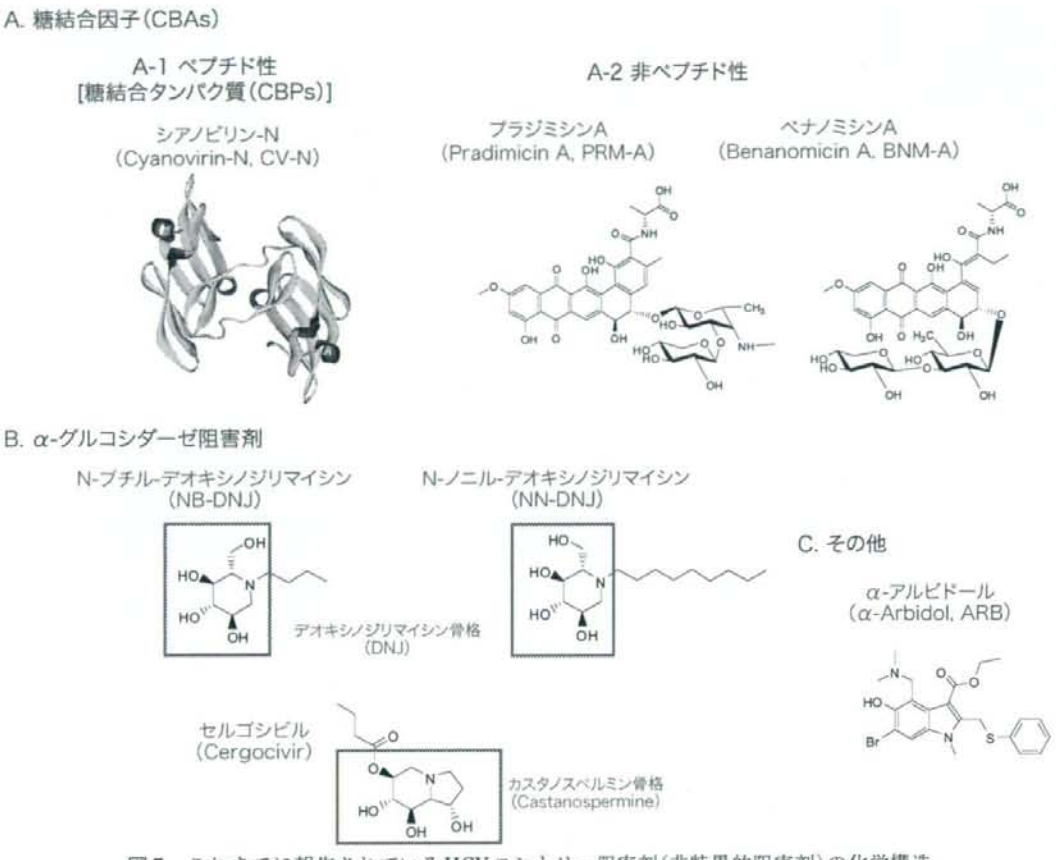

図5　これまでに報告されているHCVエントリー阻害剤(非特異的阻害剤)の化学構造

ル濃度での阻害作用)，最適化の試みがなされている．

(c) HCVエントリーに関連するその他の阻害剤

α-アルビドール Arbidol (ARB)

ARBはHCVppアッセイで，マイクロモル濃度でHCV膜融合を阻害することが知られている[15]．この薬剤は当初インフルエンザに有効な薬剤として開発され，ロシアや中国でタミフルの安価な代替え品として臨床治験が進行中である．細胞膜に親和性の高い化合物で，脂質2重膜にとけ込むことで，宿主細胞膜とウイルスエンベロープ膜との融合反応を阻害するものと考えられる．

2. 特異的HCVエントリー阻害剤

以上に述べたエントリー阻害剤は，いずれもHCVだけでなくHIVなど他のウイルス群にも作用するいわば非特異的な抗ウイルス剤である．HIV-1においては，エントリー阻害剤として，CCR5拮抗剤(CCR5はHIV-1感染に必須なコレセプターの一つ)とペプチド性の融合阻害剤の2種の特異的阻害剤が開発され，新薬として臨床応用されているが，HCVでは，これに対応するような特異的エントリー阻害剤は知られていない．

このクラスの阻害剤の開発は，HCVエン

トリー機構の解明と切り離せない．HCVエントリーは，図4に示すようなメカニズムによることが現在までに明らかにされている．HCVはまず細胞表面にあるグリコサミノグリカン(プロテオグリカンの基本骨格となる多糖類の総称)に非特異に吸着される．ついで，HCV粒子はエンベロープタンパク質E2を介してHCVエントリー受容体であるSR-B1およびCD81に結合し，ついでClaudin-1（tight junction TJタンパク質，最近第4のHCVエントリー受容体として，TJタンパク質の1つであるoccludinが報告されている)が共同して，細胞内に取り込まれると考えられている[16]．したがってこれらの受容体タンパク質の機能を阻害する薬物は，抗HCV剤としての可能性が期待されるが，現時点では，これらHCVエントリー受容体を標的とする薬剤はみいだされていない．われわれは，最近HCVccアッセイによるランダム(unbiased)スクリーニングの結果によってみいだされたHCV阻害剤の中に，HCVエントリーの特異的阻害剤と考えられる低分子化合物をいくつか同定し，現在その作用機構の解明を急いでいる．

なお，図5，表2に，これまでに報告されているHCVエントリー阻害剤の化学構造およびその性質の概略を示す．いずれもHCVだけではなくHIVを含む他のウイルスの感染も阻害するような非特異機構による侵入阻害剤であり，今後，HCVエントリーを特異的に阻害するような真の意味でのHCVエントリー阻害剤の開発が期待される．

4 おわりに

HCV治療薬開発はHIV治療薬開発が辿ってきた道を進むことになると予測される．HIVの場合にそうであったように，HCVでも，短中期的には，ウイルス側機能タンパク質を標的とする阻害剤(RNAポリメラーゼ阻害剤やプロテアーゼ阻害剤)が開発競争の中心となることは疑いない．しかし，これら薬剤が極めて高いpotencyをもっていても早晩耐性変異が出現することは不可避と考えられる．実際，急速な耐性変異株の出現が，開発途上の薬剤のいずれにおいても問題となっている．HCVはHIV-1と同程度あるいはそれ以上の極めてゲノム多様性，より高い複製能(*in vivo*でのHCVのturnover rateはHIV-1のそれを2桁以上高い10^{12}/日のレベルにある)を有しており，このようなウイルス学的な性質から考えて，耐性ウイルスの問題は，むしろHIV-1を凌ぐ深刻な問題となりうる可能性がある．

一方，宿主側因子を標的とする薬剤は，ウイルス側の変異の問題を回避できる利点が期待される(しかし，HIV-1エントリー阻害剤の一つであるCCR5拮抗剤を例にとると，ウイルス側の変異によって耐性化は全く何の障壁なく起こってしまうようにみえる)が，その一方，宿主因子を標的とする限り，副作用の可能性を念頭におかねばならない．宿主機能に障害を与えない，より特異性の高い薬剤の開発が望まれる所以である．

エイズ治療に行われているような多剤併用療法(highly active antiretroviral therapy; HAART)は，HCV治療においても現実的な治療戦略となることが予測される．臨床応用可能なHCVエントリー阻害剤が将来開発された場合，おそらくその他のクラスの阻害剤と同様，現行のPEG-インターフェロンα/リバビリンとの併用療法と組み合わせることによって副作用の強いインターフェロン・リバビリンの用量を低減し，副作用を抑え，かつその抗ウイルス作用をより強力なものとする

薬剤選択肢として位置づけられることになると考えられる．またHCVによる肝不全に対する肝移植の際に，移植片のHCV感染を防止する目的での臨床応用が考えられる．

一方また，HCV治療薬開発の問題から離れて，純粋に科学的側面で考えると，HCVの極めて複雑な受容体システムの相互の役割を解明する上で，様々なクラスのエントリー阻害剤をみいだすことは非常に重要と考えられる．われわれは，同定した阻害剤をシーズとして創薬展開を計ると同時に，それらを「探り針(プローブ)」として複雑なHCVエントリー機構全体の理解とその解明に役立てたいと願っている．国内外における今後の研究展開を期待したい．

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Replicons from genotype 1b HCV-positive sera exhibit diverse sensitivities to anti-HCV reagents

Go Nishimura[a], Masanori Ikeda[a,*], Kyoko Mori[a], Takahide Nakazawa[b], Yasuo Ariumi[a], Hiromichi Dansako[a], Nobuyuki Kato[a]

[a] Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan
[b] Gastroenterology Division of Internal Medicine, Kitasato University East Hospital, 2-1-1 Asamizodai, Sagamihara, Kanagawa, 228-8520, Japan

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abstract

Half of the population of genotype 1 HCV is resistant to current pegylated-interferon-• (PEG-IFN-•) and ribavirin therapy. The resistance to IFN therapy is an urgent problem, especially in patients with genotype 1 HCV infection. However, sensitivities among HCV strains to anti-HCV reagents including IFNs have not been thoroughly addressed. Here, we established three different subgenomic replicons (1B-4, 1B-5, and KAH5 strains) in addition to our previously established replicon (O strain). We comparatively examined the sensitivities of four replicons to IFN-• , IFN-• , IFN-• , cyclosporine A, and fluvastatin. Among the replicons, the 1B-4 and KAH5 replicons were the most sensitive and resistant, respectively to IFN-• (EC_{50}: 1.50 • M vs. 8.50 • M) and fluvastatin (EC_{50}: 2.82 • M vs. 7.87 • M), although these replicons possessed similar features in terms of genetic distance from the O strain, HCV RNA expression levels, and sensitivity to IFN-• (EC_{50}: 1.44 IU/ml vs. 1.37 IU/ml) and cyclosporine A (EC_{50}: 0.71 • g/ml vs. 0.96 • g/ml). These replicons are thus useful tools for examining the mechanism of anti-HCV activity, especially in IFN-• and statins.

1. Introduction

Hepatitis C virus (HCV) belongs to Flaviviridae family and contains a positive single-stranded RNA genome of 9.6 kb (Kato et al., 1990; Tanaka et al., 1996). The viral genome encodes a single polyprotein of approximately 3010 amino acid residues, which is proteolytically processed by host and viral proteases into at least 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Kato, 2001). HCV infection frequently causes chronic hepatitis C (CH C) and progresses to fatal cirrhosis and hepatocellular carcinoma. The current standard therapy for CH C is pegylated-interferon-• (PEG-IFN-•) and ribavirin. However, the cure rate of the therapy for the treatment of CH C is limited to approximately 50% (Firpi and Nelson, 2007). The major cause of resistance to this therapeutic approach was observed in genotype 1 HCVs. However, the mechanisms of the diverse sensitivity to IFN therapy among genotype 1 HCVs have remained unclear. Therefore, the development of more effective anti-HCV reagents is an urgent issue.

