suggest that a TRAIL autocrine loop does not play a major role for TRAIL-induced sensitization.

Although HCV replication resulted in detectable induction of apoptosis (Fig. 3, *A–H*) and cytochrome C release (Fig. 7*D*), this induction was significantly less efficient than sensitization to TRAIL-induced apoptosis (see side-by-side experiments depicted in Fig. 3, *A–H* and Fig. 8). Thus, it seems unlikely that induction of endogenous TRAIL production is responsible for the identified HCV-dependent enhancement of TRAIL-induced apoptosis in Huh7.5 cells. This hypothesis is in line with several studies demonstrating that sensitivity toward TRAIL-induced apoptosis does not correlate with TRAIL receptor expression of target cells (53, 54). Whether massive HCV-induced cell death described in another hepatoma cell line (44) or moderate virus-induced apoptosis in Huh7.5 cells in the classical HCV tissue culture model cell line Huh7.5 (our study; see Fig. 3*H* and data not shown) more accurately reflects virus-host interactions during the natural course of HCV infection remains to be determined.

Impact of HCV-mediated enhancement of TRAIL induced apoptosis on pathogenesis of HCV infection

Apoptosis of virus-infected cells is a key mechanism of viral clearance in mammals (55). Moreover, several studies have pointed to a central role of TRAIL in the elimination of viruses via induction of host cell apoptosis (40–43). In line with this concept, induction of hepatocyte apoptosis has been observed in the HCV-infected liver (4, 5). Confirming these findings, our own histopathological analyses demonstrated expression of TRAIL in $CD8^+$ T cells and $CD68^+$ macrophages in the immediate vicinity of apoptotic hepatocytes in the HCV infected liver (data not shown). Several studies have demonstrated TRAIL-dependent cell death by activated liver macrophages (30) and/or $CD8^+$ T cells (40, 41, 56). Furthermore, elimination of influenza virus in mice has been shown to require TRAIL-expressing lymphocytes (41). In hepatits B virus (HBV) infection, lymphocyte-dependent hepatocyte apoptosis has been demonstrated to depend on TRAIL (56). Taken together, these data suggest a functional impact of TRAIL-expressing mononuclear cells, including T cells and macrophages in the elimination of virus-infected hepatocytes. Interestingly, a recent study has demonstrated that another hepatotropic virus, HBV, sensitizes hepatocytes to TRAIL-induced apoptosis via the Bcl-2 protein Bax (57), and in a mouse model of adenoviral hepatitis, TRAIL-mediated apoptosis was restrained by Bcl-x_L (58). In line with these findings for other viruses including HBV, our results suggest that sensitization of TRAIL-induced apoptosis may play a key role in host antiviral defense mechanisms against HCV infection. This hypothesis is supported by our experimental finding that incubation of PHH and Huh7.5 cells with TRAIL resulted in a decrease of viral protein levels (Fig. 4, *C* and *D*; Fig. 9). This concept is further supported by the observation that IFN-dependent up-regulation of TRAIL on NK cells and macrophages seems crucial for elimination of viral infections (59). Furthermore, therapy of HCV-infected patients with pegylated IFN-α and ribavirin results in a rapid and sustained TRAIL elevation, suggesting a role of TRAIL in viral clearance (60).

Taken together, our results define a novel antiviral host defense mechanism which may play an important role for the control of HCV infection. HCV-induced TRAIL sensitization may have important implications for the pathogenesis of HCV infection and may contribute to the elimination of virus-infected hepatocytes.

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Disclosures

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Establishment of an infectious genotype 1b hepatitis C virus clone in human hepatocyte chimeric mice

Takashi Kimura,[1,2] Michio Imamura,[1,2] Nobuhiko Hiraga,[1,2] Tsuyoshi Hatakeyama,[1,2] Daiki Miki,[1,2] Chiemi Noguchi,[1,2] Nami Mori,[1,2] Masataka Tsuge,[1,2] Shoichi Takahashi,[1,2] Yoshifumi Fujimoto,[1,2] Eiji Iwao,[3] Hidenori Ochi,[2,4] Hiromi Abe,[1,2,4] Toshiro Maekawa,[4] Keiko Arataki,[5] Chise Tateno,[2,6] Katsutoshi Yoshizato,[2,6] Takaji Wakita,[7] Toru Okamoto,[8] Yoshiharu Matsuura[8] and Kazuaki Chayama[1,2,4]

Correspondence
Kazuaki Chayama
chayama@hiroshima-u.ac.jp

[1]Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

[2]Liver Research Project Center, Hiroshima University, Hiroshima, Japan

[3]Research Division, Mitsubishi Tanabe Pharma Corporation, Osaka, Japan

[4]Laboratory for Liver Disease, SNP Research Center, Institute of Physical and Chemical Research (RIKEN), Yokohama, Japan

[5]Hirosimakinen-Hospital, Internal Medicine, Hiroshima, Japan

[6]Developmental Biology Laboratory, Department of Biological Science, Graduate School of Science, Hiroshima University, Higashihiroshima, Japan

[7]Department of Virology II, National Institute of Infectious Diseases, Shinjuku-ku, Japan

[8]Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

The establishment of clonal infection of hepatitis C virus (HCV) in a small-animal model is important for the analysis of HCV virology. A previous study developed models of molecularly cloned genotype 1a and 2a HCV infection using human hepatocyte-transplanted chimeric mice. This study developed a new model of molecularly cloned genotype 1b HCV infection. A full-length genotype 1b HCV genome, HCV-KT9, was cloned from a serum sample from a patient with severe acute hepatitis. The chimeric mice were inoculated intrahepatically with *in vitro*-transcribed HCV-KT9 RNA. Inoculated mice developed viraemia at 2 weeks post-infection, and this persisted for more than 6 weeks. Passage experiments indicated that the sera of these mice contained infectious HCV. Interestingly, a similar clone, HCV-KT1, in which the poly(U/UC) tract was 29 nt shorter than in HCV-KT9, showed poorer *in vivo* infectivity and replication ability. An *in vitro* study showed that no virus was produced in the culture medium from HCV-KT9-transfected cells. In conclusion, this study developed a genetically engineered genotype 1b HCV-infected mouse. This mouse model will be useful for the study of HCV virology, particularly the mechanism underlying the variable resistance of HCV genotypes to interferon therapy.

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INTRODUCTION

Hepatitis C virus (HCV), a positive-sense, single-stranded RNA virus, infects and replicates efficiently only in the hepatocytes of humans and chimpanzees. There are many genotypes of HCV distributed worldwide (Simmonds *et al.*, 1993); among them genotype 1b is the major genotype in Asia, including Japan, and is known to be one of the most resistant genotypes to interferon (IFN) therapy (Fried *et al.*, 2002). Until recently, studies of HCV replication have long been hampered by the lack of a virus culture system. The development of HCV replicon systems has allowed the

The GenBank/EMBL/DDBJ accession numbers for the sequences of HCV-KT9 and HCV-KT1 determined in this work are AB435162 and AB426117, respectively.

study of the mechanisms of replication of HCV (Lohmann *et al.*, 1999). However, these replicons lack structural proteins, do not replicate efficiently without adaptive mutations and do not produce infectious virions. Recently, it was reported that the genotype 2a full-length JFH-1 genome replicated efficiently in Huh7 cells without adaptive mutations and produced virions that were infectious for both naïve cells and chimpanzees, as well as for a human hepatocyte-transplanted chimeric mouse (Wakita *et al.*, 2005; Zhong *et al.*, 2005; Lindenbach *et al.*, 2006). To date, five full-length genotype 1b clones, HCV-N (Beard *et al.*, 1999), Con-1 (Bukh *et al.*, 2002), HCV-J4 (Okamoto *et al.*, 1992), HCV-CG1b (Thomson *et al.*, 2001) and HCV-BK (Takamizawa *et al.*, 1991), have been demonstrated to be infectious by intrahepatic inoculation of transcribed HCV RNA into the liver of chimpanzees. Among these, only the HCV-CG1b genome is reported to produce HCV particles when transfected into Huh7 cells (Heller *et al.*, 2005).

Although the chimpanzee is a useful animal model for the study of HCV infection, there are ethical restrictions on the use of this animal. Instead, Mercer *et al.* (2001) developed a useful small-animal model for the study of HCV infection using chimeric urokinase-type plasminogen activator (uPA)/severe combined immunodeficiency (SCID) mice (which are immunodeficient and undergo liver failure) with engrafted human hepatocytes. This HCV-infected mouse model is reported to be useful for evaluating anti-HCV drugs such as IFN-α and anti-NS3 protease (Kneteman *et al.*, 2006). We have previously described methods to improve the replacement levels of human hepatocytes in this mouse model (Tateno *et al.*, 2004) and we have developed a reverse genetics system for hepatitis B virus (Tsuge *et al.*, 2005) and HCV (Hiraga *et al.*, 2007). In the present study, we report the establishment of an infectious genotype 1b HCV clone that infects and replicates efficiently in human hepatocyte chimeric mice.

METHODS

Cloning of infectious genotype 1b HCV isolate. Serum samples were obtained from a 43-year-old physician who developed severe acute hepatitis after needle stick exposure from a patient with chronic hepatitis C. On admission, the serum total bilirubin concentration was 10.0 mg dl^{-1} and the prothrombin time was 40 %. The patient tested positive for HCV antibodies by a third-generation radioimmunoassay (Ortho-Clinical Diagnostics) and for HCV RNA by RT-PCR. Serum HCV RNA was quantified using an Amplicor Monitor HCV test (Roche Diagnostics). The HCV RNA titre was 2.5×10^6 copies ml^{-1} on admission and then decreased gradually. Fig. 1 shows the serial changes in alanine aminotransferase (ALT) as a measure of liver function and HCV RNA levels in this patient. Serum samples obtained in the early phase of infection were used for cloning the full-length genome.

