray bars. Adaptive mutations are indicated by asterisks. F Luc: firefly luciferase gene; P: RNA polymerase I promoter; T: RNA polymerase I terminator. (B) Detection of NS5B and actin in Huh7.5.1 cells transfected with indicated plasmids at 4 day post-transfection. (C) Infectivity of culture supernatants from cells transfected with indicated replicon plasmids along with pCAGC-NS2/JFH1am at 4 day post-transfection.

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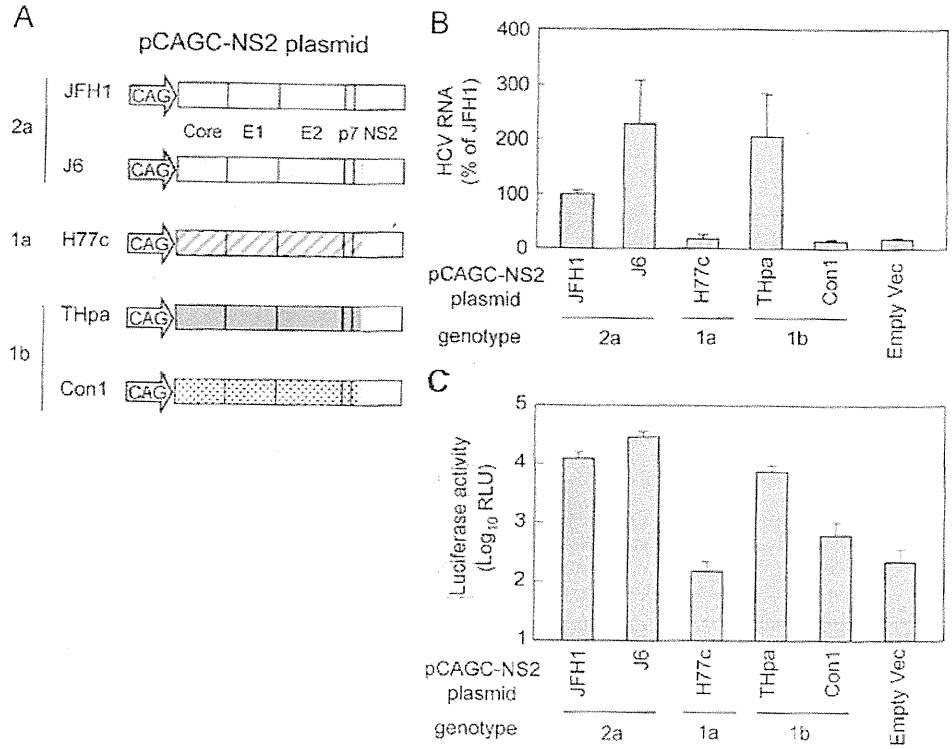

Fig. 3. HCVtcp production with structural proteins from various strains. (A) Schematic representation of plasmids used. HCV polyproteins of JFH-1, J6, H77c, THpa and Con1 strain are shown in the open box, bright gray box, box with diagonal lines, dark gray box and dotted box, respectively. (B) Relative levels of HCV RNA in the supernatant from cells transfected with indicated plasmids along with pHH/SGR-Luc. (C) Luciferase activity in cells inoculated with supernatant from cells transfected with indicated plasmids along with pHH/SGR-Luc at 4 day post-transfection.

pCAGC-NS2/THpa and pCAGC-NS2/JFH1am were used as core-NS2 plasmids for HCVtcp production carrying core-p7 derived from genotypes 1b and 2a (HCVtcp-1b and HCVtcp-2a, respectively). HCVpp derived from JFH-1 and VSVpp were generated and used for comparison. Infection with HCVtcp-1b or HCVtcp-2a was blocked by anti-ApoE antibody in a dose-dependent manner. In contrast, anti-ApoE antibody did not affect infection with HCVpp and VSVpp (Fig. 4A).

The CD81 dependence of infection was also compared between HCVtcp and HCVpp (Fig. 4B). Anti-CD81 antibody inhibited the entry of HCVtcp-1b, HCVtcp-2a, and HCVpp in a dose-dependent manner. The antibody had no effect on VSVpp infection. HCVtcp infection appears to be more sensitive to anti-CD81 antibody when compared with HCVpp infection; more than 60% inhibition was observed at 0.08 μg/mL anti-CD81 antibody for HCVtcp-1b and HCVtcp-2a, whereas approximately 50% inhibition was observed for HCVpp at 2 μg/mL antibody. Neutralization of HCVcc by anti-ApoE and anti-CD81 antibodies was also determined. Antibodies blocked HCVcc infection (Fig. 4C and D), as observed with HCVtcp. These results suggest that ApoE, as well as CD81, play an important role in HCVtcp infection. Thus, HCVtcp may be more useful for evaluating the HCV entry process than HCVpp.

Identification of novel culture-adaptive mutation in NS3 by serial passage of HCVtcp in packaging cells

The HCVtcp system was further applied to analyses of genetic changes during serial passages in target cells. As an initial attempt, supernatants of cells co-transfected with pCAGC-NS2/JFH1am and pHH/SGR were inoculated into Huh7.5.1 cells transiently transfected with pCAGC-NS2/JFH1am. However, infectious titer was lost after repeated inoculation, likely due to low HCVtcp titers and low efficiency of plasmid transduction (data not shown). To overcome this, we utilized recombinant adenovirus vectors (rAdVs) to provide core-NS2. As we were not able to obtain rAdV directly expressing core-NS2, conditional transgene expression based on a Cre-loxP strategy was employed (Kanegae et al., 1995). We constructed an rAdV containing core-NS2 gene downstream of a stuffer DNA flanked by a pair of loxP sites (AxCALNLH-CNS2). When cells were doubly infected with AxCALNLH-CNS2 and the Cre-expressing rAdV, AxCANCre (Kanegae et al., 1995), the Cre-mediated excisional deletion removed the stuffer DNA, resulting in core-NS2 expression under control of the CAG promoter (Fig. 5A). As expected, tightly regulated production of HCVtcp was observed. The cells infected with AxCANCre and AxCALNLH-CNS2 along with transduction of pHH/SGR-Luc produced HCVtcp at high levels. Production of HCVtcp was undetectable when either AxCANCre or AxCALNLH-CNS2 was not infected (Fig. 5B). The Cre-mediated rAdV expression system appears to have yielded considerably higher production of HCVtcp when compared with the settings for plasmid co-transfection.

Supernatants from cells in which core-NS2 was expressed using rAdVs and the subgenomic RNA derived from pHH/SGR replicated were inoculated into cells infected with AxCALNLH-CNS2 and AxCANCre (Fig. 6A). Blind passage was performed by sequentially transferring culture supernatants to cells infected with the above rAdVs. The two independent 10 blind passages (p10) showed virus titers of $> 1 \times 10^5$ IU/mL, which were markedly higher than those of the passage 0 (p0) stock cultures (4×10^4 IU/mL). Side-by-side infection analysis revealed that the HCVtcp p10 #1 achieved a virus titer approximately 36 times higher than that of HCVtcp p0 on the packaging cells at 6 day post-infection (Fig. 6B). Sequencing of the entire replicon in the supernatants at p10 in two independent experiments revealed

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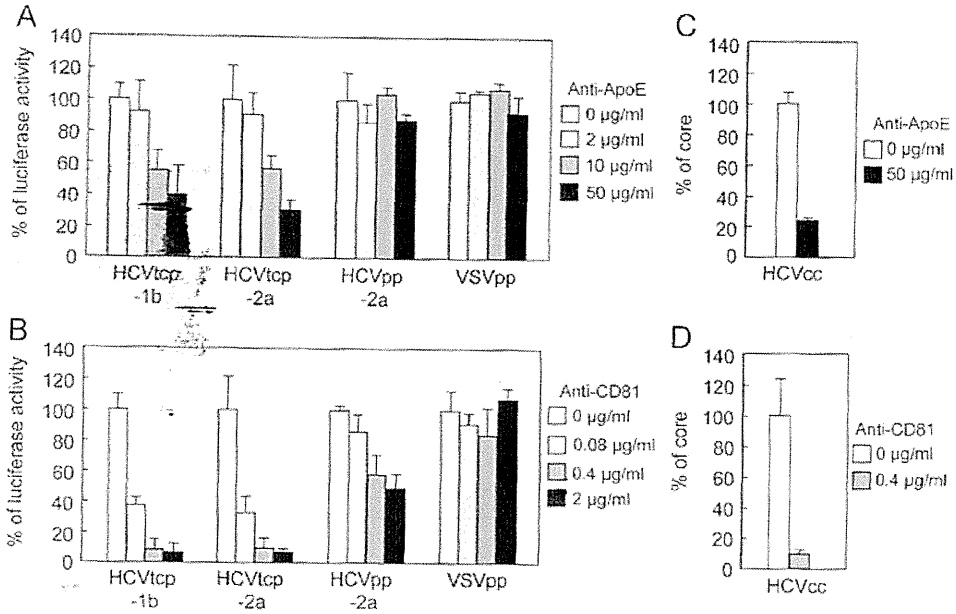

Fig. 4. Effects of anti-ApoE and anti-CD81 antibodies on HCV entry. (A) Aliquots of virus sample were incubated with increasing concentrations of anti-ApoE antibodies for 1 h and were then added to Huh7.5.1 cells. Luciferase activity was determined at 72 h post-infection and is expressed relative to activity without antibodies (white bar). (B) Huh7.5.1 cells were preincubated for 1 h with increasing concentrations of anti-CD81 antibodies, followed by inoculating virus samples. Luciferase activity was determined and expressed as shown in (A). (C) Aliquots of HCVcc were incubated with anti-ApoE antibodies for 1 h and were then added to Huh7.5.1 cells at an MOI of 0.05. Intracellular core levels were quantitated at 24 h post-infection and are expressed relative to levels without antibodies (white bar). (D) Huh7.5.1 cells were preincubated for 1 h with anti-CD81 antibodies. HCVcc infection and measurement of core proteins were performed as indicated in (C).