Since the HCV replicon system was developed by Lohmann et al. (1999), several groups have reported candidate anti-HCV reagents. Statin, a 3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, is one of the well-characterized anti-HCV reagents and its anti-HCV activity has been shown to be due to the inhibition of geranylgeranylation of host proteins (Ikeda et al., 2006; Kapadia and Chisari, 2005; Ye et al., 2003). Cyclosporine A (CsA), an immunosuppressant, is another well-characterized anti-HCV reagent that inhibits HCV RNA replication via its interaction with cyclophilins (CyPs) (Inoue et al., 2007; Nakagawa et al., 2005; Watashi et al., 2003). In addition to type I IFNs (• and •) and type II IFN (•), recently identified type III IFN (•) has been reported to possess anti-HCV activity in cell culture (Doyle et al., 2006; Marcello et al., 2006; Robek et al., 2005). Subgenomic HCV replicons have been reported since the breakthrough of the Con1(1b) replicon using different HCV strains: H77 (1a), N (1b), 1B-1 (1b), O (1b), JFH 1 (2a), and AH1 (1b) (Blight et al., 2003; Ikeda et al., 2002, 2005; Kato et al., 2003a,b; Kishine et al., 2002; Lohmann et al., 1999; Mori et al., 2008; Pietschmann et al., 2002). Moreover, a number of groups have examined anti-HCV reagents using the established replicon. However, such studies have been conducted using replicon(s) from only one or two HCV strain(s). To date, there has been no comprehensive study regarding the diverse sensitivities of anti-HCV reagents to genotype 1 HCV replicons from different strains.

To address this issue, we developed three HCV replicons from different genotype 1b HCV positive sera, in addition to our previously reported O strain (Ikeda et al., 2005). Two replicons were constructed using HCV-positive sera from healthy carriers (1B-4

* Corresponding author. Tel.: +81 86 235 7386; fax: +81 86 235 7392.
E-mail address: maikeda@md.okayama-u.ac.jp (M. Ikeda).

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and 1B-5) and one replicon was constructed using serum sampled from a case of acute hepatitis C (KAH5). These replicons contained neomycin phosphotransferase (Neo) and Renilla luciferase (RL) genes at the first cistron of the replicon with the aim of conducting a stable and highly sensitive reporter assay. In this study of four replicons, we examined the anti-HCV reagents IFN-• , IFN-• , IFN-• , CsA, and various statins (pitavastatin (PTV), fluvastatin (FLV), and rosvastatin (RSV)), and we found diverse sensitivities among the replicons. Newly developed replicons will be useful tools for the present study regarding the diverse sensitivities of genotype 1b HCVs to anti-HCV reagents, including IFNs.

2. Materials and methods

2.1. HCV-positive sera and GeneBank accession numbers

Serum O (previously described as 1B-2), 1B-4, and 1B-5 were derived from an HCV-positive healthy carrier and have been described previously (Ikeda et al., 1997). Serum KAH5 was obtained from a patient with acute hepatitis C (AH C) who provided prior informed consent. The nucleotide sequence data for 1B-4, 1B-5, and KAH5 will appear in the DDBJ, EMBL, and GeneBank nucleotide sequence databases under accession nos. AB442219, AB442220, and AB442222, respectively.

2.2. Cell cultures

Three HCV-positive sera (KAH5, 1B-4, and 1B-5 strains) were used for the development of subgenomic replicons with reporter (RL). We first established 9, 4, and 6 replicon harboring clonal cell lines derived from KAH5, 1B-4, and 1B-5 strains, respectively. Then, after characterization for these cell lines, we selected the representative clonal cell lines and designated sKAH5R (clone 6), s1B-4R (clone 2), and s1B-5R (clone 4) as sKAH5R, s1B-4R, and s1B-5R, respectively (Supplemental Fig. 1A, B, and C). sO and O cells were used as subgenomic and genome-length HCV RNA-harboring cells with a Neo gene in the first cistron, as previously described (Kato et al., 2003a; Ikeda et al., 2005). These cells were derived from a hepatoma cell line, HuH-7, and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and 0.3 mg/ml of G418 (Geneticin; Invitrogen, Carlsbad, CA). The cells were passaged twice weekly at a 5:1 split ratio. The sequences in the original subgenomic replicons were described above and appeared in the database with indicated accession numbers.

2.3. RT-nested PCR

HCV RNAs were prepared from HCV-positive sera (1B-4, 1B-5, and KAH5) using ISOGEN-LS (Nippon Gene Co., Tokyo, Japan) according to the manufacturer's protocol. These RNA samples were used for RT-PCR in order to amplify the NS2 to NS5B region (6.0 kb) of the HCV genomes. RT was performed with the OligodA23 primer, 5'-AAAAAAAAAAAAAAAAAAAAAAA-3'. The primer pair 542: 5'-GTAGAGCCCGTCGTCTTCTCTGACATGGA-3' and 9388R: 5'-ATGGCCTATTGGCCTGGAGTG-3' was employed in the first-round PCR (35 cycles). The primer pair 3295X: 5'-ATTATTCTAGACTGACA-TGGAGACCAAGATCATCAC-3' and 9357RX: 5'-ATTATTCTAGACCCG-TTCACCGGTTGGGGAGCAG-3', containing the XbaI site (underlined) was employed in the second-round PCR (35 cycles). SuperScript III reverse transcriptase (Invitrogen) and KOD-plus DNA polymerase (Toyobo, Osaka, Japan) were used for RT and PCR, respectively.

2.4. Plasmid construction

To construct an HCV replicon with RL and Neo genes, we used a previously described pRN/3-5B/KE plasmid as a cassette vector (Ikeda et al., 2005). Basically, the NS3 to NS5B region was replaced with RT-PCR products from sera with 1B-4, 1B-5, and KAH5 at SpeI (located in NS3) and BsiWI (located in NS5B) sites. The PCR products were further amplified with the primers NS3 SpeI: 5'-ATCATCACTAGTCTCACAGGCCGGGACAAGAAAC-3, containing the SpeI site (underlined); and NS5B BsiWI: 5'-CTTGGTCCGTACGGCCCAGTTGAAGAGGTACTTGC-3', containing the BsiWI site (underlined). The amplified fragments were digested with SpeI and BsiWI, and were ligated into the pRN/3-5B/KE cassette vector, which was predigested with SpeI and BsiWI.

2.5. RNA transcription

Plasmid DNAs were linearized by XbaI digestion and were used for RNA synthesis with T7 MEGAscript (Ambion) as previously described (Kato et al., 2003a).

2.6. RNA transfection and G418-resistant cells

Ten micrograms of in vitro synthesized HCV replicon RNAs were introduced into HuH-7 derived cells (OR6c cells) by electroporation, and the cells were selected in the presence of G418 (0.3 mg/ml) for 3 weeks as described previously (Mori et al., 2008).

2.7. Western blot analysis

The preparation of cell lysates, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting were performed as described previously (Kato et al., 2003a). The antibodies used in this study were those against Core, NS3, NS5A, and NS5B. • -actin antibody (AC-15, Sigma) was used as a control for the amount of protein loaded per lane. Immunocomplexes were detected with the Renaissance enhanced chemiluminescence assay (PerkinElmer Life Science, Boston, MA).

2.8. Quantification of HCV RNA

The RNAs were prepared from an HCV replicon RNA replicating cell line, and 2 • g of each total RNA was used for RT-qPCR with 5'-UTR of an HCV-specific primer pair, as described previously (Ikeda et al., 2005). Experiments were conducted in triplicate.

2.9. Northern blot analysis

Total RNA was extracted from the cultured cells using an RNeasy Mini Kit according to the manufacturer's protocol (QIAGEN). Three micrograms of total RNA were used for the analysis. HCV-specific RNA and • -actin were detected according to a previously described method (Ikeda et al., 2005).

2.10. Reagents

IFN-• and IFN-• were purchased from Sigma, and CsA was obtained from Calbiochem (San Diego, CA). IFN-• (IL-29) was purchased from WAKO. PTV was purchased from the Kowa Company, Ltd. (Tokyo, Japan). FLV was purchased from Calbiochem. RSV was obtained from AstraZeneca.

2.11. Luciferase reporter assay

For the luciferase assay, 1.0–1.5 • 10^4 HCV replicon-harboring cells were plated onto 24-well plates in triplicate and were cultured

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for 24 h. The cells were treated with each anti-HCV reagent for 72 h. Then the cells were harvested with Renilla lysis reagent (Promega, Madison, WI) and subjected to RL assay according to the manufacturer's protocol. All the luciferase assays were repeated at least three times.

2.12. Statistical analysis

Statistical comparison of the luciferase activity in various treatment groups was performed using Student's t-test. P values of less than 0.05 were considered statistically significant.

3. Results

3.1. Establishment of four subgenomic replicon-harboring cell lines using different genotype 1b HCV sera

We tried to establish replicon-harboring cells from different HCV-positive sera to assess the sensitivity of anti-HCV reagents among genotype 1b HCV strains. To this end, three sera (1B-4, KAH5, and 1B-5) were used to amplify the NS region of HCV genomes by reverse transcription-polymerase chain reaction (RT-PCR). The dicistronic replicons were designed as shown in Fig. 1A. RL and Neo genes were introduced into the first cistron and translation was driven by the HCV internal ribosomal entry site (IRES) leading to the expression of RL and Neo as a fusion protein. In the second cistron, NS3 to NS5B was translated via the encephalomyocarditis virus (EMCV) IRES (Fig. 1A). We introduced in vitro-synthesized HCV replicon RNAs (10 • g) into OR6c cells, in which HCV RNA was eliminated from OR6 cells by IFN-• treatment. After 3 weeks of G418 selection, we obtained HCV replicon-harboring cell colonies, i.e., more than 100 colonies from KAH5 and 20 colonies from 1B-4. However, no colony formation was observed among 1B-5 replicon-RNA-introduced cells. Therefore, we next attempted to perform the electroporation of a 1B-5 replicon with mutations derived from the HCV sequence in s1B-5 replicon-harboring cells, in which the replicating HCV replicon possessed only neomycin-resistant genes in the first cistron (data not shown). The mutations introduced into 1B-5 replicon were E1758D and I1851F in NS4B and R2192W and E2414Q in NS5A. Consequently, we established 9, 4, and 6 replicon-harboring cells from KAH5, 1B-4, and 1B-5, respectively, and confirmed the expression of HCV RNA and proteins. In addition to three replicon RNAs, the previously described ORN/3-5B/KE replicon RNA was also introduced into OR6c cells and selected as sOR in this study (Ikeda et al., 2005). The representative clonal cell lines, which grow healthy and stably expressed abundant HCV proteins, are used in the following experiments (Supplemental Fig. 1A, B, and C). These replicon-harboring cell lines were established from genotype 1b HCV strains: 1B-4, KAH5, O, and 1B-5 and were designated as s1B-4R, sKAH5R, sOR, and s1B-5R, respectively. We confirmed the expression of NS3, NS5A, and NS5B proteins in all replicon-harboring cells (Fig. 1B). The expression levels of HCV RNAs in the replicon-harboring cells were examined for the 5'-UTR by quantitative RT- PCR (RT-qPCR) (Fig. 1C). s1B-4R cells exhibited the highest levels of expression of HCV RNA (approximately 10^8 copies/• g total RNA), followed by sKAH5R, sOR, and s1B-5R cells (Fig. 1C). All of the replicon-harboring cells expressed HCV RNA at levels greater than at least 4 • 10^7 copies/• g total RNA. Northern blot analysis also demonstrated the presence of HCV-specific RNA with a length of approximately 9 kb in the total RNA extracts from four replicon-harboring cells (Fig. 1D). These four genotype 1b HCV replicon reporter systems were established and used for further analyses of sensitivity to anti-HCV reagents.