RNA extraction, cDNA synthesis, plasmid construction and RNA transcription. Total RNA was extracted from 100 µl serum samples using SepaGene RV-R (Sanko Junyaku) and reverse transcribed with random hexamers and ReverTra Ace reverse transcriptase (Toyobo) according to the manufacturer's instructions. PCR primers were designed based on the sequence of HCV-Con1 (GenBank accession

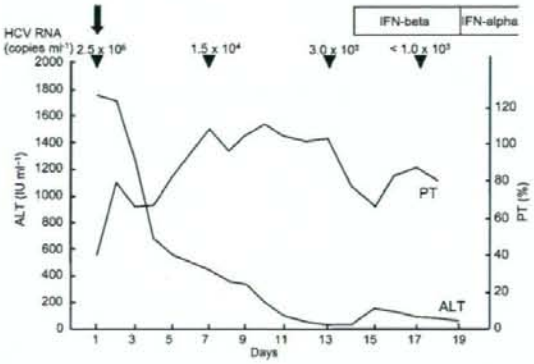

Fig. 1. Clinical course of a patient with severe acute hepatitis C. Alanine aminotransferase (ALT) and prothrombin time (PT) are shown from the day of admission (day 1). The patient was treated daily with 10^6 U IFN-β intravenously for 5 days, followed by 10^6 U IFN-α intramuscularly three times a week for 6 months. HCV RNA was measured on days 1, 7, 13 and 17 (arrowheads). A serum sample was taken on day 1 (arrow) and used to clone the full-length HCV genome.

no. AJ238799; Bukh *et al.*, 2002). Five overlapping cDNA segments (nt 1–2292, 2269–6715, 6696–9094, 7564–9404 and 9361–9605; nucleotide numbers are those of HCV-Con1) were amplified by PCR with TaKaRa LA *Taq* polymerase (Takara Biochemicals) using the above cDNA. Amplified products were separated by agarose gel electrophoresis. Nucleotide sequences were determined using a Big Dye Terminator Mix Cycle Sequencing kit (Applied Biosystems Japan) with an automated DNA sequencer (model 310; PE Biosystems). We corrected the nucleotide sequences of the obtained clones by site-directed mutagenesis and made them identical to the nucleotide sequences obtained by direct sequencing. Naturally occurring restriction enzyme cutting sites were utilized to clone each segment. We utilized the vector pBR322 and created a multiple-cloning site under the control of the T7 promoter by ligating a linker at restriction enzyme cutting sites as they appeared in order from 5′ to 3′ in the HCV sequences (Fig. 2a). Each segment of HCV was cloned into this vector to generate the full-length clones. The HCV-KT9 clone was established using the 3′-terminal fragment with the longest poly(U/UC) tract length (115 nt), which should have a high replication ability (Friebe & Bartenschlager, 2002; Yi & Lemon, 2003; You & Rice, 2008). A clone with a shorter poly(U/UC) tract length (86 nt), HCV-KT1, was also generated. A polymerase-deficient mutant with an amino acid substitution in the GDD motif (GDD→GND; HCV-KT9-GND) was generated using a Quick Change Site-Directed Mutagenesis kit (Stratagene). After digesting the plasmid with *XbaI* (New England BioLabs) at the 3′ end of the HCV cDNA, HCV RNA was transcribed using T7 RNA polymerase (MEGAscript; Ambion) at 37 °C for 3 h in a 100 µl reaction mixture, according to the manufacturer's instructions. The RNA was analysed using denaturing agarose gel electrophoresis and kept at −80 °C until use.

Construction of a phylogenetic tree. A phylogenetic tree was constructed based on the entire nucleotide sequences of 26 full-length genotype 1b clones plus HCV-KT9. The total number of synonymous and non-synonymous substitutions among the nucleotide sequences was estimated using the method of Gojobori *et al.* (1982) and a phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987).

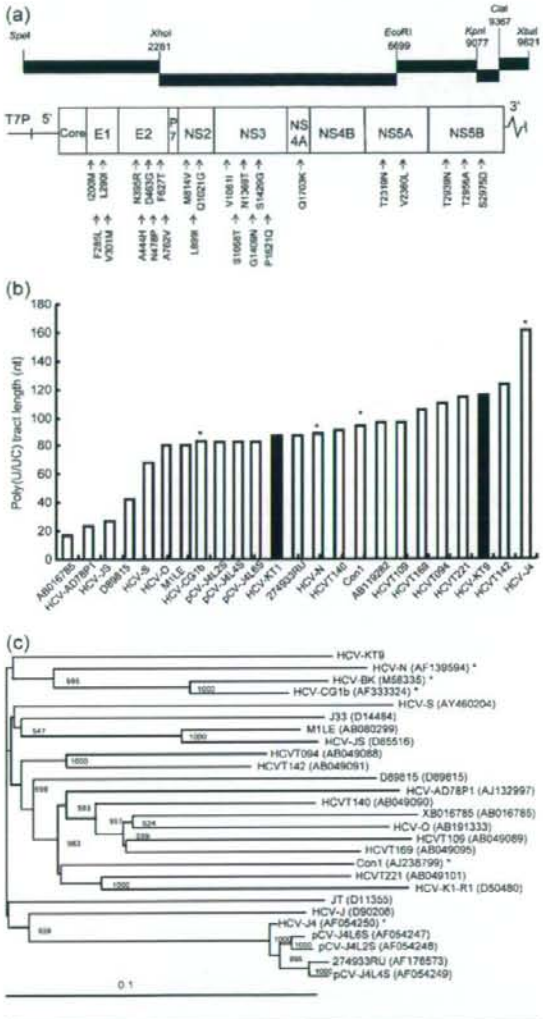

Fig. 2. (a) Schematic diagram of the organization of the cDNA clone HCV-TK9. The T7 RNA promoter (T7P) is located immediately upstream of the HCV genome. Restriction enzyme sites that were used to create clone HCV-KT9 are labelled according to their nucleotide position within the HCV sequence. Amino acid sequences unique to HCV-KT9 compared with 26 other HCV genotype 1b isolates are indicated at the bottom of the figure, with the position of the repaired amino acid residues noted within the polyprotein. (b) Length of the poly(U/UC) tracts of HCV-KT1, HCV-KT9 and 22 other HCV genotype 1b clones reported previously. Asterisks indicate clones confirmed to be infectious by experiments using chimpanzees. (c) Phylogenetic tree constructed with HCV-KT9 and 26 genotype 1b HCV whole-genome sequences. Bar, number of nucleotide substitutions per site. Asterisks indicate clones confirmed to be infectious in experiments using chimpanzees.

Intrahepatic injection experiments in human hepatocyte chimeric mice. We used methods described previously (Tateno *et al.*, 2004) to generate uPA$^{+/+}$/SCID$^{+/+}$ mice and transplant human hepatocytes. All mice used in this study were transplanted with frozen human hepatocytes obtained from the same donor. Mouse serum concentrations of human serum albumin (HSA) correlate with the repopulation index and were measured as described previously (Tateno *et al.*, 2004). Intrahepatic injection of RNA, extraction of serum samples and euthanasia were performed under ether anaesthesia. Briefly, 500 µl RNA solution containing 30 µg transcribed HCV RNA was injected into the liver of anaesthetized chimeric mice through a small abdominal incision. RNA extraction from mouse serum samples, quantification of HCV RNA and nested PCR were performed as described previously (Hiraga *et al.*, 2007). All animal protocols described in this study were performed in accordance with the guidelines of the local committee for animal experiments and under the approval of the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University.

Cell culture, RNA transfection and measurement of HCV core antigen. The human hepatoma cell line Huh7 was maintained in Dulbecco's modified Eagle's medium (Sigma) containing 10 % fetal calf serum. RNA transfection and measurement of HCV core antigen in the culture medium were performed as described previously (Wakita *et al.*, 2005).

Statistical analysis. The infectious ratio of chimeric mice was compared and the differences assessed using a χ^2 test. Differences in HCV RNA replication ability *in vitro* were analysed statistically by one-way analysis of variance followed by Scheffe's test. A P value of less than 0.05 was considered statistically significant.

RESULTS

Characteristics of genotype 1b clones HCV-KT9 and HCV-KT1

The entire genome of HCV cDNA was assembled from five DNA fragments (Fig. 2a). We obtained 24 3′-extremity clones with different poly(U/UC) tract lengths. We selected the clone with the longest (U/UC) tract because a previous study indicated that the length of poly(U/UC) tract correlates with HCV replication in an HCV replicon system (Friebe & Bartenschlager, 2002; Yi & Lemon, 2003; You & Rice, 2008). The length of the poly(U/UC) tract in the longest 3′ clone was 115 nt. The entire genome length of the HCV-KT9 clone using this longest 3′ clone was 9621 nt. We also generated the clone HCV-KT1 with a shorter (86 nt) poly (U/UC) tract to compare the replication abilities of these clones. The lengths of the poly(U/UC) tracts of 22 clones deposited in GenBank are shown in Fig. 2(b). All infectious clones had a poly(U/UC) tract longer than 80 nt. Fig. 2(c) shows a phylogenetic tree constructed using the nucleotide sequences of the 26 full-length genotype 1b clones published to date. Interestingly, the sequence of HCV-KT9 was closest to that of HCV-CG1b (GenBank accession no. AF333324), which has been reported to be infectious, and formed a cluster with two other infectious clones, HCV-N (Beard *et al.*, 1999) and HCV-BK (Takamizawa *et al.*, 1991). We compared the amino acid

sequences of HCV-KT9 with an alignment of the sequences of the 26 other genotype 1b strains. All HCV full-length clones reported from Japan were included in these 26 strains. Based on these comparisons, we identified 25 aa unique to HCV-KT9 (Fig. 2a). We found that the amino acid sequence of the IFN sensitivity-determining region in the NS5A region, which has been suggested to mediate IFN resistance via interaction with the cellular protein kinase R (Enomoto *et al.*, 1996; Gale *et al.*, 1997), was that of the wild-type.