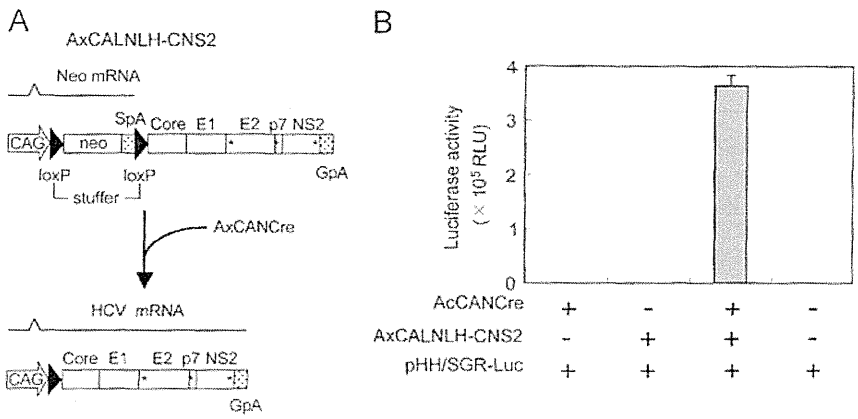

Fig. 5. Transgene activation mediated by rAdVs expressing Cre recombinase under control of CAG promoter. (A) Cre recobminase expressed by AxCANCre recognizes a pair of its target sequences loxP in AxCALNLH-CNS2, and removes the stuffer region resulting in expression of HCV core-NS2 polyprotein by CAG promoter. CAG: CAG promoter; SpA: SV40 early polyA signal; GpA: rabbit b-globin poly(A) signal. (B) Luciferase activity in Huh7.5.1 cells inoculated with 4-day post-transfection culture supernatant from cells transfected with pHH/SGR-Luc, and then infected with indicated rAdVs.

that both passaged HCVtcp had an identical nonsynonymous mutation in the NS3 region (N1586D) (Fig. 6C).

In order to examine the role of NS3 mutation identified on HCV RNA replication and on HCVtcp production, the N1586D mutation was introduced into pHH/SGR-Luc. Luciferase activities of the N1586D-mutated replicon were apparently lower than those of the WT-replicon, thus suggesting that the NS3 mutation reduced viral RNA replication (Fig. 7A). HCV RNA levels in the supernatants of cells transfected with WT- or mutant replicon plasmid along with pCAGC-NS2/JFH1am and luciferase activity in cells inoculated with supernatants from the transfected cells were then determined (Fig. 7B). The viral RNA level secreted from cells replicating the N1586D-mutated replicon was lower than that from cells replicating WT replicon (Fig. 7B, left). By contrast, a significantly higher infectivity of HCVtcp produced from the mutant replicon-cells was observed, as compared to WT replicon-cells (Fig. 7B, right), suggesting that the adaptive mutation increased the specific infectivity (almost 9-fold) of the virus particles. To further determine whether the N1586D mutation affects infectious viral assembly and/or virus release, we used the CD81-negative Huh-7 subclone, Huh7-25 (Akazawa et al., 2007), which may produce infectious particles, but is not susceptible to HCV entry due to a lack of CD81 expression, therefore allowing us to examine viral assembly and release without the influence of reinfection by produced HCVtcp. Measurement of intracellular and extracellular HCVtcp indicated that Huh7-25 cells replicating the N1586D-mutated replicon produced more infectious virus than WT in both supernatants and cell lysates (Fig. 7C). Thus, it can be concluded that the N1586D mutation contributes to enhanced infectious viral assembly, not RNA replication. We could not exclude the possibility that N1586D mutation affects virus release, since the mutation enhanced extracellular virus titers more than did the intracellular titer.

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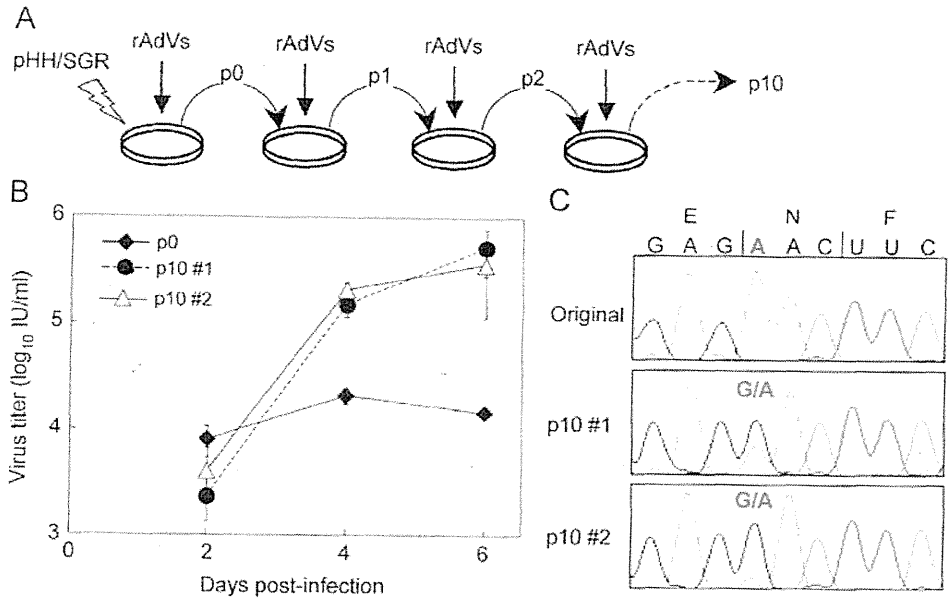

Fig. 6. Genotypic changes in HCVtcp following blind passage. (A) Experimental procedure for blind passage of HCVtcp. Huh7.5.1 cells were transfected with pHH/SGR and were doubly infected with AxCANCre and AxCALNLH-CNS2. Culture fluids were collected and were inoculated into cells infected with AxCANCre and AxCALNLH-CNS2. These procedures were repeated 10 times with two independent samples (#1 and #2). (B) Growth curves of HCVtcp p0 and p10 on Huh7.5.1 cells expressing core-NS2. Cells were infected with HCVtcp at an MOI of 0.05, and medium was collected at the indicated time points and subjected to titration. (C) Nucleotide sequences of original and blind-passaged replicons from HCVtcp. Nucleotides of mutated position are shown in red and bold.

The impact of the N1586D mutation on production of intra- and intergenotypic HCVtcp chimeras was also investigated. The N1586D mutation in the replicon enhanced the production of chimeric HCVtcp by providing core-p7 from all strains examined, although not statistically significant in THpa, and Con1 strains (Fig. 7D). Finally, to determine whether the N1586D mutation was responsible for enhancing HCVcc production, this mutation was introduced into pHHJFH1, which carries the full-length wild-type JFH-1 cDNA (Masaki et al., 2010), yielding pHHJFH1N1586D. The virus titer obtained from cells transfected with the pHHJFH1N1586D was significantly higher than that of WT (Fig. 7E), thus demonstrating that the N1586D mutation enhances yields of HCVcc, in addition to HCVtcp.

Discussion

Single-round infectious viral particles generated by *trans*-packaging systems are considered to be valuable tools for studying virus life cycles, particularly the steps related to entry into target cells, assembly and release of infectious particles. However, limited HCV strains have been applied for the efficient production of HCVtcp to date. In this study, we improved the HCVtcp system in order to enhance the productivity of infectious particles. Production of chimeric HCVtcp by providing genotype 1b-derived core-p7, in addition to intragenotypic viral proteins, was also confirmed. Furthermore, we exploited the system to investigate genetic changes during serial passage of target cells and identified a novel cell culture-adaptive mutation in NS3, which also contributes to enhance the productivity of HCVtcp.

HCVpp (Bartosch et al., 2003a; Hsu et al., 2003) has proven to be a valuable surrogate system by which the study of viral and cellular determinants of the viral entry pathway is possible. Early steps of HCV infection, including the role of HCV glycoprotein heterodimers, receptor binding, internalization and pH-dependent endosomal fusion, have been at least in part mimicked by HCVpp (Lavie et al., 2007). However, as HCVpp is generated in non-hepatic cells such as the human embryo kidney cells 293T, it is likely that the cell-derived component(s) of HCVpp differ from those of HCVcc. Hepatocytes play a role in maintaining lipid homeostasis in the body by assembling and secreting lipoproteins, including VLDL. It is highly likely that HCV exploits lipid synthesis pathways, as there is a tight link between virion formation and VLDL synthesis. Down-regulation of ApoE considerably reduces HCV production (Benga et al., 2010; Chang et al., 2007; Hishiki et al., 2010; Jiang and Luo, 2009; Owen et al., 2009). Infectivity of HCVcc is also neutralized by anti-ApoE antibodies (Chang et al., 2007). These data suggest that ApoE is important for HCV infectivity. Furthermore, Niemann-Pick C1-like 1 (NPC1L1), involving cholesterol uptake receptor, was recently identified as a host factor for HCV entry (Sainz et al., 2012). Knockdown of NPC1L1 had no effect on the entry of HCVpp whereas HCVcc entry was impaired, possibly due to different cholesterol content of these particles. Here, we found that the anti-ApoE antibody neutralized infection by HCVtcp and HCVcc, but not by HCVpp (Fig. 4A and C), thus suggesting that biogenesis and/or secretion pathways of VLDL are involved in HCVtcp similarly to HCVcc, but not in HCVpp.