3.2. Diverse activity of various IFN types on HCV replicons

IFN-• belongs to the type I IFN group and is currently used as standard therapy for patients with CH C. Therefore, first we evaluated the activity of IFN-• using the four developed replicons and a reporter assay. The s1B-4R and sKAH5R replicons showed almost equal and moderate sensitivity to IFN-• (EC_{50}: 1.44 and 1.37 IU/ml, respectively) (Fig. 2). The s1B-5R and sOR replicons, respectively, exhibited the highest (EC_{50}: 1.10 IU/ml) and lowest (EC_{50}: 2.35 IU/ml) sensitivity to IFN-• among the replicons tested (Fig. 2). We also examined the activity of IFN-• on HCV protein expression levels in these four replicons. The findings from the Western blot analysis of the sensitivity to IFN-• coincided with

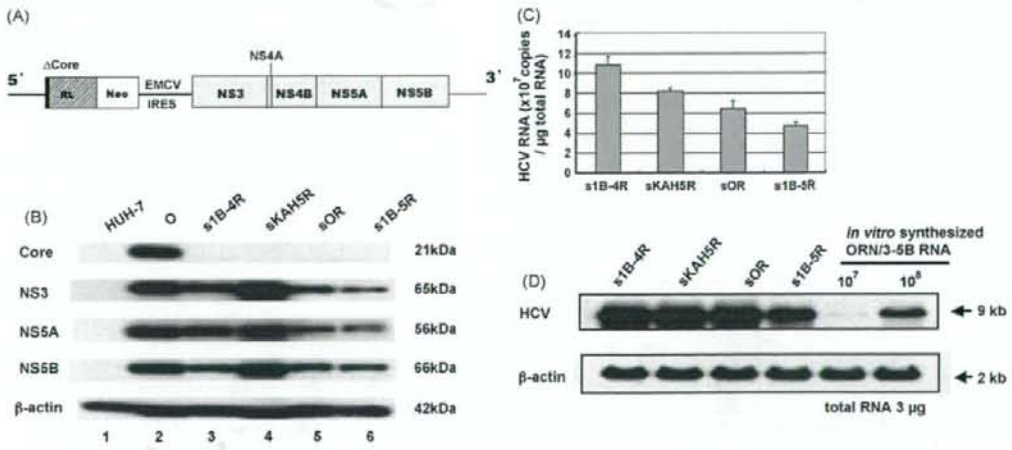

Fig. 1. The expression of HCV proteins and HCV RNAs in four replicon-harboring cell lines. (A) Schematic gene organization of subgenomic replicon RNA. The NS3 to NS5B region and 12 N-terminal amino acid residues of the Core (• C) are depicted in closed boxes. Untranslated regions, EMCV IRES, RL, and Neo genes are indicated by thin lines, thick line, shaded box, and open box. (B) Western blot analysis of HCV proteins. Production of Core, NS3, NS5A, and NS5B in HuH-7 cells (lane 1), O cells (lane 2), s1B-4R cells (lane 3), sKAH5R cells (lane 4), sOR cells (lane 5), and s1B-5R cells (lane 6) were analyzed by immunoblotting using anti-Core, anti-NS3, anti-NS5A, and anti-NS5B antibodies. (C) RT-qPCR analysis. The levels of HCV RNA in G418-resistant cells were quantified by LightCycler PCR. (D) Northern blot analysis. RNAs from s1B-4R, sKAH5R, sOR, and s1B-5R cells were used for comparison. In vitro-synthesized ORN/3-5B RNA was also used for comparative analyses.

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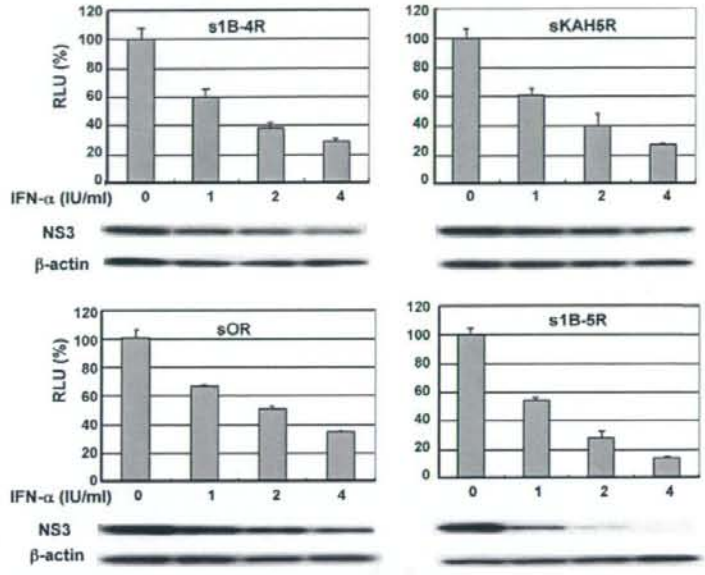

Fig. 2. The activity of IFN-• on HCV replicon RNA replication. Reporter assay and Western blot analysis for HCV replicons in IFN-• treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with IFN-• (0, 1, 2, and 4 IU/ml) for 72 h. Then, the cells were subjected to RL assay (upper panels) and Western blot analysis (lower panels). The percent relative luciferase unit (RLU (%)) was calculated with the RL activity of untreated cells assigned at a value of 100%. The data indicate means • S.D.s of triplicate samples. All of the luciferase assays were repeated at least three times. • -Actin was used as a control for the amount of proteins loaded per lane.

the results of the reporter assay. Thus, these results indicated that genotype 1b replicons possess different sensitivities to IFN-• .

Next, we examined the sensitivity of four replicons to type II IFN, IFN-• , because in our previous study, HCV (genotype 1b, AH1 strain) from a patient with AH C was found to be more resistant to IFN-• than was HCV-O (Mori et al., 2008). In this study, sKAH5R was also derived from the serum of a patient with AH C. The reporter assay revealed that sKAH5R has the lowest sensitivity to IFN-• (EC_{50}: 2.26 IU/ml) among the replicons tested (Fig. 3). To calculate the EC_{50} of IFN-• to sKAH5R, we also treated sKAH5R with IFN-• at 2 and 4 IU/ml for 72 h (data not shown). The EC_{50} of IFN-• to s1B-4R, sOR, and s1B-5R was 0.54, 0.33, and 0.21 IU/ml, respectively. The results

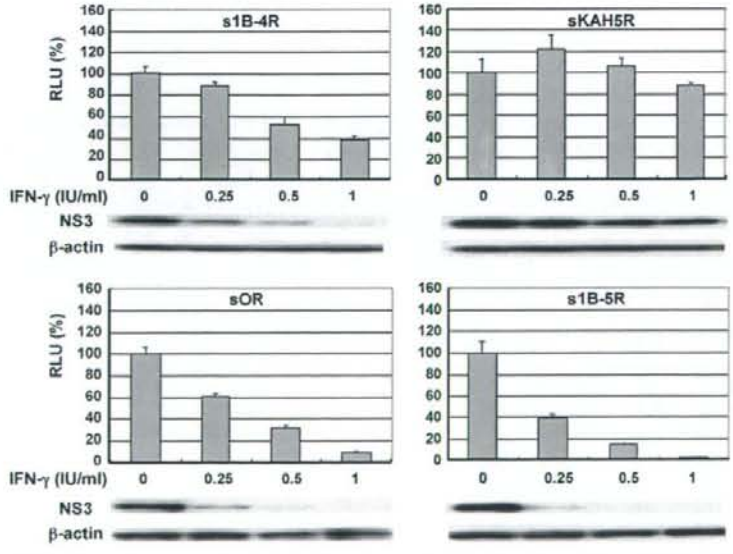

Fig. 3. The activity of IFN-• on HCV replicon RNA replication. Reporter assay and Western blot analysis for HCV replicons in IFN-• treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with IFN-• (0, 0.25, 0.5, and 1 IU/ml) for 72 h and then the cells were subjected to RL assay and Western blot analysis. All of the luciferase assays were repeated at least three times.

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of Western blot analyses of sensitivity to IFN-• coincided with those of the reporter assay. Interestingly, again in this study, the HCV RNA derived from the patient with AH C was resistant to IFN-• , as was the AH1 strain. These results may suggest that AH C in pathologic states of HCV infection may be involved in the IFN-• resistance feature of the replicon. Further studies will be needed to clarify this issue.

We analyzed a recently identified type III IFN, IFN-• , in terms of its anti-HCV activity against four HCV replicons. IFN-• shares the same Jak/Stat signaling pathway with type I IFNs, which express a common set of IFN-stimulating genes (ISGs). However, IFN-• uses distinct receptors composed of IFNLR1 and IL10R2. Here, sKAH5R and s1B-4R, respectively, exhibited the lowest and highest sensitivities to IFN-• (EC_{50}: 8.25 and 1.50 ng/ml) (Fig. 4A). Additionally, sOR and s1B-5R exhibited moderate sensitivity to IFN-• (EC_{50}: 4.48 and 4.82 ng/ml, respectively) (Fig. 4A). These diverse inhibitory activities of IFN-• were also confirmed by Western blot analysis (Fig. 4B). Moreover, s1B-4R and sKAH5R showed similar sensitivities to IFN-• . However, it was of note that these replicons exhibited different degrees of sensitivity to IFN-• , which uses a common Jak/Stat signaling pathway. These results suggest the presence of a complicated antiviral mechanism in type I and III IFNs. Recently, it was reported that IFN-• in combination with IFN-• or IFN-• enhanced anti-HCV activity (Pagliaccetti et al., 2008). Therefore, s1B-4R and sKAH5R are useful for the study in combination treatment of IFNs.