Intrahepatic injection of HCV-KT1 and HCV-KT9 RNAs into human hepatocyte chimeric mice

In the next experiments, 30 μg *in vitro*-transcribed RNA of HCV-KT1, HCV-KT9 or HCV-KT9-GND was injected into the livers of chimeric mice. Eight of 10 (80%) HCV-KT9-injected mice developed measurable viraemia at 2 weeks post-inoculation (Table 1 and Fig. 3), with the HCV RNA titre reaching 1.1×10^6 to 8.8×10^6 copies ml^{-1} at 6 weeks post-inoculation (Fig. 3). To check for the presence of infectious HCV in the serum of HCV-KT9-infected mice, each of five naïve mice was injected with 10 μl serum sample (containing 3.5×10^5 copies of HCV) obtained from an HCV-KT9-infected mouse 6 weeks after inoculation. All five naïve mice became positive for HCV RNA, as confirmed by nested PCR, at 2 weeks post-inoculation and two mice developed persistent viraemia (Fig. 4). These results indicated that the serum of HCV-KT9-injected mice contained infectious HCV. In contrast to HCV-KT9, none of the three mice injected with HCV-KT9-GND RNA developed viraemia (Table 1). These results indicated that HCV-KT9 replicates efficiently in mice livers and produces infectious virus continuously. On the other hand, only one out of seven HCV-KT1-injected mice (14%) developed measurable viraemia (Table 1 and Fig. 3). The level of viraemia was low in this HCV-KT1-infected mouse, HCV RNA was negative by nested PCR at 2 weeks after inoculation and the titre was only 2.2×10^4 copies ml^{-1} at 4 weeks post-inoculation (Fig. 3). These results confirmed the importance of the poly(U/UC) tract length in experimentally induced viraemia.

The nucleotide and amino acid sequences of the viral genome isolated from an HCV-KT9-injected mouse (Fig. 3) at 6 weeks after RNA injection were identical to the injected HCV-KT9 (data not shown). We tried to reclone the poly(U/UC) tract in the HCV-KT1-infected mouse, but it was impossible to reamplify the HCV cDNA using the remaining small amount of serum.

Table 1. Correlation between length of the poly(U/UC) tract and HCV infection

Clone	Length of poly(U/UC) tract	Number of mice			Infection ratio
		Infected	Not infected	Total	
HCV-KT1	86	1	6	7	14%
HCV-KT9	115	8	2	10	80%*
HCV-KT9-GND	115	0	3	3	0%

*P=0.015, compared with HCV-KT1.

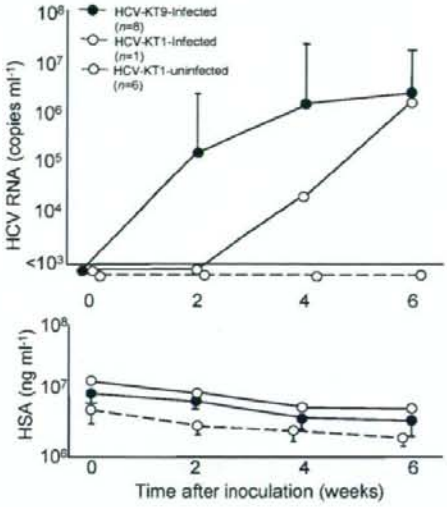

Fig. 3. Changes in HCV RNA levels and HSA concentrations in the sera of mice infected with clonal HCV. Mice were inoculated intrahepatically with 30 μg *in vitro*-transcribed HCV RNA. Eight of the ten HCV-KT9-infected mice (80%), one of the seven HCV-KT1-infected mice (14%) and none of the three HCV-KT9-GND-infected mice became positive for HCV RNA. The results for six HCV-KT1-uninfected mice are also shown. Mice serum samples were obtained every 2 weeks post-infection for analysis of HCV RNA titres. Data are shown as mean ± SD.

Analysis of virus production from HCV-KT9-transfected cells

Next, we evaluated the ability of the HCV-KT9 clone to replicate in transfected Huh7 cells. In these experiments, we used JFH-1 RNA, which is known to replicate efficiently in cell cultures, as control (Wakita *et al.*, 2005). Core protein was secreted efficiently from JFH-1 RNA-transfected Huh7 cells. In contrast, we did not observe any measurable levels of core protein in the supernatant of HCV-KT9-transfected cells (Fig. 5), suggesting a minimal replication ability of HCV-KT9 to produce and release virus into the supernatant.

DISCUSSION

In this study, we described the establishment of a genotype 1b clone, HCV-KT9, that replicated efficiently following injection of the transcribed RNA into chimeric mouse liver.

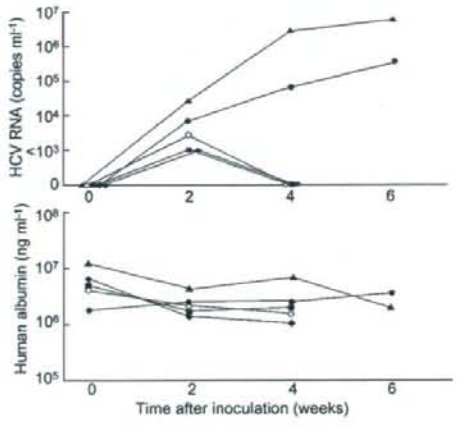

Fig. 4. Passage experiments of HCV in naïve chimeric mice. Five naïve chimeric mice were inoculated intravenously with 10 µl serum sample (containing 3.5×10^5 copies HCV) obtained from an HCV-KT9-infected mouse at week 6 post-inoculation. Serum samples were obtained at the indicated time intervals for the measurement of HCV RNA levels and HSA concentrations. Data represent the changes in five individual mice.

The key factor that determines the infectivity of HCV clones has not yet been established. We previously established a clone from HCV that replicated in a chimeric mouse after injection of serum from a chronically HCV-infected patient. However, we did not observe viraemia after intrahepatic injection of the transcribed RNA from this clone (unpublished results). In contrast, injection of HCV-KT9 RNA in the present study resulted in viraemia in eight out of ten mice (80 %). The fact that the nucleotide and amino acid sequences of the virus recovered from the infected mice were identical to those of the HCV-KT9 clone indicated that no adaptive mutation was necessary for this clone to replicate in the chimeric mouse.

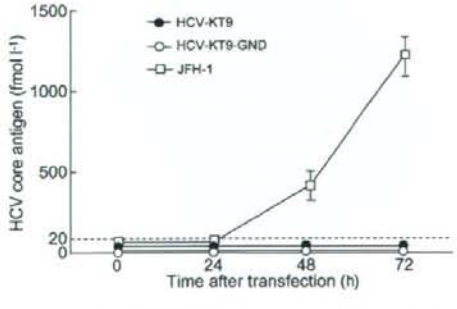

Fig. 5. Time-course studies of HCV core protein secretion into the culture medium of HCV RNA-transfected cells. Huh7 cells were transfected with 10 µg HCV-KT9, HCV-KT9-GND or JFH-1 RNA. HCV core antigen in the culture medium was measured at 24, 48 and 72 h after transfection. Data are shown as mean ± SD of HCV core protein levels obtained from three independent transfection experiments.

Interestingly, the clone was obtained from a patient with severe acute hepatitis. This is similar to JFH-1, an HCV clone with a strong replication ability in cultured cell lines, chimpanzees and chimeric mice, which was cloned from serum samples of a patient who developed acute fulminant hepatitis with a high virus titre (Wakita *et al.*, 2005). A virus that replicates in the early stage of infection may have strong replication ability, which may be lost in the chronic phase of infection.

A key amino acid substitution may be present in one (or some) of the amino acids unique to this clone (Fig. 2a). We also showed that clone HCV-KT1, which differs from HCV-KT9 only in the length of the poly(U/UC) tract, had a poorer replication ability in mice (Table 1 and Fig. 3). However, there is a possibility that a shorter poly(U/UC) tract only slows down the rate of infection, as the HCV RNA titre in the HCV-KT1-infected mouse at 6 weeks after inoculation was similar to that in HCV-KT9-infected mice (Fig. 3). It has been reported that the length and composition of the poly(U/UC) tract is important for the replication of HCV replicons (Friebe & Bartenschlager, 2002; Yi & Lemon, 2003; You & Rice, 2008). However, no replication advantage of a poly(U/UC) tract longer than 86 bp was revealed in this study. This may be due to differences *in vitro* and *in vivo*, where the innate immune response against the virus may be more robust than in cell culture.

As shown in the present study, reverse genetics of HCV has become available for studies of HCV replication. The important factors for virus replication suggested above can be analysed further using this system.

We also examined the response of HCV-KT9-infected mice to IFN treatment. Three HCV-KT9-infected mice were treated with daily intramuscular injections of 1000 IU IFN-α (g body weight)$^{-1}$ for 2 weeks. This regimen resulted in a reduction in HCV RNA levels of only 1.0 log copies ml^{-1} (data not shown). These results are consistent with our previous study, which showed a similar low-level reduction in HCV RNA in mice infected with a genotype 1a clone, and differ from our previous results in mice infected with HCV genotype 2a, which became negative for HCV RNA following daily treatment with 1000 IU IFN-α (g body weight)$^{-1}$ for 2 weeks (Hiraga *et al.*, 2007). These results are in agreement with our clinical experience that genotype 1 is more resistant to IFN therapy than genotype 2. As shown in the present study and previously (Hiraga *et al.*, 2007), reverse genetics of HCV with three genotypes, 1a, 1b and 2a, is now available. By recombination of these clones or the establishment of mutants with nucleotide and amino acid sequences similar to each other, it may be possible to clarify the mechanism underlying the variability in susceptibility of HCV genotypes to IFN.

In this study, HCV-KT9 showed no virus production ability *in vitro*. Recently, Kato *et al.* (2007) reported that the genotype 1b HCV clone CG1b replicated in Huh7.5.1 cells and produced infectious HCV. It will be of interest to create chimeric viruses of HCV-KT9 and HCV-CG1b, and to determine the mutations that are important for virus production *in vitro*.

In summary, we established an infection model of a genotype 1b HCV clone using human hepatocyte chimeric mice. This model will be useful for studies of HCV replication, particularly the mechanism underlying the variable resistance of HCV genotypes to IFN therapy.