We also observed that infectivity of HCVtcp and HCVcc is more efficiently neutralized by the anti-CD81 antibody, as compared to that of HCVpp (Fig. 4B and D). It has recently been reported that E2 of HCVcc contained both high-mannose-type and complex-type glycans, whereas most of the glycans on HCVpp-associated E2 were complex-type, which is matured by Golgi enzymes (Vieyres et al., 2010). Mutational analysis of the N-linked glycosylation sites in E1/E2 demonstrated that several glycans on E2 may affect the sensitivity of HCVpp against antibody neutralization, as well as access of CD81 to its binding site on E2 (Helle et al., 2010). The differences in sensitivity between HCVtcp and HCVpp to neutralization by anti-CD81 antibody observed here may be due to differences in carbohydrate composition of HCV glycoproteins during expression and processing of E1/E2 in cells and morphogenesis of HCVtcp and HCVpp.

By analyzing the various replicons for *trans*-packaging, we observed the highest production of HCVtcp with replicons from pHH/SGR, which lacked sequences not essential for RNA

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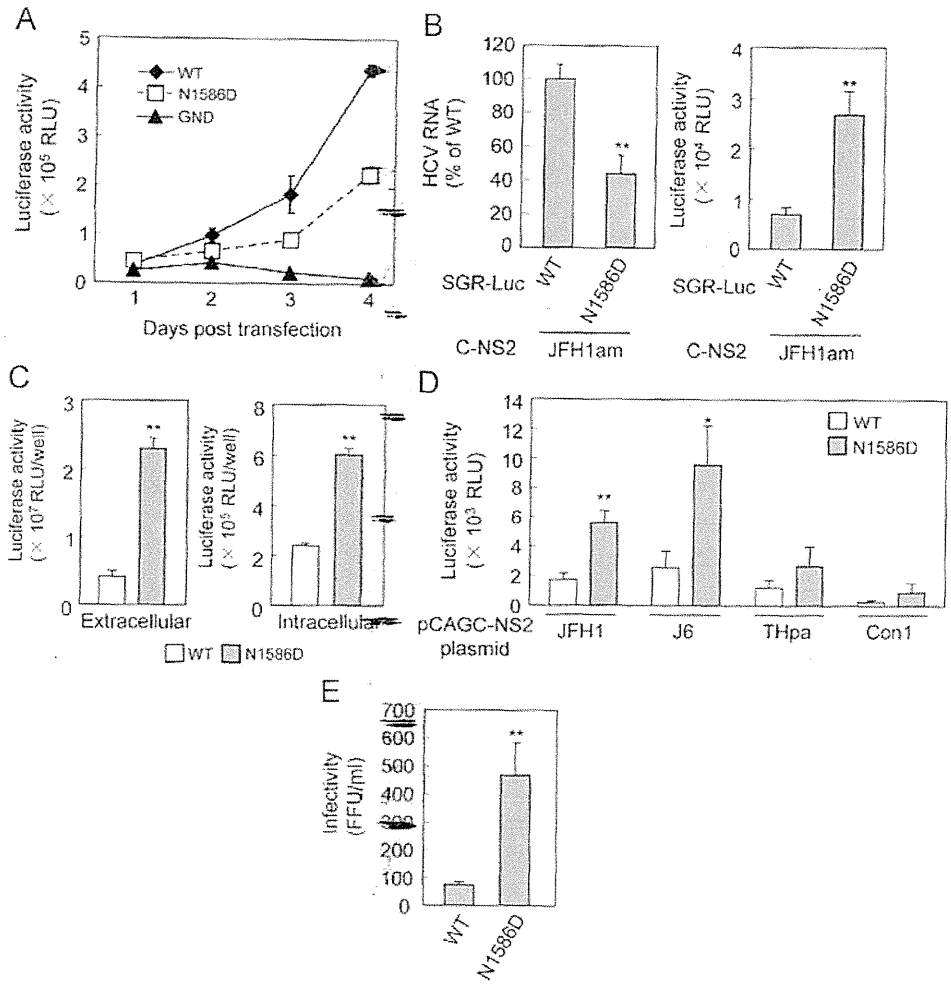

Fig. 7. Effects of N1586D mutation on RNA replication and production of HCVtcp or HCVcc. (A) RNA replication of replicons in cells transfected with pHH/SGR-Luc (WT) or N1586D mutant. Luciferase activities at 1 to 4 day post-transfection were determined. (B) Relative levels of HCV RNA in the supernatants from cells transfected with pHH/SGR-Luc (WT) or N1586D mutant plasmid along with pCAGC-NS2/JFH1am were shown in the left panel. Luciferase activities in cells inoculated with supernatants from cells transfected with indicated plasmids at 4 day post-transfection were shown in the right panel. (C) Luciferase activity in cells inoculated with supernatant and cell lysates from Huh7-25 cells transfected with pHH/SGR-Luc (WT) or N1586D mutant plasmid along with pCAGC-NS2/JFH1am at 5 day post-transfection. (D) Luciferase activity in cells inoculated with culture supernatant from cells transfected with pHH/SGR-Luc (WT) or N1586D mutant plasmid along with indicated core-NS2 plasmids at 4 day post-transfection. (E) Infectivity of supernatant from cells transfected with pHH/JFH1 (WT) or its derivative plasmid containing N1586D mutation at 6 day post-transfection. Statistical differences between WT and N1586D were evaluated using Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.005$ vs. WT.

replication, while less efficient productivity was observed from pHH/SGR-Luc, pHH/SGR-C177, pHH/SGR-C191 and pHH/SGR-C-p7/am (Fig. 2C). Differences in the replication efficiency of the replicon do not appear to be a major determinant for HCVtcp productivity, at least in the present settings, as all replicon constructs except pHH/SGR-Luc replicated at similar levels, as confirmed by Western blotting (Fig. 2B). Although the shorter viral genome sequence may offer advantages over the longer sequence, further investigation is required in order to understand the molecular mechanisms underlying viral genome packaging. By comparing pHH/SGR vs. pHH/SGR-C177, pHH/SGR-C191 and pHH/SGR-C-p7/am, it is likely that the expression of the structural protein in *cis* does not increase HCVtcp production when sufficient amounts of structural proteins are supplied in *trans*.

Blind passage of HCVtcp in packaging cells infected with rAdVs providing core-NS2 enabled us to identify a novel culture-adaptive mutation in NS3. The N-terminal third of NS3 forms a serine protease, together with NS4A, and its C-terminal two-thirds exhibits RNA helicase and RNA-stimulated NTPase activities. In addition, similarly to flaviviruses (Kummerer and Rice, 2002; Liu et al., 2002), it is now apparent that HCV NS3 is also involved in viral morphogenesis (Han et al., 2009; Ma et al., 2008), although its precise role and underlying molecular mechanism(s) have not fully been elucidated. Two cell-culture adaptive NS3 mutations which are involved in HCV assembly have been identified. The Q1251L mutation in helicase subdomain 1 resulted in approximately 30-fold higher production of HCV without affecting NS3 enzymatic activities (Ma et al., 2008). The M1290K adaptive mutation was also located in subdomain 1 of the NS3 helicase (Han et al., 2009). The N1586D mutation identified here was located in subdomain 3 of helicase. Analogous to Q1251L and M1290K, the N1586D mutation enhanced the infectious viral assembly by increasing specific infectivity without affecting the efficiency of viral RNA replication. Considering the possibility that NS3 plays a role in linking between the viral replicase and assembly sites (Jones et al., 2011), it is likely that NS3 helicase is one of the determinants for interaction with the structural proteins. Our results, together with earlier studies, suggest that chimeric and defective mutations as well as supplying the viral components in *trans*, function as selective pressures in virion assembly.

In summary, we have established a plasmid-based reverse genetics for efficient production of HCVtcp with structural

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proteins from various strains. Single-round infectious HCVtcp can complement the HCVcc and HCVpp systems as a valuable tool for the study of HCV life cycles.

Materials and methods

Cells

Huh7 derivative cell line Huh7.5.1 and Huh7-25 were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with nonessential amino acids, 100 U of penicillin/mL, 100 μg of streptomycin/mL, and 10% fetal bovine serum at 37 °C in a 5% CO_2 incubator.