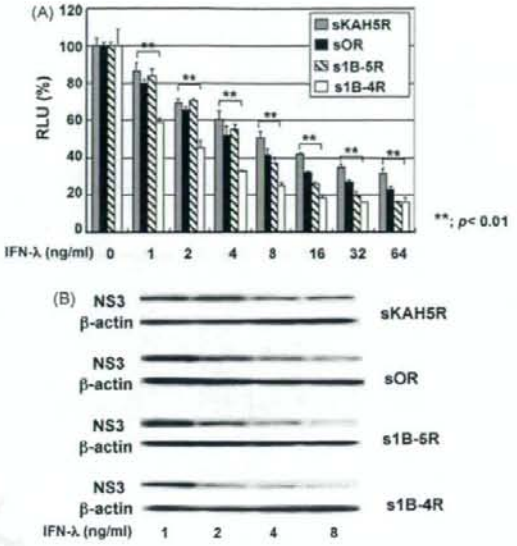

Fig. 4. Effects of IFN-• on HCV replicon RNA replication. (A) Reporter assay and Western blot analysis for HCV replicons in IFN-• treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with IFN-• (0, 2, 4, 8, 16, 32, and 64 ng/ml) for 72 h, and then the cells were subjected to RL assay (B) and Western blot analysis. Four replicon-harboring cell types were treated with IFN-• (0, 2, 4, and 8 ng/ml) for 72 h and were subjected to Western blot analysis of NS3. All of the luciferase assays were repeated at least three times.

3.3. Diverse effects of PTV but not CsA on HCV replicons

Anti-HCV reagents other than IFNs were examined in terms of their effectiveness in the presence of various replicons. As CsA is a well-characterized anti-HCV reagent, we examined the sensitivities of the replicons to CsA by reporter assay. There were no significant differences in sensitivity to CsA among the replicons (Fig. 5). The EC_{50} of CsA to s1B-4R, sKAH5R, sOR, and s1B-5R was 0.71, 0.96, 1.10, and 0.85 • g/ml, respectively. We also obtained similar results by Western blot analysis. In contrast to the findings of the IFN study, there were no significant differences in sensitivity to CsA among the genotype 1b replicons tested.

Statins, which are HMG-CoA reductase inhibitors, are yet another well-characterized anti-HCV reagent. Therefore, we

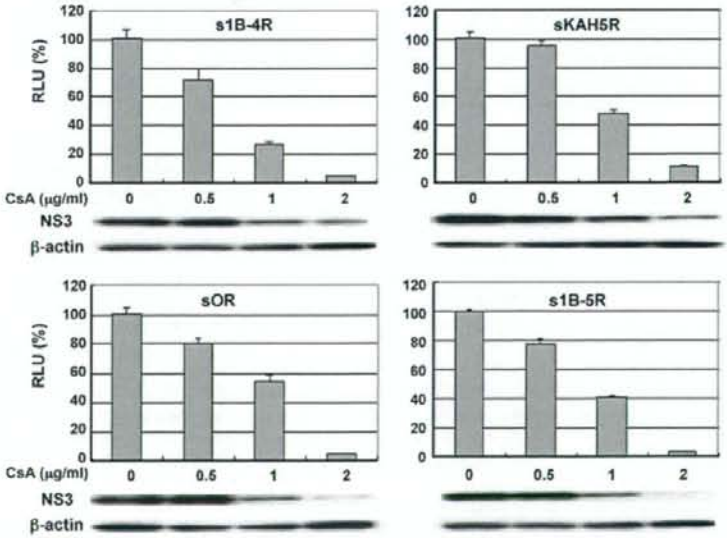

Fig. 5. The activity of CsA on HCV replicon RNA replication. Reporter assay and Western blot analysis for HCV replicons in CsA treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with CsA (0, 0.5, 1, and 2 • g/ml) for 72 h, and then the cells were subjected to RL assay (upper panels) and Western blot analysis for NS3 (lower panels), as described in Fig. 2. All of the luciferase assays were repeated at least three times.

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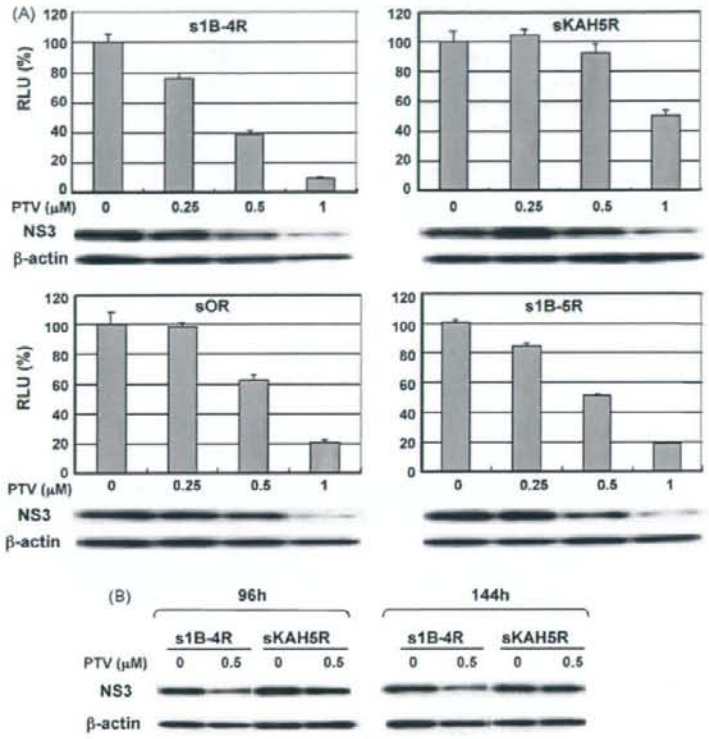

Fig. 6. The activity of PTV on HCV replicon RNA replication. (A) Reporter assay and Western blot analysis for HCV replicons in PTV treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with PTV (0, 0.25, 0.5, and 1 • M) for 72 h, and then the cells were subjected to RL assay and Western blot analysis, as described above. All of the luciferase assays were repeated at least three times. (B) s1B-4R cells and sKAH5R cells were treated with PTV (0 and 0.5 • M) for 96 and 144 h and were subjected to Western blot analysis.

examined the sensitivity of the replicons to PTV. The reporter assay revealed that sKAH5R has the lowest sensitivity to PTV (EC_{50}: 1.00 • M) among the replicons tested (Fig. 6A). The sensitivities of the other replicons to PTV were almost identical, and the EC_{50} values of s1B-4R, sOR, and s1B-5R were 0.40, 0.64, and 0.51 • M, respectively (Fig. 6A). We also obtained similar results by Western blot analysis using cell lysates at 72 h after treatment. The inhibition of HCV protein in s1B-4R persisted until 96 and 144 h after treatment with PTV (0.5 • M) (Fig. 6B). FBL2 is identified as a geranylgeranylated cellular protein required for HCV RNA replication (Wang et al., 2005). Therefore, we examined the expression levels of FBL2 in s1B-4R and sKAH5R. The expression levels of FBL2 mRNA were almost equal between both cells (Supplemental Fig. 2). This result indicates that low sensitivity of sKAH5R to statins is not due to the low expression of FBL2. Previously, we used an OR6 assay system to demonstrate that PTV inhibited genome-length HCV RNA replication, and the EC_{50} of PTV was found to be 0.45 • M (Ikeda et al., 2006; Ikeda and Kato, 2007). The EC_{50} values of PTV in three replicons other than sKAH5R were almost equal to that of PTV in OR6. These results, taken together, suggest that sKAH5R is resistant to PTV as well as to IFN-• and IFN-• .

3.4. Resistance to statins in a replicon from a patient with AH C

To further confirm that sKAH5R is resistant to statins, we examined the sensitivity of the replicons to FLV and RSV using a reporter assay. Here, sKAH5R exhibited the lowest sensitivity to FLV and RSV (Fig. 7). In the case of sKAH5R, the EC_{50} of FLV was 7.87 • M, and the EC_{50} of RSV exceeded 20 • M, because RSV was toxic to cells at concentrations of more than 20 • M. Moreover, sOR and s1B-5R showed almost equal and moderate sensitivities to both FLV and RSV. It was of note that these results were in agreement with those regarding PTV sensitivity, i.e., s1B-4R exhibited the highest sensitivity to both FLV and RSV. The EC_{50} values of FLV and RSV to s1B-4R were 2.82 and 10.12 • M, respectively. These results suggest that sKAH5R exhibits some resistance, and s1B-4R some sensitivity, to statins. Therefore, these replicons may serve as useful tools for investigating the mechanism of the anti-HCV activity of statins.

3.5. Polyclonal KAH5 replicon with a statin-resistant phenotype

sKAH5R replicon cells were found to possess the least sensitivity to statins among the replicon-harboring cells tested. However, the statin-resistant phenotype may be due to cell clonality rather than HCV strain, because the sKAH5R replicon cells used here were a cloned cell line selected from numerous G418-resistant colonies. We thus examined the sensitivity of polyclonal sKAH5R cells to statins, and then compared the results with those obtained using polyclonal s1B-4R cells in order to rule out this possibility. In polyclonal sKAH5R, the EC_{50} values of PTV and FLV were 0.88 and 6.56 • M, respectively (Fig. 8), and the EC_{50} of RSV exceeded 20 • M (Fig. 8), because RSV is toxic to these cells at concentrations of more than 20 • M. In polyclonal s1B-4R, the EC_{50} values of PTV, FLV, and RSV were 0.47, 3.41, and 10.00, respectively (Fig. 8). The polyclonal

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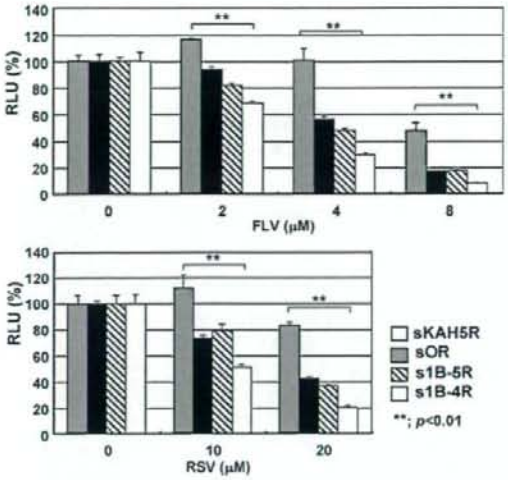

Fig. 7. HCV replicons exhibit diverse sensitivities to statins. Reporter assay of the sensitivity of HCV replicons to FLV. sKAH5R cells (light column), sOR cells (dark column) s1B-5R cells (shaded column), and s1B-4R cells (open column) were treated with FLV (0, 2, 4, and 8 • M) for 72 h (upper panel), and then the cells were subjected to an RL assay. A reporter assay of RSV sensitivity to HCV replicons was performed using RSV (0, 10, and 20 • M) (lower panel). All of the luciferase assays were repeated at least three times.

sKAH5R cells exhibited less sensitivity to PTV, FLV, and RSV than did polyclonal s1B-4R cells. These results suggest that the statin-resistant phenotype of sKAH5R is due to the KAH5 strain-specific viral factors rather than to the cell clonality of sKAH5R cells.