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Hepatitis C Virus JFH-1 Strain Infection in Chimpanzees Is Associated With Low Pathogenicity and Emergence of an Adaptive Mutation

Takanobu Kato,[1-3] Youkyung Choi,[4] Gamal Elmowalid,[1] Ronda K. Sapp,[1] Heidi Barth,[1] Akihiro Furusaka,[5] Shunji Mishiro,[2] Takaji Wakita,[3] Krzysztof Krawczynski,[4] and T. Jake Liang[1]

The identification of the hepatitis C virus (HCV) strain JFH-1 enabled the successful development of infectious cell culture systems. Although this strain replicates efficiently and produces infectious virus in cell culture, the replication capacity and pathogenesis *in vivo* are still undefined. To assess the *in vivo* phenotype of the JFH-1 virus, cell culture–generated JFH-1 virus (JFH-1cc) and patient serum from which JFH-1 was isolated were inoculated into chimpanzees. Both animals became HCV RNA-positive 3 days after inoculation but showed low-level viremia and no evidence of hepatitis. HCV viremia persisted 8 and 34 weeks in JFH-1cc and patient serum–infected chimpanzees, respectively. Immunological analysis revealed that HCV-specific immune responses were similarly induced in both animals. Sequencing of HCV at various times of infection indicated more substitutions in the patient serum–inoculated chimpanzee, and the higher level of sequence variations seemed to be associated with a prolonged infection in this animal. A common mutation G838R in the NS2 region emerged early in both chimpanzees. This mutation enhances viral assembly, leading to an increase in viral production in transfected or infected cells. *Conclusion:* Our study shows that the HCV JFH-1 strain causes attenuated infection and low pathogenicity in chimpanzees and is capable of adapting *in vivo* with a unique mutation conferring an enhanced replicative phenotype. (HEPATOLOGY 2008;48:732-740.)

Hepatitis C virus (HCV) infects approximately 170 million people worldwide and is a major causative agent of chronic liver diseases including cirrhosis and hepatocellular carcinoma.[1,2] However, the underlying biological mechanisms of pathogenesis and persistence are still not well understood. No vaccine protecting against HCV infection is currently available.[3] Therapy for HCV-related chronic hepatitis remains problematic, with limited efficacy, high cost, and substantial adverse effects.[1,4,5] Understanding the biology of this virus and the development of new therapies has been hampered by a lack of appropriate model systems for replication and infection of this virus.

Abbreviations: ALT, alanine aminotransferase; ELISpot, enzyme-linked immunosorbent spot; FFU, focus-forming unit; HCV, hepatitis C virus; HVR, hypervariable region; IFN-γ interferon gamma; JFH-1cc, cell culture generated JFH-1 virus; PBMC, peripheral blood mononuclear cell; RT-PCR, reverse transcription polymerase chain reaction; SFU, spot-forming unit; WT, wild-type.

From the [1]Liver Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD; the [2]Department of Medical Sciences, Toshiba General Hospital, Shinagawa, Tokyo, Japan; the [3]Department of Virology II, National Institute of Infectious Diseases, Shinjuku, Tokyo, Japan; the [4]Division of Viral Hepatitis, Center for Disease Control and Prevention, Atlanta; and the [5]Department of Internal Medicine, Jikei University School of Medicine (Daisan), Komae, Tokyo, Japan.

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Dr. Furusaka is currently affiliated with Department of Gastroenterology and Hepatology, Tokyo Medical University, Tokyo, Japan.

Address reprint requests to: Dr. T. Jake Liang, Liver Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892. E-mail: JakeL@bdg10.niddk.nih.gov; fax: 301-402-0491.

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Recent progress with a unique HCV genotype 2a strain, JFH-1, isolated from a case of fulminant hepatitis in Japan, has led to the development of a robust HCV infectious cell culture system.[6-9] This JFH-1 strain can replicate efficiently, produce the infectious viral particles, and show robust infection *in vitro*. However, in our previous report, the inoculation of cell culture–generated JFH-1 virus (JFH-1cc) induced only transient and attenuated infection in a chimpanzee.[8] The observed low virulence of this strain *in vivo* was unexpected but consistent, with an inverse relationship between *in vivo* and *in vitro* properties of cell culture adaptive mutations in the HCV replicon system.[10]

In this study, we performed an extensive analysis of the *in vivo* replication and pathogenicity of the JFH-1 strain by inoculating chimpanzees with JFH-1cc and patient serum from which the JFH-1 strain was isolated. Furthermore, we analyzed viral sequences during the infection to identify mutations that might represent *in vivo* adaptive mutations with unique phenotypes.

Materials and Methods

Cell Culture. Huh7 derivative cell lines Huh7.5 and Huh7.5.1 were provided by Charles Rice (Rockefeller University, New York, NY) and Francis Chisari (Scripps Research Institute, La Jolla, CA), respectively.[7,9] The Huh7 derivative clone Huh7-25 that lacks CD81 expression was reported previously.[11]

Inocula. The production of JFH-1cc has been reported previously.[12] Briefly, the full-length JFH-1 RNA was synthesized by *in vitro* transcription with linearized pJFH-1 plasmid and MEGAscript kit (Ambion, Austin, TX).[8] Ten micrograms full-length JFH-1 RNA was transfected into 3.0×10^6 Huh7 cells by electroporation, and the culture medium with JFH-1cc was harvested 5 days after transfection. The culture medium was passed through a 0.45-μm filter unit. The case of fulminant hepatitis C from which the JFH-1 strain was isolated has been reported previously.[6] An aliquot of acute-phase serum (point A as indicated by Kato et al.[6]) was used in this study. To determine the HCV RNA titers in these inocula, total RNA was extracted from 140 μL of these samples by QIAamp Viral RNA Kit (QIAGEN, Valencia, CA), and copy numbers of HCV RNA were determined by real-time quantitative reverse transcription polymerase chain reaction (RT-PCR), as described previously.[13]

Infection Study in Chimpanzees. Housing, maintenance, and care of the chimpanzees used in this study conformed to the requirement for the humane use of animals in scientific research as defined by the Institutional Animal Care and Use Committee of the Centers for Disease Control and Prevention. Chimpanzee 10273 (CH10273, female, age 5, 20 kg) was inoculated intravenously with 100 μL serum (9.6×10^6 copies) from the fulminant hepatitis patient mixed with 400 μL Dulbecco's modified Eagle's medium culture medium. Chimpanzee 10274 (CH10274, female, age 5, 22 kg) was inoculated intravenously with 500 μL Dulbecco's modified Eagle's medium culture medium containing JFH-1cc (1.4×10^7 copies). Serum and liver biopsy samples of these animals were obtained at baseline and weekly after inoculation.

Measurement of HCV RNA, anti-HCV, and Alanine Aminotransferase. HCV RNA in chimpanzees was quantitatively measured by nested RT-PCR with a sensitivity of detection of approximately 50 IU/mL (COBAS Amplicor; Roche Molecular Systems, Pleasanton, CA) and was quantified using Amplicor Monitor (Roche Molecular Systems). Serum samples were tested for anti-HCV (ORTHO version 3.0 enzyme-linked immunosorbent assay test system, Ortho-Clinical Diagnostics, Raritan, NJ). Serum alanine aminotransferase (ALT) values in chimpanzee's sera were established using a commercially available assay kit in accordance with the manufacturer's instructions (Drew Scientific, Dallas, TX). Cutoff values representing 95% confidence limit for the upper level of normal ALT activity were calculated individually for each chimpanzee using 10 pre-inoculation enzyme values obtained over a period of 4 to 6 weeks, and were 73 U/L in CH10274 and 76 U/L in CH10273.

HCV Sequencing. The total RNA was extracted from 280 μL chimpanzee sera collected at appropriate time points by the use of QIAamp viral RNA kit, and complementary DNA was synthesized by use of Superscript III (Invitrogen, Carlsbad, CA). The complementary DNAs were subsequently amplified with TaKaRa LA *Taq* DNA polymerase (Takara Mirus Bio, Madison, WI). Five separate fragments were amplified by nested PCR covering the entire open reading frame and a part of the 5′UTR of the JFH-1 strain as follows; nt 128-1829, nt 1763-4381, nt 4278-6316, nt 6172–7904, and nt 7670-9222. The sequence of each amplified fragment was determined directly. The fragment encompassing hypervariable region 1 (HVR-1) (nt 128-1829) was cloned into the pGEM-T easy vector (Promega, Madison, WI) and 10 clones from each time point were sequenced.

T-Cell Proliferation and Interferon-γ Enzyme-Linked Immunosorbent Spot Assays. The cryopreserved peripheral blood mononuclear cells (PBMCs) were used for immunological analysis. Standard T-cell proliferation assay was performed as described previously.[14] Cells were stimulated with recombinant HCV genotype 2a core or NS5a protein (Fitzgerald Industries Interna-

tional, Concord, MA) and pulsed with ^{3}H-thymidine (GE Healthcare BioSciences, Piscataway, NJ). T-cell stimulation was expressed as a stimulation index that was calculated as the ratio of average counts per minute (CPM) of antigen-stimulated proliferation over average CPM of the medium background. A sample was considered positive when the average stimulation index was greater than 5. The numbers of antigen-specific interferon gamma (IFN-γ)–producing cells were analyzed by enzyme-linked immunosorbent spot (ELISpot) assay. PBMCs were stimulated with recombinant protein antigens (HCV core and NS5a proteins) and HCV overlapping peptide pools (15mers overlapped by 10 amino acids) from core (38 peptides, amino acids 1-195) and NS3 (56 peptides, amino acids 1031-315)(Mimotopes, Raleigh, NC). The NS3 overlapping peptide pools were divided into two sets. The number of spots was counted by using a computer-assisted AID ELISpot Reader System and AID software version 3.5 (Autoimmune Diagnostika GmbH, Strassberg, Germany). Antigen-specific spot-forming unit (SFU) was calculated by subtracting the average of background values (four wells without antigen, typically fewer than 10 spots) from that of the antigen-stimulated sample. The sample was considered positive when the background-corrected SFU was greater than 10 and twice or more the mean SFU of the preinfection samples in the same animal.

To specifically evaluate the T-cell response against the NS2 region containing the G838R mutation, two peptides of 18 amino acids (NS2-G: ITLFTLTPGYKTLLGQCL and NS2-R: ITLFTLTPRYKTLLGQCL) were synthesized (Sigma-Genosys, The Woodlands, TX). PBMCs from both chimpanzees were stimulated with the wild-type (WT) and mutant peptides (2 μg/mL) and analyzed for IFN-γ production by IFN-γ ELISpot assays as described.