Plasmids

Plasmids pHHJFH1, pHH/SGR-Luc, pHH/SGR-Luc/GND and pCAG/C-NS2 were as described previously (Masaki et al., 2010). In this study, plasmid pCAG/C-NS2 was designated as pCAGC-NS2/JFH. The plasmid pCAGC-NS2/JFHam having adaptive mutations in E2 (N417S), p7 (N765D), and NS2 (Q1012R) in pCAGC-NS2/JFH was constructed by oligonucleotide-directed mutagenesis. These mutations were also introduced in pHHJFH1, resulting in pHHJFH1am. To generate core-NS2 expression plasmids with different strains of HCV, the cDNA coding core to the first transmembrane region of NS2 (33 amino acids) in pCAGC-NS2/JFH was replaced with the corresponding sequence of the J6 (Lindenbach et al., 2005), H77c (Yanagi et al., 1997), THpa (Shirakura et al., personal communication) and Con1 (Koch and Bartenschlager, 1999) strains. The THpa sequence contained the P to A mutation at 328 aa at E1 in the original TH strain. To generate pHH/SGR, pHH/SGR-Luc was digested with MluI and PmeI, followed by Klenow enzyme treatment and self-ligation to delete the luciferase coding sequence. To generate pHH/SGR-C177, pHH/SGR-C191 and pHH/SGR-C-p7/am, cDNA coding the partial core and luciferase in pHH/SGR-Luc were replaced with coding sequences for mature core (177aa), full-length core (191aa) or core-p7 polyprotein containing adaptive mutations in E2 and p7, respectively. The selected NS3 mutation (N1586D) was introduced into pHH/SGR-Luc and pHHJFH1 by oligonucleotide-directed mutagenesis.

Generation of viruses

HCVcc and HCVtcp were generated as described previously (Masaki et al., 2010). For the production of HCVpp-2a, plasmid pcDNAdeltaC-E1-E2(JFH1)am having adaptive mutations in E2 (N417S) in pcDNAdeltaC-E1-E2(JFH1) (Akazawa et al., 2007) was constructed by oligonucleotide-directed mutagenesis. Murine leukemia virus pseudotypes with VSV G glycoprotein expressing luciferase reporter (VSVpp) were generated in accordance with previously described methods (Akazawa et al., 2007; Bartosch et al., 2003a).

Luciferase assay

Huh7.5.1 cells were seeded onto a 24-well plate at a density of 3×10^4 cells/well 24 h prior to inoculation with reporter viruses. Cells were incubated for 72 h, followed by lysis with 100 μL of lysis buffer. Luciferase activity of the cells was determined using a luciferase assay system (Promega, Madison, WI). All luciferase assays were performed in triplicate.

Quantification of HCV infectivity and HCV RNA

To determine the titers of HCVtcp and HCVcc, Huh7.5.1 cell monolayers prepared in multi-well plates were incubated with dilutions of samples and then replaced with media containing 10% FBS and 0.8% carboxymethyl cellulose. Following incubation for 72 h, monolayers were fixed and immunostained with rabbit polyclonal anti-NS5A antibody, followed by Alexa Fluor 488-conjugated anti-rabbit secondary antibody (Invitrogen), and stained foci or individual cells were counted and used to calculate a titer of focus-forming units (FFU)/mL for spreading infections or infectious units (IU)/mL for non-spreading infections. For intracellular infectivity, the cell pellet was resuspended in culture media, and cells were lysed by four freeze–thaw cycles. Cell debris was pelleted by centrifugation for 5 min at 4000 rpm. Supernatant was collected and used for titration. To determine the amount of HCV RNA in culture supernatants, RNA was extracted from 140 μL of culture medium by QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA) and treated with DNase (TURBO DNase; Ambion, Austin, TX) at 37 °C for 1 h. Extracted RNA was further purified by using an RNeasy Mini Kit, which includes RNase-free DNase digestion (QIAGEN). Copy numbers of HCV RNA were determined by real-time quantitative reverse transcription-PCR as described previously (Wakita et al., 2005).

Antibodies

Mouse monoclonal antibodies against actin (AC-15) and CD81 (JS-81) were obtained from Sigma (St. Louis, MO) and BD Biosciences (Franklin Lakes, NJ), respectively. Goat polyclonal antibody to ApoE (LV1479433) was obtained from Millipore (Tokyo, Japan). Anti-NS5A and anti-NS5B antibodies were rabbit polyclonal antibody against synthetic peptides.

Neutralization assay

For neutralization experiments with anti-CD81 antibody, Huh7.5.1 cells were incubated with dilutions of anti-CD81 antibody for 1 h at 37 °C. Cells were then infected with viruses for 5 h at 37 °C. For neutralization experiments with anti-ApoE antibody, viruses were incubated with various concentrations of anti-ApoE antibody at room temperature for 1 h and cells were infected with viruses for 5 h at 37 °C. Following infection, supernatant was removed and cells were incubated with culture medium, and luciferase activity was determined at 3 day post-infection for HCVtcp and pseudotyped viruses. For neutralization experiments with HCVcc generated with pHHJFH1am, a multiplicity of infection (MOI) of 0.05 was used for inoculation, and intracellular core protein levels were monitored by ELISA (Ortho Clinical Diagnostics) at 24 h post-infection.

Immunoblotting

Transfected cells were washed with PBS and incubated with lysis buffer (50 mM Tris–HCl, pH 7.4, 300 mM NaCl, 1% triton X-100). Lysates were then sonicated for 5 min and were added to the same volume of SDS sample buffer. Protein samples were boiled for 10 min, separated by SDS-PAGE, and transferred to PVDF membrane. After blocking, membranes were probed with first antibodies, followed by incubation with peroxidase-conjugated secondary antibody. Antigen–antibody complexes were visualized using an enhanced chemiluminescence detection system (Super Signal West Pico Chemiluminescent Substrate; PIERCE, Rockford, IL), in accordance with the manufacturer's protocols.

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Generation of recombinant adenoviruses

rAdV, AxCANCre, expressing Cre recombinase tagged with nuclear localization signal under CAG promoter was prepared as described previously (Baba et al., 2005). The target rAdV AxCALNLH-CNS2 expressing HCV core-NS2 polyprotein with adaptive mutations in E2, p7 and NS2 was generated as follows. Cosmid pAxCALNLwit2 is identical to pAxCALNLw (Sato et al., 1998), except that both the terminal sequences of the rAdV genome are derived from pAxCAwit2 (Fukuda et al., 2006). The core-NS2 fragment obtained from pCAGC-NS2/JFH1am by StuI-EcoRI digestion and subsequent Klenow treatment was inserted into the SwaI site of pAxCALNLwit2. The resultant cosmid pAxCALNLH-CN2it2 was digested with PacI and transfected into 293 cells to generate rAdV AxCALNLH-CNS2.

Preparation of packaging cells for HCVtcp

Huh7.5.1 cells were coinfected with AxCANCre at an MOI of 1 and AxCALNLH-CNS2 at an MOI of 3 for expression of JFH-1 core-NS2 polyprotein containing the adaptive mutations in E2, p7 and NS2.

RNA preparation, RT-PCR and sequencing

Total cellular RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA), and subjected to reverse transcription with random hexamer and Superscript III reverse transcriptase (Invitrogen). Three fragments of HCV cDNAs that cover the entire HCV subgenomic replicon genome, were amplified by nested PCR with TaKaRa Ex Taq polymerase (Takara, Shiga, Japan). Amplified products were separated by agarose gel electrophoresis, and were used for direct DNA sequencing.

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A Cell-Based, Microplate Colorimetric Screen Identifies 7,8-Benzoflavone and Green Tea Gallate Catechins as Inhibitors of the Hepatitis C Virus

Hidesuke Fukazawa,*[,a] Tetsuro Suzuki,[b] Takaji Wakita,[c] and Yuko Murakami[a]

[a] *Department of Bioactive Molecules, National Institute of Infectious Diseases; 1–23–1 Toyama, Shinjuku-ku, Tokyo 162–8640, Japan:* [b] *Department of Infectious Diseases, Hamamatsu University School of Medicine; 1–20–1 Handayama, Higashi-ku, Hamamatsu 431–3192, Japan; and* [c] *Department of Virology II, National Institute of Infectious Diseases; 1–23–1 Toyama, Shinjuku-ku, Tokyo 162–8640, Japan.*

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We describe a cell-based, microplate colorimetric screen for anti-hepatitis C virus (HCV) drugs that exploits the HCV-JFH1 viral culture system. Antiviral activity was assessed by measuring protection against the HCV-JFH1-induced cytopathic effect (CPE) in Huh7.5.1 cells using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) viability assay. The use of serum-free medium substantially sensitized Huh7.5.1 cells to HCV-induced CPE, causing sufficient cell death to perform colorimetric assays for anti-HCV activity in 96-well plates. As a proof of concept, we carried out a pilot screen of an inhibitor library and identified cyclosporin A and tamoxifen, two compounds with reported anti-HCV activity. Using the assay, we discovered the anti-HCV properties of the plant flavonoids epigallocatechin gallate (EGCG) and 7,8-benzoflavone (α-naphthoflavone). Other gallate-type catechins and flavones also displayed anti-HCV activity, but 5,6-benzoflavone (β-naphthoflavone), flavanone, and non-gallate catechins were inactive. EGCG apparently acted mainly on HCV entry, although it may also block other steps. In contrast, 7,8-benzoflavone was presumed to inhibit later stages of the HCV life cycle. This assay is simple, reliable and cost-effective; does not require any specially engineered cell lines or viruses; and should be useful in the identification of compounds with anti-HCV activity.

Key words hepatitis C virus; 7,8-benzoflavone (α-naphthoflavone); epigallocatechin gallate

More than 170 million people worldwide are chronically infected with the hepatitis C virus (HCV) and are at risk for developing liver diseases such as cirrhosis and hepatocellular carcinoma. Vaccines against HCV are not currently available; furthermore, the standard interferon/ribavirin combination therapy is not effective in approximately half of HCV-infected patients, and it has considerable side effects.[1,2] Thus, there is an obvious and urgent need for new agents that can enhance or replace current HCV therapies.