3.6. Second generation of sKAH5R possessed less sensitive phenotype to PTV than that of s1B-4R

To further demonstrate that the statin-resistant phenotype of sKAH5R is not due to the clonal specificity of the cells, we developed the second generation of sKAH5R and s1B-4R. Total RNAs from sKAH5R and s1B-4R were introduced into naïve OR6c cells. The second generation of sKAH5R and s1B-4R, designated as ssKAH5R and ss1B-4R, respectively, were selected as the polyclonal cells after 3 weeks G418 selection. ssKAH5R revealed less sensitive to PTV than ss1B-4R (EC_{50}: 0.76 • M vs. 0.43 • M) (Fig. 9A). These results further support that the viral factor plays the major role in the statin-resistant phenotype of sKAH5R.

On the contrary, there was no significant difference between ssKAH5R and ss1B-4R in the sensitivity to IFN-• (EC_{50}: 4.1 ng/ml vs. 3.5 ng/ml) (Fig. 9B). These results suggest that cellular factors are dominant in the sensitivity to IFN-• .

Table 1
EC_{50} of anti-HCV reagents to HCV replicons.

	s1B-4R	sKAH5R	sOR	s1B-5R
IFN-• (IU/ml)	1.44	1.37	2.35	1.10
IFN-• (IU/ml)	0.54	2.26	0.33	0.21
IFN-• (ng/ml)	1.50	8.25	4.48	4.82
CsA (• g/ml)	0.71	0.96	1.10	0.85
PTV (• M)	0.40	1.00	0.64	0.51
FLV (• M)	2.82	7.87	4.53	3.81
RSV (• M)	10.12	ND	17.52	17.10

ND: not determined.

4. Discussion

In the present study, we established an HCV replicon reporter assay system using four genotype 1b HCV strains (1B-4, KAH5, O, and 1B-5). Genotype 1 HCV infection accounts for most cases of resistance to current PEG-IFN-• and ribavirin therapy. However, in most previous reports, anti-HCV reagents have been assessed in terms of their effects using replicon(s) derived from only one or two HCV strain(s). Therefore, in order to further evaluate the anti-HCV activity of various reagents among the genotype 1b HCVs, we performed a comparative study using the present replicon reporter assay system, which was found to a precise, highly sensitive, and time-sparing assay compared to assays involving the quantification of HCV RNA. The EC_{50} values of anti-HCV reagents in four genotype 1b replicons are summarized in Table 1.

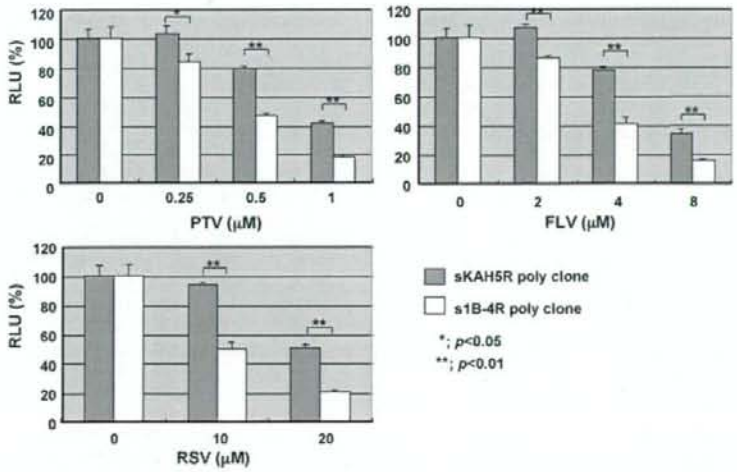

Fig. 8. Diverse sensitivities of polyclonal replicons to statins. Reporter assay of the sensitivity of polyclonal sKAH5R and s1B-4R replicons to PTV, FLV, and RSV. Polyclonal sKAH5R cells and polyclonal s1B-4R cells were treated with PTV (0, 0.25, 0.5, and 1 • M), FLV (0, 2, 4, and 8 • M), and RSV (0, 10, and 20 • M) for 72 h and then were subjected to RL assay. All the luciferase assays were repeated at least three times.

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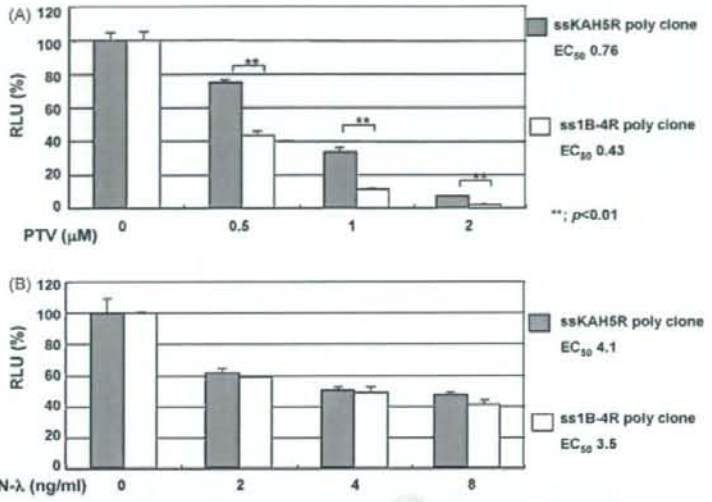

Fig. 9. The sensitivities of the second generation of sKAH5R and s1B-4R to PTV and IFN-• . Polyclonal second generations of sKAH5R (ssKAH5R) and s1B-4R (ss1B-4R) were treated with PTV (0, 0.5, 1, and 2 • M) (A) and IFN-• (0, 2, 4, and 8 ng/ml) (B) for 72 h and then were subjected to RL assay. All the luciferase assays were repeated at least three times.

Here, sOR exhibited the lowest level of sensitivity to IFN-• (EC_{50}: 2.35 IU/ml). In the clinical setting, high titers of HCV RNA are among the determining factors for IFN resistance. However, the sensitivity to IFN-• was found to be greater in the case of s1B-4R and sKAH5R than in sOR, although HCV RNA titers were higher than those of sOR. These results suggest that factor(s) other than the HCV RNA level may be involved in conferring sensitivity to IFN-• , and the genetic background of sOR may serve as a candidate for interpreting differences in IFN-• sensitivity among the genotype 1b HCVs tested. Previously the structural region of HCV was shown to be involved in viral resistance to type I IFN (Taylor et al., 1999). Therefore, the development of genome-length HCV RNA reporter systems from 1B-4, 1B-5, and KAH5R strains in addition to our developed OR6 cells will overcome the limitation of subgenomic replicon and will become powerful tool for the study of anti-HCV reagents including IFNs. Now we are planning to develop the genome-length HCV RNA reporter systems using these three HCV strains.

sKAH5R exhibited the lowest level of sensitivity to IFN-• (EC_{50}: 2.26 IU/ml) among the replicons tested, as it is approximately 10 times more resistant to IFN-• than s1B-5R (EC_{50}: 0.21 IU/ml). KAH5 was the only HCV strain derived from a patient with AH C in the present study. In our previous study, an AH1 strain derived from a patient with AH C also exhibited lower sensitivity to IFN-• (EC_{50}: 1.9 IU/ml) than did O strain (EC_{50}: 0.3 IU/ml) in the genome-length HCV RNA replication system (Mori et al., 2008). In both subgenomic and genome-length HCV RNA replication systems, HCV strains from patients with AH C possess less sensitivity to IFN-• than do HCV strains from healthy carriers. These results suggest that the NS region of HCV derived from AH C may be involved in IFN-• resistance.

In 2003, IFN-• was identified by two groups at the same time, and this novel IFN was classified as type III IFN. IFN-• shares the Jak/Stat signaling pathway with the type I IFNs, although they bind to distinct membrane receptors, i.e., type I IFNs bind to the heterodimer of IFNAR1 and IFNAR2, whereas type III IFNs bind to the heterodimer of IFNLR1 and IL10R2 (Uze and Monneron, 2007). Therefore, we expected to obtain similar IFN-• sensitivity results in the four replicons tested here. However, unexpectedly, the profiles of replicon sensitivity to IFN-• and IFN-• differed. The sensitivity of s1B-4R to IFN-• was approximately five times greater than that of sKAH5R, although the sensitivities of these replicons to IFN-• were almost identical. There are several possible interpretations of these unexpected findings. First, an unidentified branched signaling pathway may account for variation in the anti-HCV activity of IFN-• . Second, expression levels of the receptor for IFN-• may vary. The second generation replicon assays suggest that the cellular factors may be dominant in the sensitivity to IFN-• . Further study will be needed to clarify this issue. The anti-HCV activity of IFN-• has already been reported by several groups using HCV RNA-harboring cells (Doyle et al., 2006; Marcello et al., 2006; Robek et al., 2005). The present study was the first to demonstrate the diverse anti-HCV activities of IFN-• on HCV replicons.

In the case of CsA, we did not observe any significant differences among the genotype 1b replicons. Using a genome-length HCV RNA replication system, we recently demonstrated that an AH1 strain obtained from an AH C patient showed greater sensitivity than did an O strain (Mori et al., 2008). There are two possible explanations for this high sensitivity to CsA in the replicon derived from the AH C case: first, a high degree of sensitivity to CsA may not be a common feature in HCV from AH C and may instead be strain-dependent; second, a particular structural region of HCV from AH C may be responsible for this high level of sensitivity to CsA. However, further study will be needed to fully account for these findings.

Respectively, sKAH5R and s1B-4R exhibited the lowest (EC_{50}: 1.00• M) and highest (EC_{50}: 0.40• M) levels of sensitivity to PTV among the replicons tested. We also confirmed that sKAH5R and s1B-4R possessed the lowest and highest levels of sensitivity, respectively in both FLV and RSV treatment. Therefore, resistance to statins may be a unique feature of sKAH5R. It should be noted that we obtained these results using polyclonal sKAH5R and polyclonal s1B-4R cells. The polyclonal sKAH5R cells were less sensitive to PTV, FLV, and RSV than were the polyclonal s1B-4R cells. Therefore, the statin-resistant phenotype of sKAH5R cells is due to a KAH5 strain-specific characteristic, rather than to the clonality of the cells. The second generation of replicon harboring cells, ssKAH5R and ss1B-4R, further supported that the viral factor plays the major role in the statin-resistant phenotype of sKAH5R. These two cell lines with contrasting sensitivity to statins promise to be useful for

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determining the statin resistance-responsible region of HCV and also for investigating the anti-HCV mechanism of statins in general.