Production of JFH-1 G838R Mutant Virus. The full genome JFH-1 construct with G838R mutation in the NS2 region was generated by site-directed mutagenesis. The replication-deficient clone of JFH1 generated by introducing a point mutation into the GDD motif of the NS5B to abolish the RNA-dependent RNA polymerase activity was used as a negative control (JFH-1 GND).[8]

Quantification of HCV RNA and HCV Core Antigen. To determine the amount of HCV, total RNA was extracted with QIAamp Viral RNA Kit from 140 μL culture medium, or with RNeasy mini kit (QIAGEN, Valencia, CA) from cell pellet. Copy numbers of HCV RNA were determined by real-time quantitative RT-PCR as described. HCV core antigen (Ag) in culture supernatant was quantified by highly sensitive enzyme immunoassay (Ortho HCV core antigen ELISA Kit, Ortho Clinical Diagnostics, Tokyo, Japan).[15] To determine intracellular HCV core Ag, the cell pellet was resuspended with 100 μL radioimmune precipitation assay buffer containing 1% sodium dodecyl sulfate, 0.5% NP40, 10 mM Tris-HCl (pH 7.4), 1 mM ethylenediaminetetra-acetic acid, 150 mM NaCl, and Complete Mini protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), then sonicated 10 minutes and subjected to the Ortho HCV core antigen enzyme-linked immunosorbent assay after centrifugation.

Titration of HCV Infectivity. To assess the intracellular infectivity, cells were harvested by treatment with trypsin-ethylenediaminetetra-acetic acid and pelleted by centrifugation. Cell pellets were resuspended with 500 μL Dulbecco's modified Eagle's medium with 10% fetal bovine serum and lysed by four freeze–thaw cycles. The supernatant was collected after centrifugation and passage through a 0.45-μm filter. These cell lysates and culture supernatants were serially diluted fivefold and inoculated into naïve Huh7.5.1 cells seeded at 1×10^4 cells/well in 96-well flat-bottom plates and assayed for focus-forming unit (FFU) by anti-core immunofluorescence as described previously.[16]

Statistical Analysis. Data from repeated experiments were averaged and expressed as mean ± standard deviation. Statistical analysis was performed using the Mann-Whitney test. P values of less than 0.05 were considered statistically significant.

Results

Clinical, Virological, and Immunological Profiles of JFH-1–Infected Chimpanzees. Chimpanzee 10273 (CH10273) was inoculated with patient serum containing 9.6×10^6 copies of HCV RNA. Chimpanzee 10274 (CH10274) was inoculated with 1.4×10^7 copies of JFH-1cc in culture medium. In both chimpanzees, HCV RNA became detectable in serum by RT-PCR 3 days after inoculation. Viremia was low, with titers of approximately 10^3 copies/mL. Serum ALT levels were within normal limits, and histological observation of liver biopsy showed no evidence of hepatitis (Fig. 1). In CH10273, HCV RNA in serum fluctuated but persisted for 34 weeks after inoculation, and anti-HCV was detected from 20 weeks after inoculation (Fig. 1A). In CH10274, serum HCV RNA disappeared at 9 weeks after inoculation, and no anti-HCV seroconversion was observed (Fig. 1B).

Immunological analysis for T-cell proliferation and IFN-γ production showed that HCV-specific immune responses were induced in both animals (Fig. 1). Their responses corresponded to the profiles of viremia and remained at low levels after disappearance of viremia. The

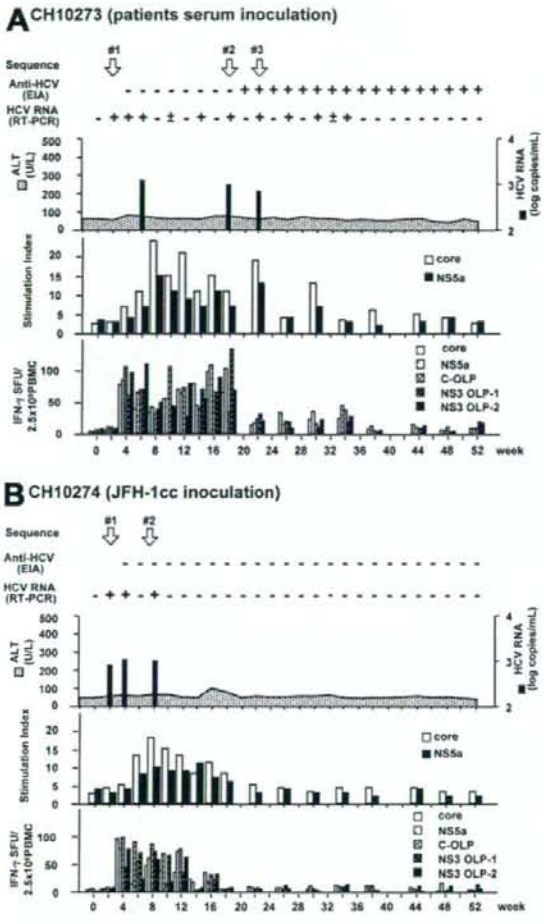


Fig. 1. Infection profiles and T cell immune responses in patient serum-inoculated and JFH-1cc-inoculated chimpanzees. (A) Chimpanzee CH10273 was inoculated with patient serum containing 9.6×10^6 copies of HCV. (B) Chimpanzee CH10274 was inoculated with JFH-1cc containing 1.4×10^7 copies of HCV. White arrows indicate the time points at which HCV sequences were determined. T cell proliferation assay results against HCV core and NS5a are shown as stimulation index (middle panel). IFN-γ responses against HCV core and NS5a proteins or overlapping peptide pools of core and NS3 are shown as SFU per 2.5×10^5 cells (bottom panel).

T-cell proliferative responses against the HCV core and NS5a proteins became positive 4 weeks after inoculation and continued up to 30 and 18 weeks in CH10273 and CH10274, respectively. Likewise, the IFN-γ responses against HCV structural and nonstructural antigens were detected 4 weeks after inoculation and maintained 34 weeks and 16 weeks in CH10273 and CH10274, respectively (Fig. 1).

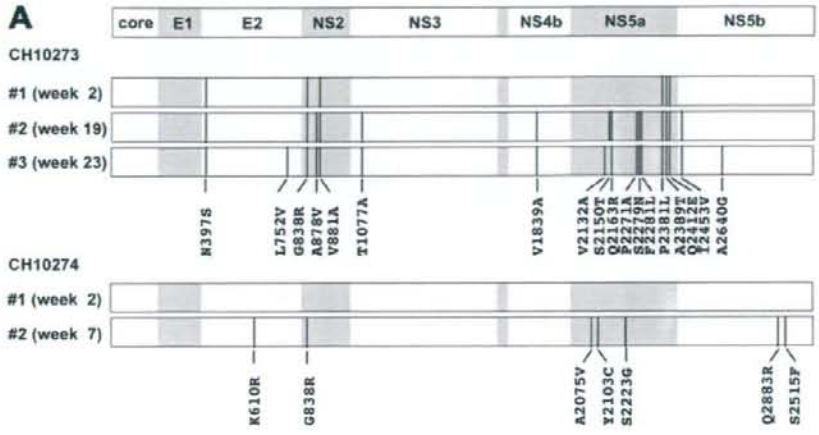
HCV Sequence Analysis. To investigate the difference and evolution of infected viruses, HCV sequences in both chimpanzees were determined directly at multiple time points as indicated in Fig. 1. In CH10273, HCV sequences were determined with sera collected at weeks 2, 19, and 23. Nineteen synonymous and six nonsynonymous mutations were already observed at week 2, and the number of mutations increased gradually with time (Table 1). Conversely, CH10274 showed no mutation at the earliest time point of infection (week 2) but subsequently developed four synonymous and seven nonsynonymous mutations at week 7 (Table 1). The mutated amino acids in the JFH-1 genome were distributed in E2, NS2, NS5a, and NS5b regions (Fig. 2A). Among these mutations, only one mutation, G838R in NS2, was identified as a common mutation between the two chimpanzees. To assess the complexity of the quasispecies, the amplified fragment encompassing HVR-1 was cloned and 10 clones in each time point were sequenced. In both animals, HVR populations of isolated HCV indicated similarly low complexity of heterogeneity (Fig. 2B). HCV clones isolated from CH10273 contained one HVR-1 mutation N397S at the earliest time point of infection, and this mutation could not be found in clones of the inoculum (Fig. 2B). To exclude the possibility of PCR artifact, sequences were confirmed by independent analyses. To ensure that the common NS2 mutant was not present as a minor species at the earliest time point of CH10274 (week 2), cloning (15 clones) and sequencing was performed and showed the WT sequence.

Effect of the NS2 Mutation on HCV Life Cycle. To assess whether this NS2 mutation could be a result of cytotoxic T-lymphocyte escape, which has been described in acutely HCV-infected chimpanzees,[17] we tested the T cell response of PBMCs from various time points during the infection against 18-mer peptides encompassing this region (both the WT and mutant sequences were tested). No T cell response could be detected against either the WT or mutant peptides throughout the infection, therefore making cytotoxic T-lymphocyte escape mutation highly unlikely. To assess the phenotype of the observed common mutation, G838R in the NS2 region, JFH-1 construct with this mutation was generated (JFH-1

Table 1. Sequence Evolution of JFH-1 in Chimpanzees

	Synonymous Mutations*	Non-synonymous Mutations*	Total
CH10273			
#1 (week 2)	19	6	25
#2 (week 19)	33	15	48
#3 (week 23)	35	17	52
CH10274			
#1 (week 2)	0	0	0
#2 (week 7)	4	7	11

*Compared with the consensus JFH-1 sequence.