Screening programs using HCV replicon-based systems have successfully identified compounds that act on HCV RNA replication. However, replicon systems do not reproduce the entire HCV life cycle, and they cannot isolate inhibitors of many important steps such as viral entry, assembly, and egress. HCV cell culture infection models that recapitulate the entire viral life cycle *in vitro* have greatly enhanced the opportunity for HCV drug discovery. Several reports, including ours, have demonstrated that this model can overcome the limitations of the HCV replicon system and enable the discovery of compounds that target various stages of the HCV life cycle.[3–8]

Using the HCV cell culture system, we previously developed a tube-capture-reverse transcription-polymerase chain reaction (RT-PCR) assay for screening HCV inhibitors and identified bisindolylmaleimides and indolocarbazoles as inhibitors of HCV replication.[4] Here, we describe another screening method for the detection of anti-HCV activity. This assay measures the inhibition of the HCV-induced cytopathic effect (CPE) in Huh 7.5.1 cells. We show that the use of serum-free medium improves the sensitivity of the assay and permits the identification of drug candidates that may be overlooked in other assays due to their serum-binding capacities. The validated assay was used to evaluate a compound library, in which it successfully identified the anti-HCV activities of cyclosporin A and tamoxifen.

Many biological properties, including anti-oxidative, anti-inflammatory, anti-tumorigenic, anti-bacterial, and anti-viral activities, have been documented for plant flavonoids, particularly the green tea catechin epigallocatechin gallate (EGCG).[9] We tested various flavonoids in the assay and found that EGCG and 7,8-benzoflavone (α-naphthoflavone) inhibit HCV infection. Other gallate-type catechins were also active, but non-gallate catechins and 5,6-benzoflavone (β-naphthoflavone) did not exhibit prominent anti-HCV activity. EGCG has recently been shown to block HCV entry.[10] Our results using the JFH1 viral culture system also suggested that EGCG mainly targets virus entry but implied that it may also act on other stages of the HCV life cycle. In contrast, 7,8-benzoflavone appeared to inhibit post-entry phases.

The assay described here is simple, reliable and cost-effective, does not require any specially engineered viruses or cell lines, and should allow the high-throughput screening of HCV drug candidates.

MATERIALS AND METHODS

Materials Epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate, cyclosporin A and tamoxifen were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Catechin gallate and gallocatechin gallate were purchased from Nagara Science Co., Ltd. (Nagase, Japan). Flavone, flavanone, 5,6-benzoflavone, and 7,8-benzoflavone were obtained from Wako Pure Chemicals (Osaka, Japan). The SCADS Inhibitor Kit I was provided by the Screening Committee of

The authors declare no conflict of interest.

*To whom correspondence should be addressed. e-mail: fukazawa@nih.go.jp

Anticancer Drugs, supported by a Grant-in-Aid for Scientific Research on Innovative Areas in the Scientific Support Programs for Cancer Research, from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The insulin-transferrin-selenium-X (ITS-X) supplement was purchased from Invitrogen (Grand Island, NY, U.S.A.). The anti-NS5 monoclonal antibody was obtained from Austral Biologicals (San Ramon, CA, U.S.A.), and antibodies against extracellular-regulated kinase 1 (ERK1) and ERK2 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Anti-core monoclonal antibody (2H9) has been described previously.[11] Huh 7.5.1 cells were kindly provided by Francis V. Chisari.[12] The pJFH1 plasmid, containing a full-length cDNA corresponding to the JFH1 isolate, was used to produce HCV in culture.[11] To prepare virus stock for screening, naïve Huh 7.5.1 cells were infected with the passaged supernatant virus, and the medium was collected 7 d post-infection and stored at −80°C until use.[4] The cells harboring the genotype 1a full-genomic replicon (RCYM1)[13] and the genotype 2a subgenomic replicon (clone 4-1)[14] have been described previously.

HCV CPE Inhibition Assay Huh 7.5.1 cells were suspended in 1 : 1 Dulbecco's modified Eagle's medium : Nutrient Mixture F-12 (DMEM/F12) supplemented with 0.5% ITS-X and seeded in wells of collagen-coated 96-well plates at a density of 5000 cells per well in a volume of 125 μL. The cells were allowed to attach to the plates, and 15 μL of test compounds in DMEM/F12 were added to the wells. The cells were then infected with HCV-JFH1 virus stock at a multiplicity of infection (MOI) of 1. A duplicate plate without the virus was prepared in parallel to assess the cytotoxicity of the compounds. After 4 d, cell growth was monitored using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. MTT solution (15 μL at 5 mg/mL in phosphate buffered saline) was added and incubated for 4 h. The resulting formazan was extracted by adding 100 μL of 20% sodium dodecyl sulfate (SDS) in 10 mM HCl, and the absorbance was monitored after 24 h at 570 nm with a reference wavelength of 690 nm.

Immunoblotting Analysis Huh 7.5.1 cells were seeded in 24-well collagen-coated plates at a density of 1.5×10^5 cells per well and infected with HCV-JFH1 at an MOI of 0.5. Drug treatment was initiated at various timepoints before and after infection. At 48 h after infection, cells were fixed with 10% trichloroacetic acid and lysed with 9 M urea, 2% Triton X-100 and 2% lithium dodecyl sulfate. The lysates were neutralized with 2 M Tris and analyzed by immunoblotting. Replicon cells were seeded in 24-well collagen-coated plates at a density of 5.0×10^4 cells per well, treated with the compounds for 72 h, and processed for immunoblotting as described above.

RESULTS

Assay Development As reported by Sekine-Osajima,[15] infection with HCV-JFH1 induces CPE in Huh 7.5.1 cells. We postulated that compounds with anti-HCV activity would protect cells from CPE and improve cell viability. Thus, by measuring the recovery of cell growth from HCV-induced CPE in microtiter well plates, it should be possible to screen for inhibitors of HCV.

To increase the sensitivity of the primary screen and identify as many compounds with anti-HCV activity as possible, we reduced the concentration of serum in the assay medium; many compounds are known to bind to serum proteins and thus may not show activity in media containing high concentrations of serum. We found that Huh 7.5.1 cells can proliferate on a collagen-coated surface in DMEM/F12 supplemented only with insulin and transferrin at a rate fully comparable to the growth in serum-containing medium.

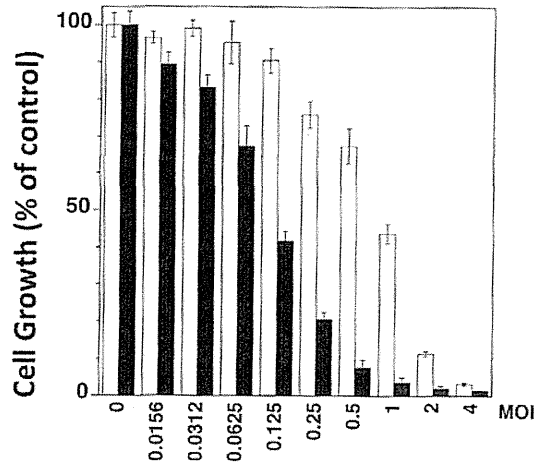

Fig. 1. HCV-Induced CPE in Serum-Free (Closed Bars) and Serum-Supplemented (Open Bars) Medium

Huh 7.5.1 cells were suspended in serum-free or serum-supplemented medium and seeded in 96-well plates at a density of 5000 cells per well. Cells were infected with HCV-JFH1 at various MOIs and cultured for 4 d. Cell growth was monitored by MTT assay and is presented as percentage of control (uninfected) cells. The values presented are the means±S.D. of at least triplicate wells.

The infection of Huh 7.5.1 cells cultured in serum-free medium with JFH1 caused marked CPE. HCV-mediated CPE was also observed in serum-supplemented medium, but cells were less susceptible and required several-fold higher viral titers to achieve equivalent growth inhibition (Fig. 2). We chose an MOI of 1 in serum-free medium as the condition for screening. Although there is some serum carry over from the virus stock, at this MOI, the final serum concentration is generally reduced more than 100-fold compared to serum-supplemented medium. Cell growth usually decreased to less than 10% of the control, as measured by MTT assay.

To test the ability of the assay to identify HCV inhibitors, we performed a test screen using an inhibitor kit obtained from the Screening Committee of Anticancer Drugs (SCADS inhibitor kit I). This kit consists of 92 compounds with various biological activities. Cyclosporin A and tamoxifen, two compounds with known anti-HCV activity, were identified as HCV inhibitors in the assay, providing a proof-of-concept for anti-HCV screening. As shown in Fig. 3, infection with JFH1 at an MOI of 1 reduced the growth of Huh 7.5.1 cells to less than 7% of the control in this experiment. In the presence of 2 μM cyclosporin A and tamoxifen, cell growth recovered to 73% and 71% of the control, respectively.