In the present study, we demonstrated the diverse profiles of four HCV replicons to anti-HCV reagents. sKAH5R showed the lowest sensitivity to IFN-•, IFN-•, and statins (PTV, RSV, and FLV). In contrast, s1B-4R exhibited the highest level of sensitivity to IFN-• and statins (PTV, RSV, and FLV). sKAH5R and s1B-4R possessed a sensitive and a resistant phenotype to various anti-HCV reagents. The nucleotide sequences in the NS3-NS5B regions of 1B-4, 1B-5, and KAH5 strains showed differences of 6.5%, 8.6%, and 6.1%, respectively, from those of the O strain. Similarly, the amino acid sequences in the NS3–NS5B regions of 1B-4, 1B-5, and KAH5 strains showed differences of 2.6%, 4.7%, and 2.5%, respectively, from those of the O strain. Phylogenetic analysis revealed that O, 1B-4, and KAH5 strains formed the cluster different from 1B-5 strain (Supplemental Fig. 3). These data indicate that sKAH5R and s1B-4R are at a similar genetic distance from the O strain. These two replicons were also found to possess similar features in terms of HCV RNA expression levels and sensitivity to IFN-• . Therefore, sKAH5R and s1B-4R are expected to be useful tools for comparative analyses of anti-HCV determining factors of HCV, especially as regards IFN-• and statins.

In conclusion, we established an HCV replicon reporter assay system with four different genotype 1b HCV strains. This replicon system is a useful tool for investigating differences in sensitivity to anti-HCV reagents among genotype 1b HCV strains, and it is expected to increase the rate of resolution of HCV cases otherwise resistant to current IFN therapy.

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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.antiviral.2009.01.007.

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ORIGINAL ARTICLE

Genetic variability and diversity of intracellular genome-length hepatitis C virus RNA in long-term cell culture

Nobuyuki Kato · K. Abe · K. Mori · Y. Ariumi · H. Dansako · M. Ikeda

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Abstract Hepatitis C virus (HCV) is known to circulate persistently in vivo as a complex population of different but closely related viral variants. To understand the quasispecies nature of HCV, we performed genetic analysis of intracellular HCV RNAs obtained in long-term cell culture of genome-length HCV-RNA-replicating cells. The results revealed that genetic mutations in HCV RNAs accumulated in a time-dependent manner, and that the mutation rates of HCV RNAs were 3.5–4.8 × 10^{-3} base substitutions/site/year. The mutation rates of nonstructural regions that are essential for RNA replication were lower than those of structural regions. The genetic diversity of HCVs was also enlarged in a time-dependent manner. Furthermore, we found that the GC content of HCV RNA was increased in a time-dependent manner. These results suggest that an HCV-RNA-replicating cell culture system would be useful for analysis of the evolutionary dynamics and variations of HCV.

Introduction

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. Such persistent infection has now become a serious health problem, with more than 170 million people worldwide currently infected with HCV [23]. HCV is an enveloped, positive single-stranded RNA (9.6 kb) virus belonging to the family *Flaviviridae*, and the HCV genome encodes a large polyprotein precursor of approximately 3,000 amino acid (aa) residues [9]. This polyprotein is cleaved by a combination of the host and viral proteases into at least 10 proteins in the following order: core, envelope 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [5, 6, 8].

The most characteristic feature of the HCV genome is its remarkable diversity and variation. To date, more than six genotypes and multiple subtypes, which show more than 20% difference at the nucleotide level compared with any of the other subtypes, have been identified worldwide [4, 19]. An approximately 5–8% difference at the nucleotide level is observed within a single genotype [8]. Furthermore, an approximately 1% difference at the nucleotide level is also observed among HCV genomes in an individual [20]. Regarding variations of the HCV genome, three reports using specimens from chimpanzees [16, 18] and a human patient [17] have estimated that the mutation rate of the HCV genome was 1.4–1.9 × 10^{-3} base substitutions/site/year. Since the selective pressure of the immune system functions in vivo [10, 24], an experimental system of HCV replication is needed to define the actual mutation frequency of HCV RNA.

We considered that the cell-culture-based HCV replicon system developed in 1999 [15] would be useful as an experimental system for analysis of the genetic variations and diversity of HCV, since it has been shown that HCV subgenomic RNA (so-called replicon RNA) containing the NS3–NS5B regions could autonomously and efficiently replicate in a human hepatoma cell line, HuH-7, using this

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N. Kato (✉) · K. Abe · K. Mori · Y. Ariumi · H. Dansako · M. Ikeda
Department of Tumor Virology,
Okayama University of Graduate School of Medicine,
Dentistry, and Pharmaceutical Sciences, 2-5-1 Shikata-cho,
Okayama 700-8558, Japan
e-mail: nkato@md.okayama-u.ac.jp

HCV replicon system [3]. The replicon RNA is a selectable, bicistronic HCV RNA with the first cistron, the neomycin phosphotransferase (Neo^R) gene, being translated under control of the HCV internal ribosome entry site (IRES) and the second cistron, the NS3–NS5B regions, being translated under control of the encephalomyocarditis virus (EMCV) IRES. Therefore, we previously performed genetic analyses of HCV variation and diversity using HCV replicon systems [11, 13] developed using two HCV strains, 1B-1 and HCV-O [12]. In that study, HCV-replicon-harboring cells were cultured for 18 months (1B-1 strain) or 12 months (HCV-O strain), and, using these cell cultured specimens, the mutation rates of both HCV replicons were estimated to be approximately 3.0×10^{-3} base substitutions/site/year. The genetic diversity of both replicons was also enlarged during long-term cell cultures [12]. However, it is unclear that the obtained results reflect the variations and diversity of the whole HCV genome, since the HCV replicon lacks the core–NS2 regions (half of the HCV genome). Furthermore, information regarding the genetic variation and diversity of the core–NS2 regions is needed in order to understand the dynamics of the whole HCV genome. To clarify this point, recently established genome-length HCV RNA (HCV-O strain)-replicating cell lines, HuH-7-derived O, OA, OB, OD, and OE [1, 7], were used for this study. There is no evidence that infectious HCV particles are released into the supernatants of genome-length HCV-RNA-replicating cells (O–OE). Since genome-length HCV-RNAs possessing cell-line-specific adaptive mutations that enhance the efficiency of RNA replication efficiently replicated in these five kinds of cells, we cultured these cells for 2 years and comprehensively analyzed the variations and diversity of the whole intracellular HCV genome. Here, we report the evolutionary HCV dynamics occurring in long-term replication of genome-length HCV RNAs.

Materials and methods

Cell cultures

The O, OA, OB, OD, and OE cells supporting genome-length HCV RNAs were cultured in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10%) and G418 (0.3 mg/ml). These cells were passaged every 7 days for 2 years.

Northern blot analysis

Total RNAs from the cultured cells were prepared using an RNeasy extraction kit (Qiagen, Hilden, Germany). Total RNA (3 μg) was used to detect the genome-length HCV RNA and β-actin mRNA (for check the amount of RNA). Northern blotting and hybridization were performed using a positive-stranded HCV-genome-specific RNA probe (NS5B region) and a β-actin-specific probe, as described previously [12].

Quantification of HCV RNA

The reverse transcription (RT)-quantitative PCR (RT-qPCR) analysis for HCV RNA was performed using LightCycler PCR as described previously [8]. Experiments were done in triplicate.

Western blot analysis

The preparation of cell lysates, SDS-PAGE, and immunoblotting analysis with a PVDF membrane were performed as described previously [6]. The antibodies used to examine the expression levels of HCV proteins were those against core [CP9, CP11, and CP14 monoclonal antibodies (Institute of Immunology, Tokyo); a polyclonal antibody (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science, Japan)], E1 and NS5B (generous gifts from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science, Japan). The epitopes of CP9, CP11, and CP14 were located within aa positions 39–74, 21–40, and 5–40 of the core protein, respectively. Anti-β-actin antibody (AC-15; Sigma, St. Louis, MO) was also used to detect β-actin as an internal control. Immunocomplexes on the membranes were detected by enhanced chemiluminescence assay (Renaissance; Perkin-Elmer Life Sciences, Boston, MA).

RT-PCR and sequencing

To amplify genome-length HCV RNA, RT-PCR was performed separately in two fragments as described previously [7]. Briefly, one fragment covered from 5′-UTR to NS3, with a final product of approximately 5.1 kb, and the other fragment covered from NS2 to NS5B, with a final product of approximately 6.1 kb. These fragments overlapped at the NS2 and NS3 regions and were used for sequence analysis of the HCV open reading frame (ORF) after cloning into pBR322MC [11]. SuperScript II (Invitrogen, Carlsbad, CA) and KOD-plus DNA polymerase (Toyobo, Osaka, Japan) were used for RT and PCR, respectively. Plasmid inserts were sequenced in both the sense and antisense directions using Big Dye terminator cycle sequencing on an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA). The nucleotide sequences of each of the three independent clones obtained were determined.

Molecular evolutionary analysis

Nucleotide and deduced amino acid sequences of the clones obtained by RT-PCRs were analyzed by neighbor-joining analysis using the program GENETYX-MAC (Software Development, Tokyo, Japan).

Results

Efficient replication of genome-length HCV RNA is maintained in long-term cell culture

To prepare the specimens for the genetic analysis of HCV, genome-length HCV-RNA-replicating O, OA, OB, OD, and OE cells were cultured for 2 years. The cell-line-specific and conserved adaptive mutations, K1609E, E1202G, P1115L, Q1112R, and P1115L, in the NS3 region were detected in the O, OA, OB, OD, and OE cells, respectively, when these cell lines were established [1, 7]. Using the specimens obtained at 0, 1, and 2 years in culture of O, OA, OB, OD, and OE cells, the levels of genome-length HCV RNAs were examined by Northern blot analysis (Fig. 1a) and RT-qPCR analysis (Fig. 1b). As shown in Fig. 1a, genome-length HCV RNAs approximately 11 kb long were detected in all specimens except that from HuH-7 parental cells, although the strength of the detected bands was weak in some cases. However, RT-qPCR analysis revealed that at least approximately 2×10^7 copies/μg RNA were present in the cultured cells (Fig. 1b). The results of RT-qPCR were well correlated with those of Northern blot analysis. The levels of HCV proteins (core, E1, and NS5B) were also examined by Western blot analysis. The E1 and NS5B proteins were also detected in all specimens except that from HuH-7 cells, although the levels of E1 protein were rather different among the specimens (Fig. 1c). In contrast, core protein was not detected in OB1, OB2, and OE2 cells, when the mixture of three kinds of monoclonal antibodies (CP9, CP11, and CP14) was used for the analysis. Even when polyclonal anti-core antibody was used, core protein was still not detected in OB2 cells. In addition, the strength of bands detected in the Western blot analysis was decreased in a time-dependent manner. These results suggest that sequence variations within the epitopes of the anti-core or E1 antibody, but not the anti-NS5B antibody, have occurred during the long-term cell culture.