B

Patient Serum:	HVR Sequence GTTTVGGAVARSTNVIAGVFSHGPQQN	No./Clones
(Inoculum) :	.....P.....	1/20
:	.....I.....	1/20
:	.....S.....	1/20
CH10273;		
Direct #1 :	.....S.....	
Cloning #1 :	.....S.....	9/10
:	S.....S.....	1/10
Direct #2 :	.....S.....	
Cloning #2 :	.....S.....	9/10
:	.....S.....R..	1/10
Direct #3 :	.....S.....	
Cloning #3 :	.....S.....	10/10
CH10274;		
Direct #1 :	.....	
Cloning #1 :	.....	9/10
:	.....A.....	1/10
Direct #2 :	.....	
Cloning #2 :	.....	10/10

Fig. 2. HCV sequence analyses. (A) Distribution of amino acid substitutions in patient serum-inoculated (CH10273) and JFH-1cc-inoculated (CH10274) chimpanzees. Positions of amino acid substitutions are indicated as vertical bars, and the mutated amino acids are shown at the bottom of each panel. The amino acid numbers correspond to the JFH-1 sequence. (B) HVR-1 populations in patient serum (inoculum) and chimpanzees. HVR-1 sequence in patient serum has been reported previously.[6] HVR-1 sequences determined by direct sequencing (Direct) or cloning and sequencing (Cloning) (10 clones at each time) in each animal are shown. Investigated time points (#1, 2, and 3) are indicated in Fig. 1. Identical amino acids are indicated as dots.

G838R). Viral replication and production of the JFH-1 G838R mutant was compared with that of the WT JFH-1 (JFH-1 WT) by transfecting the *in vitro* transcribed full-length genome RNA into Huh7.5.1 cells. HCV RNA levels in culture media of JFH-1 WT and JFH-1 G838R transfected cells were $2.96 \times 10^6 \pm 1.63 \times 10^5$ and $1.69 \times 10^7 \pm 3.61 \times 10^5$ copies/mL on day 3, and $2.67 \times 10^6 \pm 3.69 \times 10^5$ and $1.14 \times 10^7 \pm 2.23 \times 10^5$ copies/mL on day 5, respectively ($P < 0.05$)(Fig. 3A). In JFH-1 WT and JFH-1 G838R transfected cells, intracellular HCV RNA levels were $1.14 \times 10^8 \pm 1.36 \times 10^7$ and $3.66 \times 10^8 \pm 1.20 \times 10^7$ copies/well on day 3, and $1.67 \times 10^8 \pm 3.94 \times 10^7$ and $2.23 \times 10^8 \pm 1.90 \times 10^7$ copies/well on day 5, respectively ($P < 0.05$) (Fig. 3A). Thus, JFH-1 G838R could produce HCV RNA approximately fivefold higher than the JFH-1 WT in culture media and transfected cells (days 3 and 5, $P < 0.05$).

To confirm this observation, an infection study was also conducted with cell culture–generated viruses. After transfection of JFH1 WT and JFH-1 G838R genome RNA, viruses in culture media were harvested, and FFU of these viruses were titrated. The same titer of JFH1 WT or JFH-1 G838R viruses was inoculated into naïve Huh7.5.1 cells (9×10^2 FFU, multiplicity of infection = 0.003). After infection, HCV RNA titer in culture medium and infected cells was determined. Consistent with the transfection study, HCV RNA levels in culture media of JFH-1 G838R virus–infected cells were threefold to sixfold higher than those of JFH-1 WT virus (days 3 and 5, $P < 0.05$; Fig. 3B). Intracellular HCV RNA level on

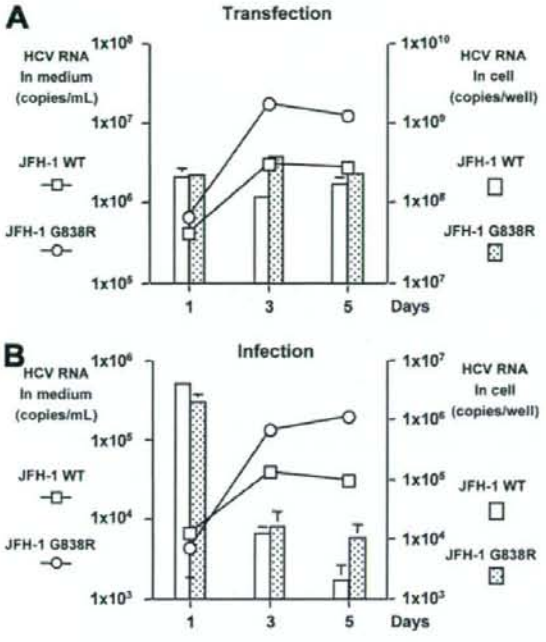


Fig. 3. Comparison of viral replication between JFH-1 WT and JFH-1 G838R in Huh 7.5.1 cells. At various times, HCV RNA was measured in culture media and cells by transfecting the same amount of *in vitro* transcribed full genome RNA (A) and by infecting the same FFU of JFH-1cc at a multiplicity of infection of 0.003 (B). Means of triplicate samples ± standard deviations are shown.

day 5 also appeared to be higher (fivefold) in JFH-1 G838R–infected cells ($P < 0.05$). Based on these data, JFH-1 G838R replicates more efficiently than the WT.

To further investigate the mechanism of this enhanced replication, we reasoned that this mutation could affect any of the viral RNA synthesis, assembly, or secretion steps. To distinguish among these possibilities, we used Huh7-25 cells, a Huh7 cells–derived cell line lacking CD81 expression.[11] This cell line cannot be reinfected by HCV but can support and produce infectious HCV on transfection with the HCV genome, therefore allowing us to address this question without the confounding effect of reinfection. HCV RNA levels of JFH-1 G838R–transfected cells in culture media were eightfold higher on day 1 and threefold higher on day 3 compared with those of JFH-1 WT transfected cells (Fig. 4A, $P < 0.05$). On day 5, the HCV RNA level was still higher in JFH-1 G838R–transfected cells, but the difference was less. The HCV RNA levels of the replication-deficient clone, JFH-1 GND, transfected cells were substantially lower than both NS2 mutant-transfected and WT-transfected cells (Fig. 4A). Similarly, HCV core Ag in culture media showed a significant difference between JFH-1 WT–transfected and JFH-1 G838R–transfected cells (days 1, 3, and 5, $P < 0.05$)(Fig. 4B). HCV core Ag of JFH-1 GND–transfected cells was under the detection limit. In contrast to culture media data, intracellular HCV RNA and core Ag levels in JFH-1 G838R–transfected cells were similar to or slightly lower than those of JFH-1 WT–transfected cells. Therefore, the G838R mutation does not appear to affect RNA replication and probably enhances either the assembly or secretion step.

To distinguish between these two possible effects, we determined the infectivity titer of intracellular viral particles in transfected cells as reported previously.[18] On day 3 after transfection, the intracellular infectivity titer in JFH-1 G838R–transfected cells was approximately four-

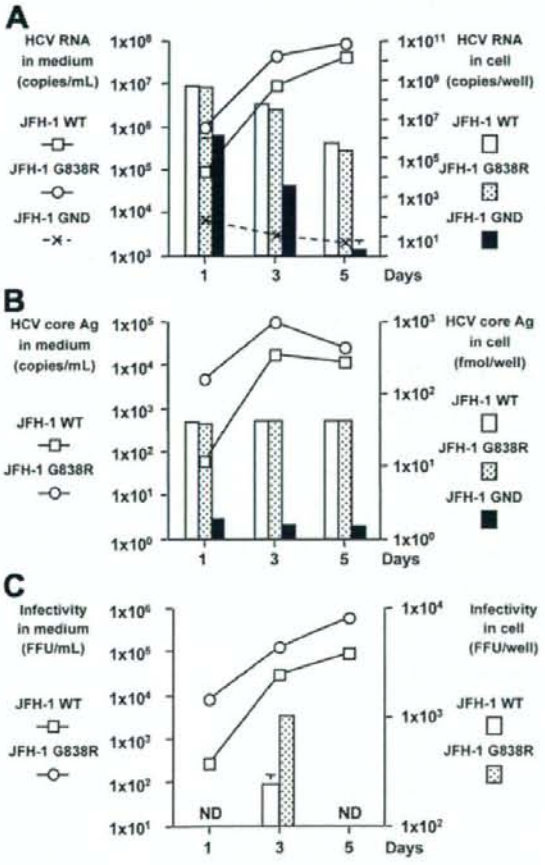


Fig. 4. Comparison of viral replication among JFH-1 WT, JFH-1 G838R, and JFH-1 GND in Huh 7-25 cells. At various times, HCV production was assessed in culture media and cells by transfecting the same amount of *in vitro* transcribed full genome RNA. HCV RNA titer (A), HCV core Ag level (B), and infectivity titers (C) are shown. The data are expressed as means of triplicate samples ± standard deviations. ND, not done.

Table 2. Specific Intectivity and Virus Secretion in Huh7-25 Cells

Clone	Intracellular HCV RNA (copies/well)	Intracellular Intectivity* (FFU/well)	Specific Intracellular Infectivity* (FFU/copies)	Extracellular HCV RNA* (copies/well)	Extracellular Infectivity* (FFU/well)	Specific Exracellular Infectivity (FFU/copies)	Infectious Virus Secretion (extra/intra)
JFH-1 WT	$4.40 \times 10^7 \pm 1.58 \times 10^7$	$2.27 \times 10^2 \pm 5.17 \times 10^1$	$1.09 \times 10^{-5} \pm 2.58 \times 10^{-6}$	$1.83 \times 10^7 \pm 1.95 \times 10^6$	$6.17 \times 10^3 \pm 9.61 \times 10^2$	$3.37 \times 10^{-4} \pm 1.38 \times 10^{-3}$	7.20 ± 2.83
JFH-1 G838R	$2.19 \times 10^7 \pm 1.11 \times 10^6$	$9.89 \times 10^2 \pm 5.02 \times 10^1$	$9.05 \times 10^{-5} \pm 2.76 \times 10^{-6}$	$5.14 \times 10^7 \pm 3.48 \times 10^6$	$2.69 \times 10^4 \pm 6.96 \times 10^3$	$5.33 \times 10^{-4} \pm 1.83 \times 10^{-4}$	6.87 ± 2.07

The data are from day 3 after HCV RNA transfection of the Huh7-25 cells.
*$P < 0.05$ comparing JFH-1 WT and G838R.

fold higher than that in JFH-1 WT–transfected cells ($P < 0.05$, Fig. 4C and Table 2). Moreover, specific intracellular infectivity of JFH-1 G838R–transfected cells was approximately eightfold higher than that in JFH-1 WT–transfected cells ($P < 0.05$, Table 2). Specific infectivity in culture medium was determined as the ratio of infectious virus (FFU) over HCV RNA copies. Specific infectivity of the JFH-1 G838R viruses was not significantly different from that of JFH-1 WT (Table 2). Finally, the rate of secretion was determined by the ratio of extracellular FFU over the intracellular FFU (Table 2), and no difference was observed between JFH-1 WT and G838R-transfected cells. Based on these data, the G838R mutation in JFH-1 enhances the assembly step of HCV.