Identification of Plant Flavonoids as Inhibitors of HCV We then used the assay to evaluate the potential anti-HCV activities of various compounds. As shown in Figs. 3 and 4, various plant flavonoids, including the green tea gallate catechin EGCG and 7,8-benzoflavone, reversed the CPE of HCV. The growth of Huh 7.5.1 cells recovered from less than 7% to 90% and 84% in the presence of 10 μM EGCG or 2.5 μM 7,8-benzoflavone, respectively. The morphology of cells infected in the

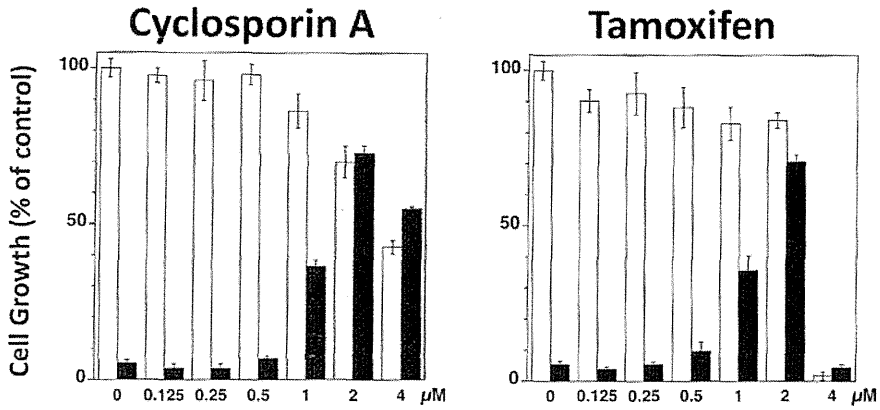

Fig. 2. Cyclosporin A and Tamoxifen Protect Huh 7.5.1 Cells from HCV-Induced CPE

Huh 7.5.1 cells were seeded in 96-well plates and treated with compounds in the absence (open bars) or presence (closed bars) of HCV-infection at an MOI of 1. Cell growth is expressed as percentage of mock-infected cells without inhibitors. The values presented are the means±S.D. of quadruplicate wells.

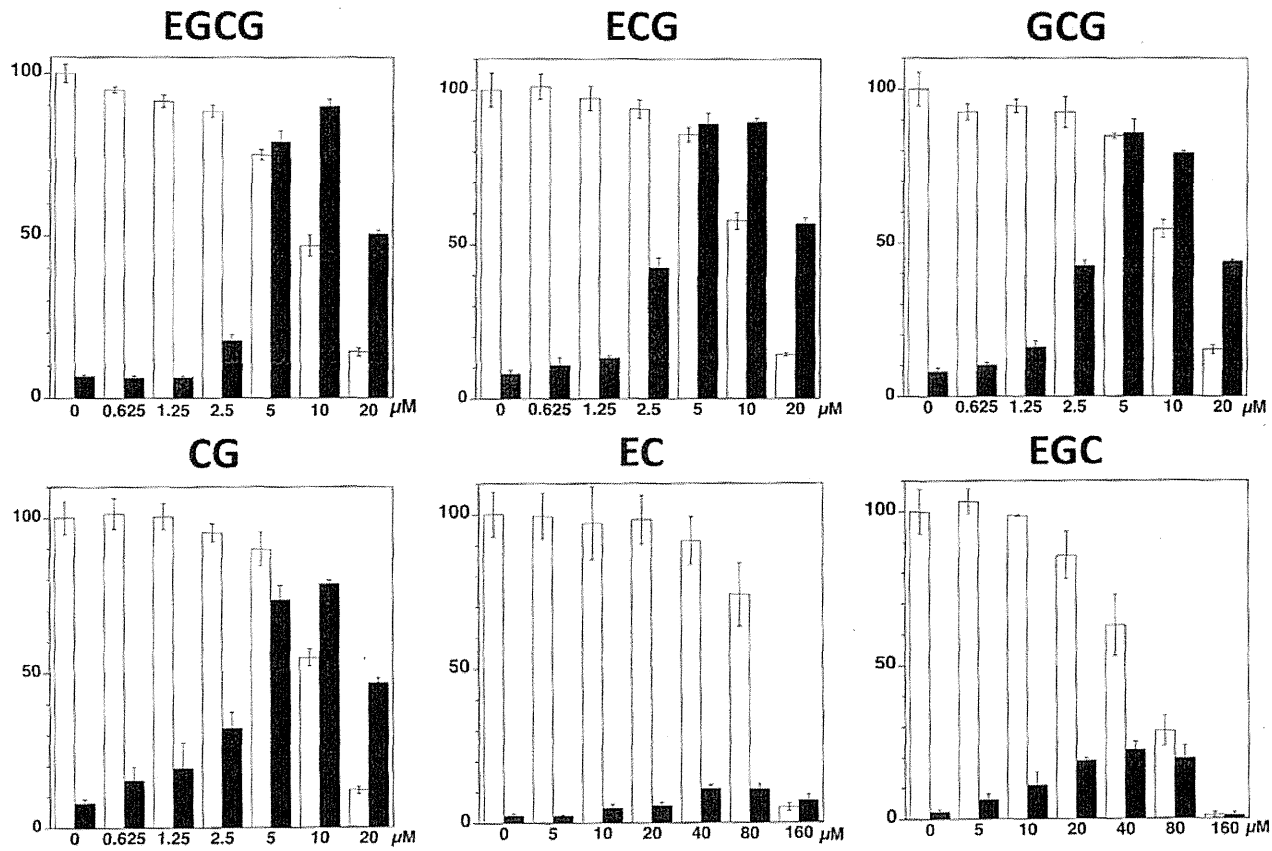

Fig. 3. The Effect of Green Tea Catechins on HCV-Induced CPE

Huh 7.5.1 cells were treated with compounds in the absence (open bars) or presence (closed bars) of HCV-infection at an MOI of 1. Cell growth is presented as a percentage of mock-infected cells without inhibitors. EGCG, epigallocatechin gallate; ECG, epicatechin gallate; GCG, gallocatechin gallate; CG, catechin gallate; EC, epicatechin; EGC, epigallocatechin. The values presented are the means±S.D. of triplicate wells.

presence of EGCG or 7,8-benzoflavone was indistinguishable from that of control cells without virus (not shown). Other gallate-type catechins (catechin gallate, gallocatechin gallate, and epicatechin gallate) were approximately as active as EGCG (Fig. 3). Flavone also displayed a protective effect (Fig. 4), although only at concentrations approximately 10-fold higher than that of 7,8-benzoflavone, but 5,6-benzoflavone, flavanone (not shown), and the non-gallate-type catechins epicatechin and epigallocatechin did not show significant anti-HCV activity (Fig. 3).

Effects of Serum on Anti-HCV Activities of Various Compounds We next performed the assay in a medium containing 5% FBS to assess the effects of serum on the anti-HCV activities of the identified compounds. To obtain CPE comparable with that observed in serum-supplemented medium (Fig. 3), cells were infected at an MOI of 4 in serum-supplemented medium.

In the absence of HCV infection, cyclosporin A was

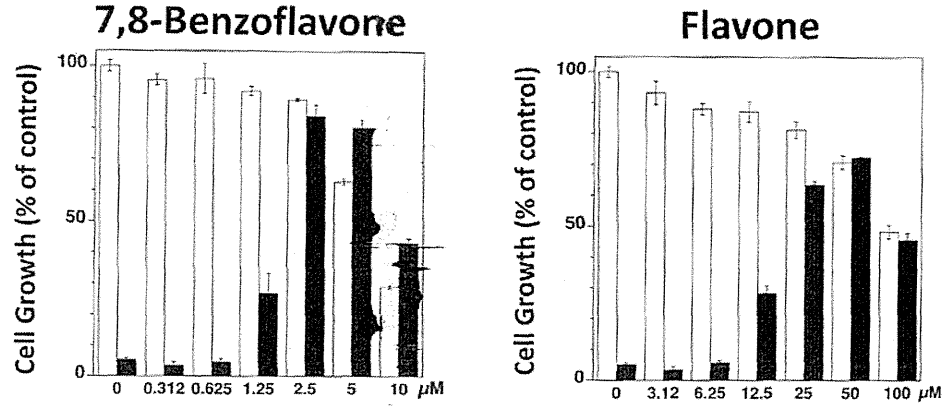

Fig. 4. The Effects of Flavonoids on CPE Induced by HCV

Huh 7.5.1 cells were treated with compounds in the absence (open bars) or presence (closed bars) of HCV-infection at an MOI of 1. Cell growth is presented as a percentage of mock-infected cells without inhibitors. The values presented are the means±S.D. of triplicate wells.