Genetic variations of genome-length HCV RNAs during long-term cell culture

The determined nucleotide sequences of genome-length HCV RNAs were compared with those of the original ON/C-5B RNA (Gene Bank accession no. AB191333) [7] used for the establishment of the O, OA, OB, OD, and OE cell lines. The results revealed that the numbers of base substitutions in genome-length HCV RNAs increased in a time-dependent manner (Fig. 2). These substitutions were considered to be mutations that occurred during the intracellular replication of genome-length HCV RNA. Based on the results after 2 years in culture, the apparent mutation rates of genome-length HCV RNAs in O, OA, OB, OD, and OE cells were calculated to be 3.5 ± 0.4, 4.5 ± 1.4, 4.8 ± 0.6, 4.3 ± 0.5, $4.2 \pm 0.4 \times 10^{-3}$ base substitutions/site/year, respectively. These values suggest that the genetic evolution of HCV in these different cell lines occurs at similar rates during long-term RNA replication. The deduced aa substitution rates in HCV ORFs among these cell lines are well correlated with the mutation rates of HCV RNAs (Fig. 2). We further examined whether or not the mutation rates are similar throughout the HCV genome. For this analysis, genome-length HCV RNA was divided into three parts: the 5′-terminus to the EMCV IRES region (1,938 nts), the core to the NS2 region (3,078 nts), and the NS3 to the NS5B region (5,955 nts). The results revealed that the mutation rates in the NS3–NS5B regions were lower than those of the other regions, although the 5′-terminus to the EMCV IRES region in the OA and OE cell lines showed mutation rates similar to that for the NS3–NS5B regions (Fig. 3). These results suggest that the NS3–NS5B regions, which are essential for RNA replication, are evolutionally limited. The conserved aa substitutions (mutated in all three clones sequenced) are summarized in Table 1 (core–p7 regions) and Table 2 (NS2–NS5B regions). Eight aa substitutions (K12N, Q1112R, P1115L, K1609E, A1738T, K2280E, D2292E, and D2415G) were commonly detected in at least two different cell lines. Approximately 57% of aa substitutions detected in this study were found in the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp; Nagoya City University, Japan).

Classification of mutations occurring in genome-length HCV RNAs during the long-term cell culture

We examined the numbers of synonymous and non-synonymous mutations with transition or transversion in three divided regions (Neo^R, core–NS2, and NS3–NS5B regions). The results revealed that the frequencies of aa substitutions in the NS3–NS5B regions were lower than those in the core–NS2 regions, and that the rate of transition mutations in genome-length HCV RNA was greater than the rate of transversion mutations (Supplementary Table S1), as previously reported for the replicon system [12].

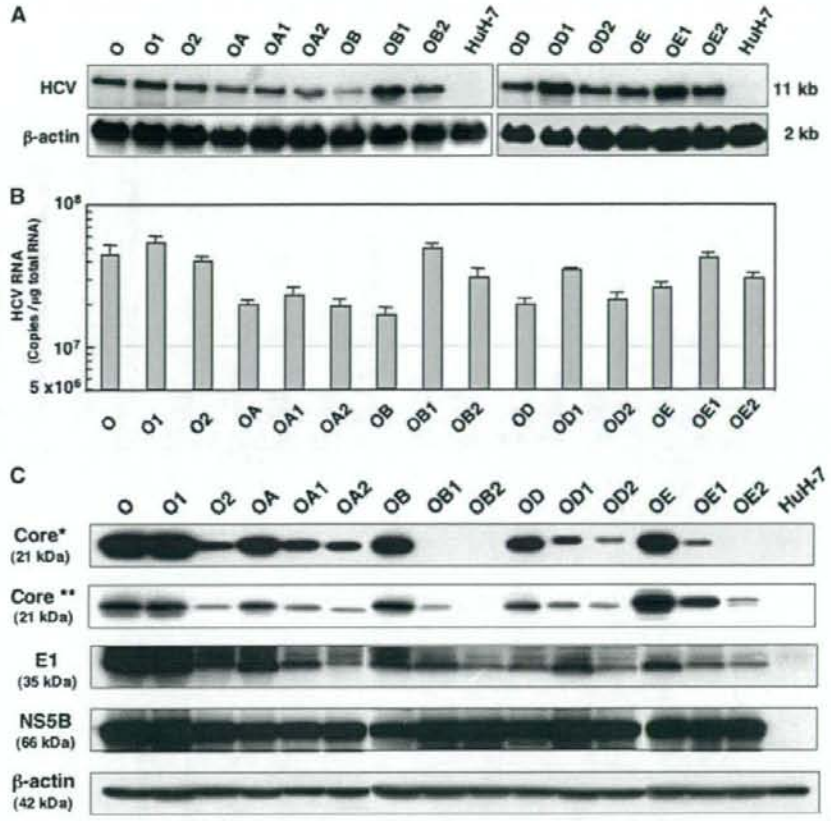

Fig. 1 Characterization of cells containing replicating genome-length HCV RNA in long-term cell culture. **a** Northern blot analysis. Total RNAs from O, OA, OB, OD, and OE cells after 1 year (O1, OA1, OB1, OD1, and OE1) and 2 years (O2, OA2, OB2, OD2, and OE2) in culture, as well as total RNAs from the parental O, OA, OB, OD, and OE cells were used for the analysis. HuH-7 cells were used as a negative control. In vitro-synthesized ON/C-5B RNA [1] was used as a size marker (11 kb). **b** Quantitative analysis of intracellular genome-length HCV RNA. The total RNAs from the cells used for Northern blot analysis were also used for comparison. The levels of intracellular genome-length HCV RNA were quantified by Light-Cycler PCR. **c** Western blot analysis. The cellular lysates from the cells used for Northern blot analysis were also used for comparison. Core, E1, and NS5B were detected by Western blot analysis. *β*-actin was used as a control for the amount of protein loaded per lane. A *single star* indicates that the mixture of three kinds (CP9, CP11, and CP14) of anti-core monoclonal antibodies was used for detection. A *double star* indicates that the anti-core polyclonal antibody was used for detection

Also regarding the mutation patterns, U → C and A → G mutations were the most and second-most frequent mutations, and these mutations were two to three times more frequent than C → U and G → A mutations (Supplementary Table S2) as previously reported in the replicon analysis [12]. The rarest mutation was C → G in 1- and 2-year cultures (Supplementary Table S2). As a result, we observed that the GC content of HCV RNA gradually increased in a time-dependent manner. The increase in GC content was observed in all genome-length HCV RNAs obtained from cultured cell lines (Fig. 4).

Genetic diversity of genome-length HCV RNA arising during long-term cell culture

Based on the sequence data of all clones obtained after 2-year culture, we examined the genetic diversities of genome-length HCV RNAs by the construction of phylogenetic trees. The results revealed that the genetic diversities of genome-length HCV RNAs were expanded at both the nucleotide and aa sequence levels, as previously reported in the replicon analysis [12], and that the three clones derived from each cell line were clustered and located at similar genetic distances from the origin

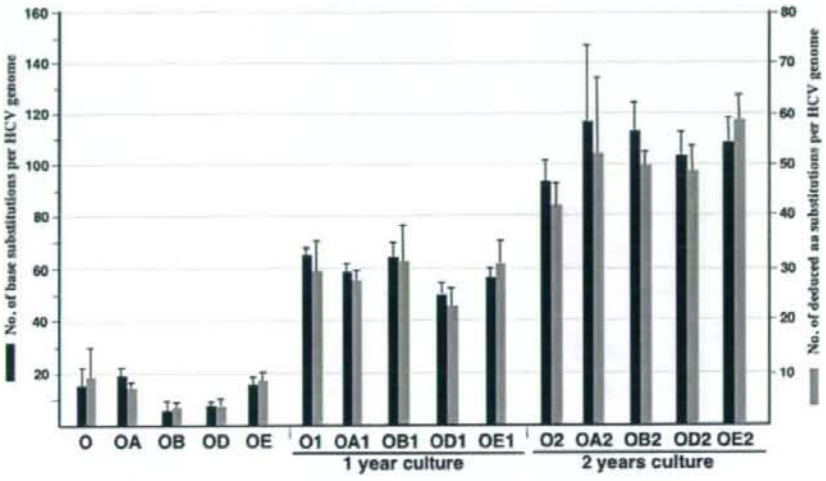

Fig. 2 Genetic variations occurring in long-term replication of genome-length HCV RNAs. The *left vertical line* indicates the mean numbers of base substitutions detected in three clones of genome-length HCV RNA, by comparison with the original sequences (ON/C-5B) [7]. The *right vertical line* indicates the mean numbers of aa substitutions deduced from each of three clones of genome-length HCV RNA, by comparison with the original aa sequences (ON/C-5B) [7]

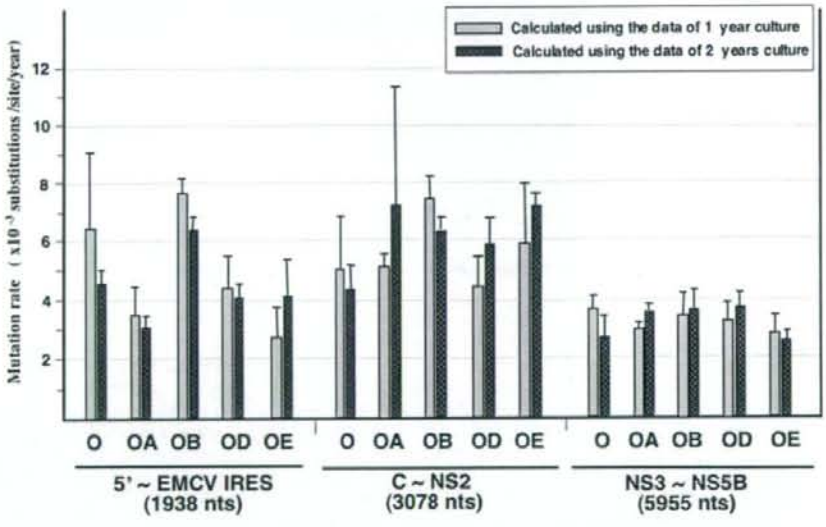

Fig. 3 Mutation rates of genome-length HCV RNAs in long-term cell culture. The mutation rates of three regions (5′–EMCV-IRES, Core–NS2, and NS3–NS5B) of genome-length HCV RNAs (O, OA, OB, OD, and OE) were calculated using the sequence data obtained from 1- or 2-year cell culture. The *vertical line* indicates the means of the mutation rates calculated using the nucleotide sequences of three clones of genome-length HCV RNAs, by comparison with the original sequences (ON/C-5B) [7]

(ON/C-5B) at both the nucleotide and aa sequence level (Supplementary Fig. S1). These results indicate that the quasispecies nature of genome-length HCV RNA has been steadily acquired over long-term intracellular RNA replication.

Discussion

In the present study, we analyzed the genetic evolution and dynamics of HCV in long-term culture of five kinds of genome-length HCV-RNA-replicating cells, and demonstrated that the genetic mutations of HCV accumulated in a time-dependent manner, and the genetic diversity of HCV also increased with time. These results will be useful for understanding the quasispecies nature of HCV in patients with chronic hepatitis C.