Discussion

Although HCV-associated fulminant hepatitis is rare, several cases have been reported.[6,19-25] The HCV JFH-1 strain was isolated from one of these cases, and its unique characteristic of robust replication in cell culture might be related to the cause of fulminant hepatitis. Previously, HCV from a patient with fulminant liver failure has been shown to cause severe acute hepatitis with high viremia in a chimpanzee, although its molecular clone could not replicate in culture cells and did not induce severe hepatitis in the chimpanzee.[26,27] In our previous study, JFH-1cc induced a transient and attenuated infection in a chimpanzee.[8] The infection profile was different from the typical course of HCV infection either with patient sera or infectious RNA molecules in chimpanzees.[28-32] Because this observation was unexpected, we reasoned that the lower virulence of this strain *in vivo* might be related to the age of the chimpanzee. The chimpanzee used in the previous study was older (>25 years of age), and older chimpanzees typically do not develop significant disease on HCV infection. Another possible cause was the characteristics of the viral inoculum. JFH-1cc inoculated in the chimpanzee was monotypic because it was generated in culture cells. The original JFH-1 virus replicating in the fulminant hepatitis patient existed as a mixture of various viral species and might induce a different outcome *in vivo*. Thus, to elucidate the pathogenesis and replication capacity of the original JFH-1 strain *in vivo*, the patient serum and the JFH-1cc were inoculated into juvenile chimpanzees (5 years old). However, both chimpanzees showed attenuated infection with low-titer viremia, no ALT elevation, and absence of histological hepatitis during the acute phase of infection. Therefore, the manifestation of fulminant hepatitis of the original patient was likely a result of host factors, with the caveat that humans and chimpanzees might respond differently to HCV infection.

Similar to our previous study, the chimpanzee inoculated with monotypic JFH-1cc showed a short duration of infection and absence of seroconversion. Conversely, the chimpanzee inoculated with the patient serum showed a longer course of infection and developed anti-HCV antibodies. Immunological analysis with T-cell proliferation and IFN-γ ELISpot assays showed that HCV-specific immune responses were similarly induced in both animals and abated with the disappearance of viremia. Consistent with the longer viremia, the chimpanzee inoculated with the patient serum had a longer duration of detectable HCV immune response (Fig. 1). These differences could be explained by the sequence variations of the infecting HCV. In the chimpanzee inoculated with the patient serum, the infecting HCV showed a low sequence complexity but exhibited some sequence diversity already at week 2. The infecting HCV had a sequence alteration in the HVR-1 (N397S), but this sequence alteration could not be found in any of the 20 clones of the inoculum (Fig. 2B).[6] In addition, the NS2 G838R mutation was also not detected by cloning (six clones) and sequencing of the inoculum. Thus, this infecting HCV was probably selected from a minor species in the patient serum. It has been reported that minor clones in human serum were

selected during HCV infection in chimpanzees.[33] The selected clones were in the lighter fraction of the sucrose density gradient of the inoculum, which is devoid of immunoglobulins. Similar selection might have occurred in our study. The dominant clones in the inoculum might not be infectious because of binding to neutralizing antibodies. As a result, the infection-competent minor clone, selected during the infection, became the dominant species. Furthermore, this infecting minor clone could persist longer, although the characteristics of this clone and mechanisms for persistence are still unknown. HCV clones in CH10273 showed several other mutations at 2 weeks postinfection and accumulated additional mutations in E2, NS2, NS3, NS4b, NS5a, and NS5b regions over time (Fig. 2). Some of these regions contain known T-cell epitopes, although the major histocompatibility complex haplotype of this animal is unknown. In this chimpanzee, heterogeneity of the inoculating viruses might have contributed to the emergence of escape mutants from the host immune system, resulting in a prolonged infection. Similar observations have been reported in acute HCV infection in chimpanzees and humans.[34-36]

In HCV strains isolated from these two chimpanzees, one common mutation G838R in the NS2 region was identified. This mutation has not been reported among the adaptive mutations emerged in the JFH-1 virus passaged in cell culture.[37-39] This mutation likely arose *de novo* because one of the chimpanzees was inoculated with a molecular clone, and the week 2 sample did not harbor this mutation. NS2 is a membrane-associated cysteine protease, composed of three transmembrane domains and a protease domain.[40] Although the NS2 region is dispensable for RNA replication, it is essential for production of infectious virus in cultured cells.[41-43] Furthermore, the significance of this region has been shown in the establishment of replication-competent and infection-competent intergenotypical chimeric viruses.[44,45] The identified common mutation G838R was at the end of the first transmembrane domain,[46] and mutations in the transmembrane domains have been shown to improve the yield of infectious virus production in several studies.[45,47] Thus, some advantage of this mutation in HCV replication and production could be expected. This mutation was shown to enhance HCV production in Huh7.5.1 cells. Detailed analysis with CD81-negative Huh7-25 cell demonstrated that viral assembly was affected by this mutation. Production of infectious virus in JFH-1 G838R–transfected cells was eightfold higher than that in the JFH-1 WT–transfected cells. Thus, this mutation enhances the assembly of infectious virus particle in cultured cells, and as a result, increases infectious virus production in the culture medium. This mutation represents the first identified *in vivo* adapted mutation that is not immunologically mediated and probably confers a replication advantage to the virus *in vivo*. This adaptive mutation, unlike the other adaptive mutations reported *in vitro* with poor infectivity *in vivo*, likely results from a highly biologically relevant event in the dynamic interaction between HCV and host. Finally, it is possible that compensatory mutations in other regions of the virus may contribute to the overall biological adaptive response of the virus *in vivo*.

This study demonstrates that the HCV JFH-1 strain either generated in cell culture as a monotypic virus or obtained from patient serum is associated with attenuated infection in chimpanzees; however, the virus can rapidly evolve with adaptive mutations to facilitate propagation of the virus in a susceptible host.

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Induction of Incomplete Autophagic Response by Hepatitis C Virus via the Unfolded Protein Response

Donna Sir,[1] Wen-ling Chen,[1] Jinah Choi,[1] Takaji Wakita,[2] T.S. Benedict Yen,[3] and Jing-hsiung James Ou[1]

Autophagy is important for cellular homeostasis and can serve as innate immunity to remove intracellular pathogens. Here, we demonstrate by a battery of morphological and biochemical assays that hepatitis C virus (HCV) induces the accumulation of autophagosomes in cells without enhancing autophagic protein degradation. This induction of autophagosomes depended on the unfolded protein response (UPR), as the suppression of UPR signaling pathways suppressed HCV-induced lipidation of the microtubule-associated protein light chain 3 (LC3) protein, a necessary step for the formation of autophagosomes. The suppression of UPR or the suppression of expression of LC3 or Atg7, a protein that mediates LC3 lipidation, suppressed HCV replication, indicating a positive role of UPR and the incomplete autophagic response in HCV replication. ***Conclusion:*** **Our studies delineate the molecular pathway by which HCV induces autophagic vacuoles and also demonstrate the perturbation of the autophagic response by HCV. These unexpected effects of HCV on the host cell likely play an important role in HCV pathogenesis.** (HEPATOLOGY 2008;48: 1054-1061.)

Autophagy is important for removing long-lived proteins and damaged organelles in cells. During autophagy, double-membrane vesicles form to sequester part of the cytoplasm. These double-membrane vesicles, also known as autophagosomes, subsequently fuse with lysosomes to form autolysosomes for the degradation of their contents for recycling.[1] Many genes that are important for autophagy have been identified. Among them is microtubule-associated protein light chain 3 (LC3), whose covalent linkage to phosphatidylethanolamine by the ubiquitin-activating enzyme E1-like protein Atg7 is necessary for the formation of autophagosomes.[2]

Hepatitis C virus (HCV) is a positive-stranded RNA virus with a genome size of 9.6 Kb. Infection by this virus can lead to liver cirrhosis and hepatocellular carcinoma. Based on their genetic relatedness, different HCV isolates have been grouped into six major genotypes and many more subtypes. The HCV genome codes for a polyprotein, which is proteolytically cleaved to generate the mature protein products.[3]

Recently, a cell culture system for efficient HCV propagation using the JFH1 strain, which belongs to HCV genotype 2a, has been developed.[4-7] In this system, the HCV JFH1 RNA or its derivative was transfected into human hepatoma cells to direct the replication and release of infectious HCV particles, which could then initiate the next round of infection. In this report, we use this HCV RNA transfection/infection system to study HCV–host interactions. Our results indicate that HCV induces the accumulation of autophagosomes by activating unfolded protein response (UPR). However, HCV does not enhance autophagic protein degradation. Importantly, this induction of autophagosomes enhanced HCV replication. The persistent induction of the UPR and the

Abbreviations: BAF, bafilomycin A1; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; ER, endoplasmic reticulum; GFP, green fluorescence protein; HCV, hepatitis C virus; LC3, microtubule-associated protein light chain 3; mRNA, messenger RNA; PERK, phosphorylated extracellular signal-regulated kinase; qRT-PCR, quantitative real-time polymerase chain reaction; siRNA, small interfering RNA; Tg, thapsigargin; UPR, unfolded protein response.

From the [1]Department of Molecular Microbiology and Immunology, University of Southern California, Los Angeles, California; the [2]Department of Virology II, National Institute of Infectious Diseases, Shinjuku, Tokyo, Japan; and the [3]Pathology Service, Veterans Affairs Medical Center, and Department of Pathology, University of California, San Francisco, San Francisco, CA.

Dr. Choi is currently affiliated with the School of Natural Sciences, University of California, Merced, CA.

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Address reprint requests to: Jing-hsiung James Ou, Department of Molecular Microbiology and Immunology, University of Southern California, Keck School of Medicine, Los Angeles, CA 90033; E-mail: jamesou@hsc.usc.edu; fax: 323-442-1721.