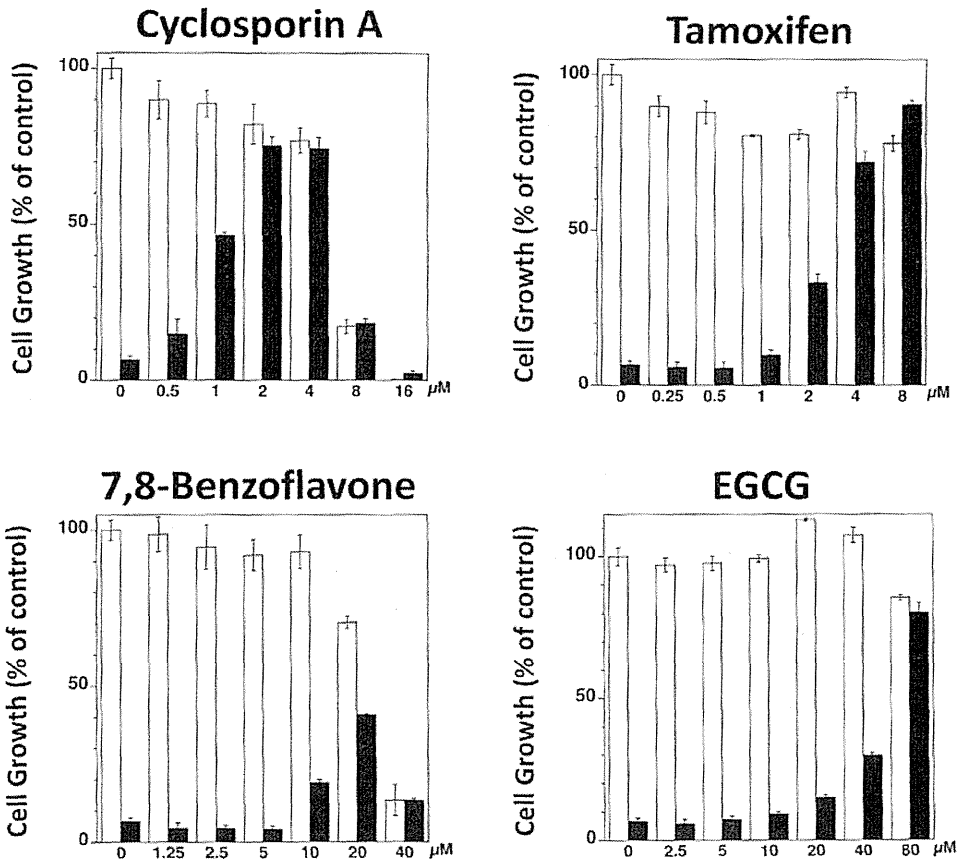

Fig. 5. The Effects of Serum on the Anti-HCV Activity and Cytotoxicity of Cyclosporin A, Tamoxifen, EGCG and 7,8-Benzoflavone

Huh 7.5.1 cells were suspended in serum-supplemented medium and seeded in 96-well plates. Cells were then treated with compounds in the absence (open bars) or presence (closed bars) of HCV-infection at an MOI of 4. Cell growth is presented as a percentage of mock-infected cells without inhibitors. The values presented are the means±S.D. of triplicate wells.

somewhat more toxic to Huh 7.5.1 cells in serum-free medium than in serum-supplemented medium. However, growth recovery from HCV-induced CPE did not appear to be noticeably influenced by the increase in MOI (not shown) or by the addition of serum (Fig. 5). Other compounds did show anti-HCV activity and cytotoxicity in the presence of serum but required higher concentrations to achieve equivalent activities.

In serum-free medium, tamoxifen showed the highest HCV-inhibitory activity at 2 μM (Fig. 2). The increase in MOI from 1 to 4 slightly reduced the anti-HCV activity of tamoxifen (not shown). In 5% serum, the most effective dose of tamoxifen shifted to highest tested concentration of 8 μM, although the maximum observed growth improvement (6.6% to 91%) was better than that in serum-free medium. The cytotoxic concentrations also changed. In serum-free medium, tamoxifen was highly toxic to Huh 7.5.1 cells at concentrations

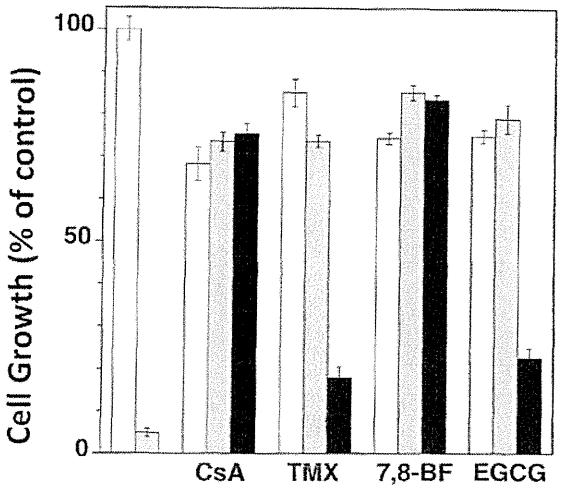

Fig. 6. The Effects of Time of Addition on the Anti-HCV Activity of Cyclosporin A (CsA), Tamoxifen (TMX), 7,8-Benzoflavone (7,8-BF) and EGCG

Compounds were added immediately before (shaded bars) or 2h after (closed bars) HCV infection, and cell growth was monitored after 4d. Open bars represent cell growth in the absence of HCV-infection. Cell growth is presented as a percentage of mock-infected cells without inhibitors. The values presented are the means±S.D. of quadruplicate wells. The concentrations used were: cyclosporin A, 2 μM; tamoxifen, 5 μM; 7,8-benzoflavone, 2.5 μM; EGCG, 5 μM.

greater than 4 μM (Fig. 2), but in the presence of serum, the cells still showed 78% growth at 8 μM (Fig. 5).

At 2.5 μM, 7,8-benzoflavone clearly protected cells from HCV-induced CPE in serum-free medium, but was completely inactive in the presence of 5% serum. Even at 20 μM, cell growth only recovered from 6.6% to 41% in serum-supplemented medium. The cytotoxic concentrations also changed; the 50% growth inhibitory concentrations in the absence of HCV infection without and with serum were 4.2 μM and 26 μM, respectively.

Among the compounds tested, EGCG was most influenced by serum addition and increased MOI. For example, 5 μM EGCG stimulated recovery from 7% to 80% at an MOI of 1 (Fig. 3), but no significant improvement could be observed at an MOI of 4 (not shown). The highest anti-HCV activity of EGCG at an MOI of 4 was observed at 20 μM (recovery from 3.1% to 78% of control), and its activities were further attenuated by the addition of serum. In 5% serum-supplemented medium, growth only recovered from 6.1% to 15% upon the addition of 20 μM EGCG, and the optimum concentration was 80 μM or higher (Fig. 5).

The results indicate that serum proteins may mask the anti-viral activities of some compounds and that the use of serum-free medium can improve the sensitivity of a cell-based screen.

EGCG and 7,8-Benzoflavone Target Different Steps of the HCV Life Cycle To determine which step of the HCV life cycle is blocked by flavones, we examined the effect of the time of addition. Compounds were added either immediately before HCV infection or at 2h after infection, when the entry process had presumably been completed. The activity of entry inhibitors would be expected to decrease when added after the completion of entry, whereas compounds that interfere with post-entry steps should still be effective.

The protective effects of tamoxifen and EGCG decreased when added at 2h after infection, suggesting that these compounds mainly target the early phases of infection (Fig. 6). By contrast, the anti-HCV activities of cyclosporin A and 7,8-benzoflavone were not affected by time of addition.

Immunoblot analysis of the HCV core protein supported the results of the MTT assay. Compounds were added at various time points before and after infection, and the levels of the HCV core protein were analyzed at 48h after infection. As shown in Fig. 7, tamoxifen and EGCG completely blocked core expression if added at or before the time of infection, but addition later than 1h post-infection resulted in markedly decreased efficacy. On the contrary, 7,8-benzoflavone and cyclosporin A were still effective even when added 4h after infection. Our data suggested that EGCG and tamoxifen mainly inhibited the early steps of the HCV life cycle, such as attachment and entry, whereas 7,8-benzoflavone and cyclosporin A blocked later stages.

Effects of Inhibitors on Replicon Cells We next compared the effects of cyclosporin A, tamoxifen, EGCG and 7,8-benzoflavone on cells that harbor a genotype 2a subgenomic or a genotype 1b full-genomic replicon. Cells were

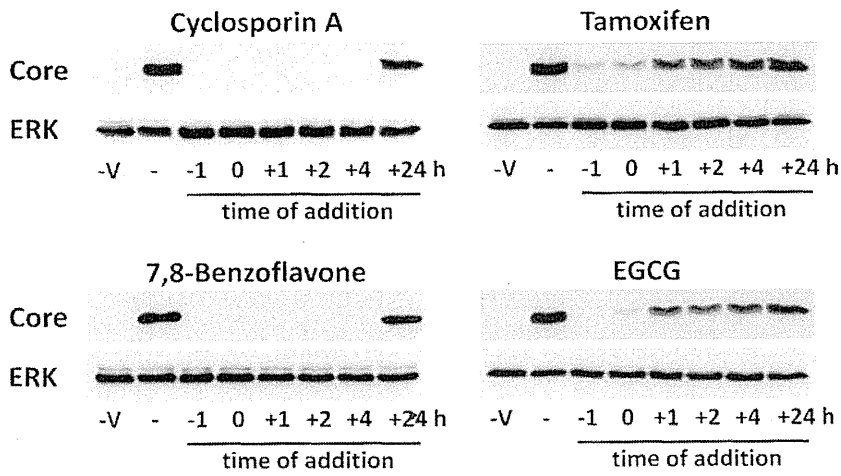

Fig. 7. The Effect of Time of Compound Addition on the Expression of Core Protein

Huh 7.5.1 cells were seeded in 24-well plates. Compounds were added at the indicated times before and after infection. Cells were fixed 48h after infection and analyzed by immunoblotting with an anti-core antibody; anti-ERK antibody was used to confirm equal loading. −V indicates control without infection.