Previously, we reported that the genetic mutation rate of HCV replicons (subgenomic RNA) was approximately 3.0×10^{-3} base substitutions/site/year in both the 5′ terminus–EMCV IRES region and the NS3–NS5B regions [12]. The NS3–NS5B regions in this study showed mutation rates ($2.8–3.8 \times 10^{-3}$ base substitutions/site/year) similar to those of the replicons in the previous study; however, the mutation rates of the 5′ terminus to the EMCV IRES region in O and OB cells were over 6.0×10^{-3} base substitutions/site/year, suggesting that genetic mutations in this region occur independently

Table 1 Conserved aa substitutions occurring during long-term replication of genome-length HCV RNAs (I)

Region	aa Substitution	Observed cells	Region	aa Substitution	Observed cells	Region	aa Substitution	Observed cells
Core	S2G	OB2	Core	N163S	O2	E2	A457T[a]	OB1, OB2
	K6N	OB2		N163T	OE1, OE2		D463H[a]	OE, OE1, OE2
	K10R[a]	OB2		L169S	OD1, OD2		W469R	OE2
	K10E	OA2		F174S	O2		Y485H	OA1, OA2
	K12N[a]	OA1, OA2		F177S	OB1, OB2		Y485C	OD2
		OE2					G504S	OE2
	N16D	OE1, OE2	E1	N205T	O2		Y507H	OA2
	F24V	OB2		D218G[a]	OB2		L537P	OB2
	V34A	OD2		M219V[a]	OB1, OB2		M555V[a]	OB1
	R40G	O2		I220V[a]	OE1, OE2		T595A[a]	OA2
	L44M[a]	OE2		C226R[a]	OE2		K595A[a]	OA2
	T49A[a]	OE2		L242I[a]	OE2		K596N	OD1, OD2
	K67M[a]	O2		T257A[a]	OA1, OA2		L603S	OE2
	P71S[a]	O2		I258K	OE, OE1, OE2		C607S	OA1, OA2
	A75P	OE2		L264S[a]	O2		G649E	OE2
	A77T	OB2		C272R[a]	OE2		D658G[a]	OD1, OD2
	A77P	OD2		S273P[a]	O2		T670A[a]	OB2
	E89V[a]	O2		R296H[a]	OD1		I674T[a]	O2
	R101C	OE2		Q302R[a]	OA1, OA2		L689S	OA1, OA2
	T110M[a]	OE1, OE2		V313A[a]	OB2		N695S	OE2
	L119S	OB2		Y361C[a]	OA2		V713L	OA1, OA2
	K121R	OD1, OD2		Y361H	OE, OE1, OE2		Y718H	OB1, OB2
	I123T[a]	O2		V381D	OA2		Y718C	OD1
	F130L[a]	OA1, OA2					L722P	O2
	L133F	OB2	E2	G389R	OE2			
	L139P	OE1, OE2		F437S	OD1, OD2	p7	F771S	OE2
							L796P	O2

[a] aa Substitutions found in the Hepatitis Virus Databasse (http://s2as02.genes.nig.ac.jp; Nagoya City University, Japan)

among these cell lines. It was also noticed that the mutation rates ($4.3–7.4 \times 10^{-3}$ base substitutions/site/year) in the core–NS2 regions became higher than those in the NS3–NS5B regions due to frequent mutations in the core, E1, and E2 regions (Table 1). It was particularly difficult to detect the core protein by Western blot analysis due to the genetic changes within epitopes for anti-core antibodies (Fig. 1c). These results suggest that the structural region including the core, E1, and E2 regions is not required for persistent intracellular RNA replication, although approximately 42% of the aa substitutions detected in this study were observed in HCV-infected persons (Table 1). However, since we have recently found that DDX3 DEAD-box RNA helicase, which binds to the N-terminal domain (aa 1–59) of the core protein, is required for efficient replication of genome-length HCV RNA in O cells [2], none of the mutations detected in the core region should impair the interaction with DDX3. Furthermore, 6 and 9N-glycosylation sites in the E1 and E2 proteins, respectively, were completely conserved even after 2 years in culture, indicating that the E1 and E2 proteins may also affect the efficiency of RNA replication. Therefore, we speculate that the aa substitutions detected in the structural region do not reflect all of the random mutations occurring in long-term RNA replication. In contrast to the numerous aa substitutions in the structural region, the hypervariable region (HVR) 1 located in the N-terminal region of the E2 protein showed only one aa substitution (G389R in OE2 cells). This finding supports our previous proposition that an immunosurveillance system is involved in the genetic mutation in HVR1 [10]. In addition, no aa substitutions were detected in HVR2 (aa 474–480) of the E2 protein.

We showed that the mutation rates of HCV RNAs were $3.5–4.8 \times 10^{-3}$ base substitutions/site/year. However, our observed mutation rates of the HCV RNAs were 1.8–3.4 times higher than those previously obtained in chimpanzees [16, 18] and a human patient [17] with chronic hepatitis C. Since the selective pressures of the humoral immune responses [10] targeting the envelope proteins and cellular immune responses [24] targeting all HCV proteins

Table 2 Conserved aa substitutions occurring during long-term replication of genome-length HCV RNAs (II)

Region	aa Substitution	Observed cells	Region	aa Substitution	Observed cells	Region	aa Substitution	Observed cells
NS2	M814T[a]	OE1, OE2	NS4B	L1724I[a]	OB2	NS5A	D2377G[a]	OA2
	I885V[a]	O2		A1738T[a]	OA2		V2385H[a]	OD2
	F886L	OB2			OD1, OD2		S2387P	OD1, OD2
	E887G[a]	O2			OE2		L2391P	OA2
	T889A	OB2		I1797V[a]	OB2		W2405R[a]	OE2
	I891V[a]	O2		P1822S	OE2		E2414G[a]	OB2
	L902F[a]	OB2		V1880A	OA1, OA2		D2415G[a]	OA, OA1, OA2
	M939V[a]	OE, OE1, OE2						OD1, OD2
			NS5A	S1975T[a]	OE2		C2418R[a]	OA2
NS3	Q1112R[a, b]	O1, O2		H2218R[a]	OE2			
		OB2		H2219R[a]	OB2	NS5B	N2529S[a]	OB2
		OD, OD1, OD2		S2221F[a]	OA2		N2536S[a]	OB2
	P1115L[a, b]	OB, OB1, OB2		N2248D[a]	OB2		V2757A[a]	OB2
		OE, OE2		K2280E[a]	OB2		K2860R[a]	OB2
	N1148S[a]	OE2			OD1, OD2		R2963Q[a]	OB2
	E1202G[a, b]	OA, OA1, OA2		A2284T	OB2		W2990R	O2
	T1531A[a]	OA2		D2292E[a]	OB2		V3002A	O2
	D1581E[a]	OA2			OE2			
	K1609E[a, b]	O, O1, O2		V2340M[a]	OA2			
		OE2		S2342P[a]	OD2			
	I1612T[a]	OD1, OD2		G2371A[a]	OE2			
	I1641M[a]	OD1, OD2		G2376S[a]	OD1, OD2			

[a] aa Substitutions found in the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp; Nagoya City University, Japan)

[b] Adaptive mutations detected in O, OA, OB, OD, and OE cells when these cell lines were established [1, 7]

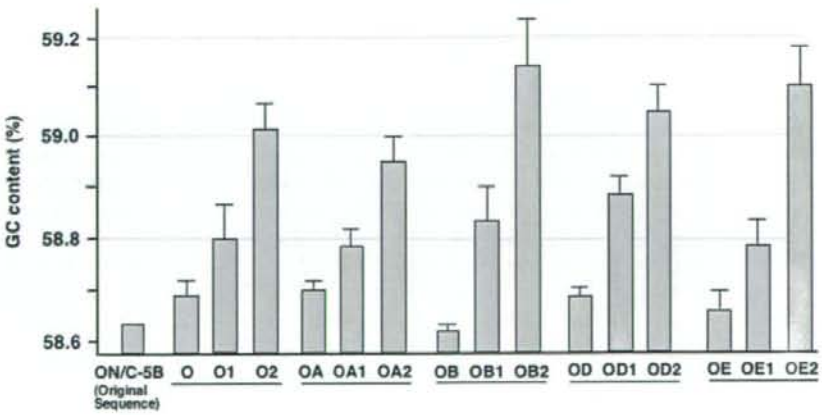

Fig. 4 Increased GC content of genome-length HCV RNAs in long-term cell culture. The GC content of genome-length HCV RNAs (O–O2, OA–OA2, OB–OB2, OD–OD2, and OE–OE2) was calculated. The values indicate the means of three clones of each genome-length HCV RNA

function in vivo, the mutation rates obtained in this study using the cell culture system without the immunological pressure would be reasonable values as a potential mutation rate of HCV in RNA replication.

It is noteworthy that none of the aa substitutions were detected in the N-terminal half (242 aa of aa 1,976–2,217) of the NS5A protein after 2 years in cell cultures. This finding suggests that this region is the most critical for maintenance of RNA replication. It is interesting that this region corresponds to domain I (aa 1,973–2,185), which has been shown to complex with a zinc ion [21] and exists as a dimer [22]. Since the mutation of four cysteine residues essential for binding to zinc ions results in the complete inhibition of RNA replication [21], the complete

conservation of domain I in this study suggests that the intact form of domain I is required for efficient RNA replication. Genetic analysis in further long-term cell cultures will specifically clarify the critical domains required for the maintenance of RNA replication.

The unexpected phenomenon in this study was the time-dependent increase of the GC content of the HCV genome. After 1 year in culture, the GC content increased 0.14% (mean of five cell culture lines), corresponding to 15 nts per HCV genome, and during the next 1 year in culture, the GC content increased an additional 0.24% (mean of five cell culture lines), corresponding to 26 nts per HCV genome. Consequently, approximately 40 nts per HCV genome changed to a G or C residue during the 2 years in culture. The HCV genome may gradually change to an energetically stable form during RNA replication. The other possibility is that the increase in GC content may be due to an increase in G- and C-ending codons, except AGG and TTG codons, for efficient expression in human cells (codon optimization) [14]. However, our study revealed that the increase of G- and C-ending codons other than codons AGG and TTG was only 16–18% of the increase of GC content observed during the 2-year cultures of O–OE cells. To understand the mechanism underlying the increase of the GC content of genome-length HCV RNA during long-term RNA replication, further long-term cell cultures will be needed.

This study demonstrated that a single HCV genome could exhibit a quasispecies nature after 2 years in cell culture with RNA replication. Such quasispecies populations of HCV obtained by long-term cell culture may be useful not only for further analysis of the genetic variations and diversity of HCV but also for analysis of the sensitivity of reagents such as interferon against HCV.

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