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perturbation of the autophagic response likely play an important role in HCV pathogenesis.

Materials and Methods

Cell Cultures, DNA Plasmids, and Small Interfering RNAs. Huh7.5 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% nonessential amino acids (the "growth medium"). Huh7.5 cells that stably expressed GFP-LC3 were produce by transfecting Huh7.5 cells with pEGFP-LC3[8] followed by G418 selection. The plasmids pJFH1 and pJFH1/GND used had been described before.[9] Stable Huh7 cells that contained the HCV Con1 subgenomic RNA replicon has also been described.[10] The two phosphorylated extracellular signal-regulated kinase (PERK) small interfering RNAs (siRNAs)[11] and LC3 siRNA[12] were synthesized at the USC Microchemical Core Facility. IRE1α, ATF6, and Atg7 siRNAs were purchased from Qiagen (Germantown, MD), and the negative control siRNA was purchased from Invitrogen (Carlsbad, CA). Cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) per the manufacturer's instructions. The siRNA sequences are shown in Supplementary Table 1.

In Vitro Transcription of HCV RNA and Electroporation of Huh7.5 Cells. Plasmids pJFH-1 and pJFH-1/GND were linearized with the restriction enzyme Xba1 for RNA synthesis using the MEGA-script kit (Ambion, Foster City, CA). Huh7.5 cells were then electroporated with the HCV RNA. Unless specifically indicated, all the analyses were conducted on cells between 2 and 5 days after electroporation.

Confocal Microscopy and LysoTracker-Red Staining. Cells were fixed with 4% formaldehyde and incubated with the rabbit anti-HCV core antibody and then with the rhodamine-conjugated goat anti-rabbit antibody for confocal microscopy. For LysoTracker red staining, cells were treated with 50 nM LysoTracker Red DND-99 (Invitrogen, Carlsbad, CA) at 37°C for 3 hours. Depending on the experiments, cells might be nutrient starved in Hank's balanced salt solution or treated with 2.5 mM dithiothreitol (DTT) in the growth media for the induction of endoplasmic reticulum (ER) stress at 37°C for an additional 20 minutes in the presence of LysoTracker-red. The colocalization coefficient, which measures the fraction of green fluorescent protein (GFP) pixels that are also positive for LysoTracker-red, was performed on randomly selected GFP-positive cell images ($n > 20$) using the Zeiss LSM 510 imaging software. A colocalization coefficient of 1 means complete colocalization, whereas 0 means no colocalization.

Long-Lived Protein Degradation Assay. Cells were labeled with L-[4,5-^{3}H] leucine (50 μCi/mL) for 24 hours in growth media, rinsed with DMEM, and further incubated in growth media for 24 hours. Cells were then rinsed with DMEM or, for nutrient starvation, with Hank's balanced salt solution, and treated with or without 200 nM bafilomycin A1 in DMEM or Hank's balanced salt solution at 37°C for 1 or 4 hours. Cell lysates and media were collected at the end of treatment, precipitated with trichloroacetic acid and analyzed by scintillation counting. The protein degradation rate was determined by the following equation: (trichloroacetic acid–soluble counts in media) / (total counts in media and cell lysates) × 100%. Cells treated with thapsigargin (Tg) were analyzed using the same procedures, with the exception that Tg was added into the growth media 12 hours after the removal of the ^{3}H-leucine.

Quantitative Reverse Transcription Polymerase Chain Reaction. Total cellular RNA was analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) using the TaqMan Gold RT-PCR Kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. HCV JFH1 primers 5′-TCTGCGGAACCGGTGAGTA-3′ (sense) and 5′-TCAGGCAGTACCACAAGGC-3′ (antisense) and the probe 5′-CACTCTATGCCCGGCCATTTGG-3′ were used for the qRT-PCR. Control glyceraldehyde 3-phosphate dehydrogenase primers and its probe were purchased from Applied Biosystems (Foster City, CA).

Analysis of XBP1 Messenger RNA Splicing by IRE1. Total cellular RNA was reverse transcribed and amplified using the sense primer (5′-CCTTGTAGTTGAGAACCAGG-3′) and antisense primer (5′-GGGGCTTGGTATATATGTGG-3′). The PCR products were further digested with the restriction enzyme Pst1. For internal control, the complementary DNA of the β-actin messenger RNA (mRNA) was also amplified using the sense primer (5′-ATCTGGCACCACACCTTCTACAATGAG-3′) and the antisense primer (5′-CGTCATACTCCTGCTTGCTGATCC-3′).

Results

Induction of Autophagosomes by HCV. We transfected the HCV JFH1 RNA or its replication defective GND mutant RNA into Huh7.5 cells to study HCV–host interactions. The GND mutant RNA served as a negative control, because it cannot replicate and is rapidly degraded in transfected cells. By performing electron microscopy, we found that HCV JFH1 induced the accu-

mulation of membrane vesicles with the morphology of autophagosomes (Supplementary Fig. 1A), which were rarely observed in GND RNA transfected cells (Supplementary Fig. 1B). To investigate whether HCV could indeed induce the accumulation of autophagosomes, we established stable Huh7.5 cells that expressed the GFP-LC3 fusion protein. Cells transfected by the GND RNA displayed no detectable HCV core protein signal and in general a weak and diffused cytoplasmic signal of the GFP-LC3 fusion protein (Fig. 1A). In contrast, most of the cells transfected by the JFH1 RNA stained strongly for the HCV core protein with most of them (approximately 80% at day 5 posttransfection) also displaying bright and punctuate GFP-LC3 signal (Fig. 1B). At a higher magnification, many of the core protein signals were often found to be circular, which is consistent with previous reports that the core protein is associated with lipid droplets. Importantly, the GFP-LC3 signals were also often found to be circular, consistent with their localization on autophagosomal membranes (Fig. 1C). Circular GFP-LC3 signals were also observed when JFH1 cells were stained for the HCV NS5A protein (Supplementary Fig. 2).

These observations indicated that HCV could induce the accumulation of autophagosomes. This possibility was further confirmed by western blot analysis of LC3, which is converted from the cytosolic form (LC3-I) to the lipidated, autophagosome-associated form (LC3-II) during autophagy. Although little LC3-II could be detected in mock transfected cells or cells transfected with the GND mutant RNA, a significant amount of LC3-II was detected in cells transfected with the JFH1 RNA (Fig. 2A). The ability of HCV to induce the lipidation of LC3 is not limited to the HCV JFH1 isolate; a similar result was observed in Huh7 cells harboring a high replication level of the subgenomic HCV RNA replicon derived from the genotype 1b Con-1 virus (Fig. 2B).[10]

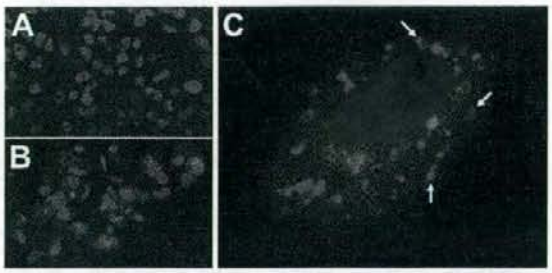

Fig. 1. Induction of autophagic vacuoles by HCV. Stable GFP-LC3 cells were transfected by the HCV GND RNA (A) or the HCV JFH1 RNA (B, C). Green, the GFP-LC3 signal; red, the HCV core protein; blue, 4′,6-diamidino-2-phenylindole staining of nuclei. Cells were analyzed 5 days posttransfection. Arrows in C highlight the circular GFP-LC3 signals.

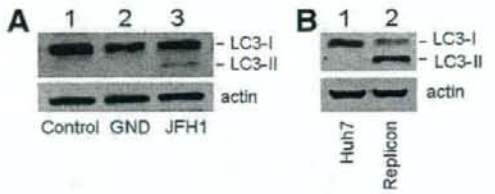

Fig. 2. Induction of LC3 lipidation by HCV. (A) Huh7.5 cells were mock-transfected (lane 1), transfected with the HCV GND RNA (lane 2) or with the HCV JFH1 RNA (lane 3), and lysed 5 days posttransfection for western blot analysis using the rabbit anti-LC3 antibody (MBL International, Woburn, MA) (top panel). Actin (bottom panel) was also analyzed to serve as a loading control. (B) Control Huh7 cells (lane 1) or stable Huh7 cells containing the HCV Con-1 subgenomic RNA replicon (lane 2) were lysed and analyzed by western blotting for LC3 (top panel) and actin (bottom panel).

Lack of Enhancement of Autophagy by HCV. The results shown in Figs. 1 and 2 indicated that HCV could induce the accumulation of autophagosomes in cells. To investigate whether HCV could also enhance autophagic protein degradation, we analyzed the degradation rate of long-lived proteins in cells. Huh7.5 cells were metabolically labeled with ^{3}H-leucine for 24 hours followed by the chase of another 24 hours to allow the degradation of labeled, short-lived proteins. Cells were then rinsed and incubated in fresh media in the presence and absence of bafilomycin A1 (BAF), a vacuolar adenosine triphosphatase inhibitor that suppresses the fusion between autophagosomes and lysosomes.[13] The protein degradation rate was determined by measuring the amount of trichloroacetic acid–soluble radiolabel released into the medium per hour. The overall protein degradation rates and those sensitive to BAF and hence mediated by autophagy are shown in Fig. 3A and 3B, respectively. As shown in the figures, nutrient starvation, which induces autophagy and served as the positive control, increased both overall and BAF-sensitive protein degradation rates. Interestingly, HCV JFH1 did not increase, but rather slightly reduced, overall and BAF-sensitive protein degradation rates. These results suggested that, although HCV JFH1 was able to enhance the accumulation of autophagosomes, it did not enhance autophagic protein degradation. To further confirm this observation, we analyzed the p62/SQSTM1 protein level in cells. The p62/SQSTM1 protein binds to LC3 and is degraded by autophagy.[14] There was a continuous increase, albeit small, of the p62/SQSTM1 protein level in cells during the first 24 hours after the transfection of the HCV RNA (Fig. 3C). In contrast, nutrient starvation reduced the p62 level by approximately 65%. These results again indicated that HCV JFH1 did not enhance autophagic protein degradation.