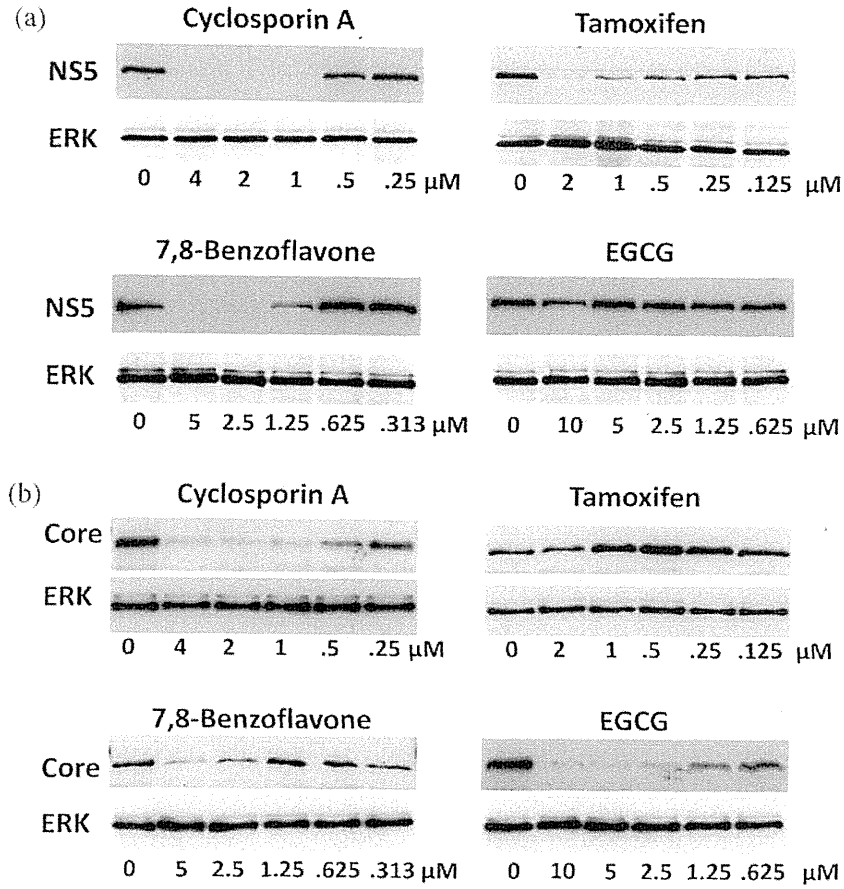

Fig. 8. The Effects of Cyclosporin A, Tamoxifen, EGCG and 7,8-Benzoflavone on the Expression of Viral Proteins in Replicon Cells

Replicon cells were treated for 72 h with the indicated concentrations of compounds and processed for immunoblotting. (a) NS5 in genotype 2a subgenomic replicon cells. (b) Core in genotype 1b full genomic replicon cells. Filters were probed with anti-ERK antibody to confirm equal loading.

treated with the inhibitory compounds for 72 h, and the levels of NS5 or core proteins were analyzed by immunoblotting. Replicon replication is completed within cells, so compounds that target steps other than RNA replication, such as attachment or entry, should not show activity. We hypothesized that cyclosporin A and 7,8-benzoflavone would be effective against replicon cells and that tamoxifen and EGCG would not be effective.

As expected, treatment with cyclosporin A and 7,8-benzoflavone noticeably reduced the level of NS5 in 2a subgenomic replicon cells (Fig. 8a). EGCG showed little effect, but tamoxifen appeared to show a moderate level of activity. In the genotype 1b full-genomic replicon cells, cyclosporin A and 7,8-benzoflavone also reduced the amount of core protein, although 7,8-benzoflavone appeared to be somewhat less effective (Fig. 8b). However, tamoxifen was completely inactive and even appeared to enhance core expression at some concentrations. Unexpectedly, EGCG displayed considerable activity, implying that it may inhibit stages of the HCV life cycle other than entry. The results indicate that the efficacy and even the mechanism of action of inhibitors may differ with viral genotype.

DISCUSSION

We established a microtiter plate method for anti-HCV drug discovery that measures the increased viability of infected cells. This method enables the screening of inhibitors that target all stages of the HCV life cycle, including steps that cannot be recognized by replicon systems, such as viral attachment, entry, and egress. An assay based on a similar principle using an engineered cell line has recently been described.[7,16] In that study, an HCV NS3-4A protease-cleavable derivative of Bid, which renders cells highly susceptible to HCV infection, was expressed in Huh 7 cells to increase the sensitivity of the assay. Here, we showed that the use of serum-free medium substantially enhanced HCV-induced CPE, and obviated the need for specially engineered cells. The mechanism of increased CPE in serum-free medium is not clear, but it is likely that serum proteins and/or lipids confer resistance to HCV-infection.

Many compounds are known to bind to serum proteins and may not show activity in a cell-based assay in the presence of high concentrations of serum. For example, EGCG has been reported to form water-soluble complexes with bovine serum albumin.[17] We showed that the anti-HCV activity of EGCG is diminished in the presence of serum. Thus, serum-free medium offers various benefits to screening programs. It increases the sensitivity of the assay by allowing the use of lower MOIs and by enabling the detection of compounds with high serum protein-binding capacity. Compounds that bind to serum proteins with high affinity may have undesirable

pharmacodynamic properties, but the object of primary screening is to identify as many potentially active compounds as possible, and pharmacodynamic properties can be improved by chemical modifications. Although serum-free medium does have certain negative aspects such as enhanced toxicity of some compounds, we anticipate that its use will expand the range of identified compounds and increase the possibility of discovering anti-HCV drug candidates.

In our trial screening, we identified active compounds with several different modes of action, including the plant flavonoids EGCG and 7,8-benzoflavone. The green tea catechin EGCG is most likely the best-studied plant flavonoid, and it is known to exert multiple biological effects. It has been reported to inhibit various viruses through a number of mechanisms. The documented anti-viral activities of EGCG include the inhibition of the influenza virus,[18] human immunodeficiency virus type 1 (HIV-1),[19,20] Epstein–Barr virus,[21,22] herpes simplex virus,[23] and hepatitis B virus.[24] *In vitro*, EGCG inhibited various viral enzymes, such as adenovirus protease adenain,[25] influenza A RNA polymerase,[26] and HIV-1 integrase.[27] EGCG bound to CD4 on T cells and prevented its interaction with HIV gp120.[28] EGCG has been shown to possess activity against the HCV NS3 serine protease[29] and NS5B,[30] although the actual impact of these effects on HCV replication have not been confirmed.

EGCG has recently been reported to inhibit the entry of HCV.[10] EGCG inhibited the entry of all genotypes tested (1a, 1b and 2a), but it was much less active against vesicular stomatitis virus (VSV) entry. The present study also suggested that the attenuation of the replication of genotype 2a HCV by EGCG is mainly due to entry inhibition. We tested the effect of EGCG on the entry of other genotypes using pseudoparticles and, as reported, observed that it inhibited the entry of all HCV genotypes (not shown). In our hands, however, the entry of VSV was blocked at comparable concentrations, demonstrating that EGCG was not specific to HCV. It has also been reported that EGC, EC, and ECG had no obvious inhibitory activity on HCV.[10] Although EGC and EC were not effective in our assay, ECG and the other gallate-type catechins GCG and CG showed anti-HCV activities similar to that of EGCG. Time-of-addition experiments suggested that these gallate-type catechins all mainly target viral entry, at least for genotype 2a HCV (not shown). It is likely that the gallate moiety is essential for the anti-HCV activity of catechins. The influence of the third hydroxyl group in the B-ring appeared to be marginal, at least in the present study.

EGCG had little effect on the expression of HCV proteins in genotype 2a replicon cells, a context that does not involve viral entry, further supporting the theory that EGCG is an entry inhibitor. However, EGCG substantially reduced the level of HCV proteins in genotype 1b replicon cells, implying that it may also target post-entry phases. Given that green tea catechins display diverse biological effects, it is conceivable that the mechanism of anti-HCV action is multimodal and that the main target may differ with genotypes.

In contrast to EGCG, 7,8-benzoflavone inhibited HCV replication even when added after the completion of the entry phase, and it effectively reduced the amount of NS5 in genotype 2a replicon cells, suggesting that it acts on post-entry phases. Furthermore, 7,8-benzoflavone displayed genotype selectivity and was not as active against the genotype 1b replicon. 7,8-Benzoflavone has been reported to inhibit aromatase[31] and breast cancer resistant protein (BCRP)[32] and modulate aryl hydrocarbon receptor (AhR) signaling.[33,34] Whether any of these biological activities are associated with the anti-HCV activity of 7,8-benzoflavone remains to be elucidated.

Other plant flavonoids with reported anti-HCV activity include naringenin[35] and quercetin.[36] Although the mechanisms of action of these compounds are not completely defined, naringenin acted as a PPAR α agonist to block virus assembly.[37] The inhibition of HCV particle production by quercetin was attributed at least partially to the reduction of HSP40 and HSP70 that is potentially involved in IRES translation.[36] More recently, quercetin was found to inhibit NS3 activity.[38] In our assay, 7,8-benzoflavone was more active than naringenin or quercetin in inhibiting HCV (not shown). We are currently testing various plant flavonoids to gain insight into the structure/activity relationship.

In conclusion, using the JFH1-HCV viral culture system, we developed a simple and cost-effective microplate colorimetric assay that will allow the high-throughput screening for HCV inhibitors that target various phases of the viral life cycle. The use of serum-free medium sensitized Huh 7.5.1 cells to HCV-induced CPE and eliminated the need for specially engineered cells or viruses. We anticipate that this assay will facilitate the discovery of compounds with anti-HCV activity and assist drug development. We have extended the screening to large chemical libraries and revealed anti-HCV activity in a wide variety of compounds.

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