Indirect immunofluorescence assay

The inoculated cells were fixed with methanol and immunostained with a mouse monoclonal anti-core antibody and a rabbit polyclonal anti-NS5A antibody [22], followed by an Alexa Fluor 555-conjugated anti-mouse secondary antibody (Life Technologies Japan Ltd.).

Transmission electron microscopy (EM)

Cells were fixed with 1.5% glutaraldehyde in 1.0% cacodylate buffer, pH 7.4, for 5 min, and then post-fixed with 2% OsO_4 in phosphate buffer, pH 7.4, for 1 hour. The cells were dehydrated in ethanol and embedded in Epon. Ultrathin sections were double stained and examined at an accelerating voltage of 80 keV. Immuno-EM (IEM) were performed by using the labeled-(strept) avidin-biotin (LAB) kit according to the manufacturer's instructions (Zymed laboratories, San Francisco, CA) as described previously [27].

Statistical Analysis

Assays were performed at least four independent experiments. Data are expressed as the mean ± SD. Statistical analysis was performed using Student's t test.

Results

Anti-HCV effects of GL

To assess the anti-HCV effects of GL, HCVcc-infected cells were treated with various concentrations of GL for 72 hours, and then the levels of HCV core antigen and infectivity of the medium were determined. HCV core antigen levels were reduced by 29% with 500 μM GL (Figure S1). As shown in Figure 1A, infectivity of supernatant following GL treatment at 3, 30, or 500 μM was reduced by 12, 62, or 71% of the control levels, respectively. The calculated 50% effective concentration (EC_{50}) was 16.5 μM. There was no effect on cell viability after these treatments (Figure 1B). These results suggest that GL effectively inhibited the production of infectious HCV.

HCV propagates in hepatocytes throughout its lifecycle, including the stages of attachment, entry, uncoating, translation, genome replication, assembly, budding, and release. To investigate which step of the HCV lifecycle GL inhibited, we used the HCVpp system for evaluating attachment and entry, and the HCV replicon system for translation and genome replication. Treatment of HCVpp2a with GL resulted in a moderate reduction of luciferase activity in the cells infected with HCVpp, with an EC_{50} value of 728 μM (Figure 1C). On the other hand, there was no significant reduction of luciferase activity in the cells infected with HCVpp1b (Figure 1D) and VSVpp (Figure 1E). No cytotoxic effects of GL were observed (data not shown).

Huh7 cells harboring the type-2a subgenomic replicon were treated with various concentrations of GL for 72 hours. Relative luciferase activities of GL-treated cells were inhibited in a dose-dependent manner with an EC_{50} value of 738 μM (Figure 1F). A similar result was obtained by using the type-1b subgenomic replicon (data not shown). We also transfected HCV RNA lacking E1E2 (JFH1delE1E2) and monitored the effect of GL on HCV replication to avoid reinfection of Huh7 cells. There was no significant reduction of HCV RNA titers in the cells (Figure 1G). There was no significant cytotoxicity seen following these treatments (data not shown).

To investigate the effect of GL on entry, HCV particles were treated with increasing concentrations (0 to 1500μM) of GL. The viral samples were then used to inoculate Huh7 cells cultured in GL-containing medium. Several hours post-infection, medium was replaced with DMEM without GL. The levels of HCV core antigen in the medium were determined at 72 h postinfection (p.i.). There was no significant reduction of HCV production (Figure 1H). These results indicated that GL did not inhibited HCV entry and replication significantly.

Effects of GL on infectious HCV particle release

To further assess whether GL treatment affects other steps of the viral lifecycle, we analyzed infectious HCV particle assembly and release following GL treatment. Supernatant or crude cell lysates of HCVcc-infected cells treated with GL were used to inoculate naïve Huh7 cells to determine extra- and intracellular specific infectivity, respectively. Specific infectivity was determined as the ratio of infectious virus titer to HCV core antigen level, as described previously [28]. As shown in Figure 2A, the extracellular specific infectivity titer was inhibited by 57% by GL at a concentration of 500 μM, on the other hand, the intracellular specific infectivity titer was increased 3.8-fold over that of controls at the same concentration of GL (Figure 2B). There was no significant cytotoxicity following these treatments (data not shown).

It has been previously reported that virus assembly takes place around lipid droplets (LDs) [29]. By immunofluorescence staining, we examined the subcellular co-localization of HCV core (Figure 2C) or NS5A (Figure 2D) with LDs in HCVcc-infected cells with or without GL treatment. Un-infected cells were shown in Figure 2E. We observed HCV proteins colocalized with LDs (Figure 2C and 2D). Intensity profiles along the line segments, shown on the bottom of the images, demonstrated that core proteins were tightly colocalized with LD in the HCVcc-infected cells treated with GL, when compared with untreated cells (Figure 2C lower panel). We quantified the size of LDs in HCV-infected cells (Figure 2D) and un-infected cells (Figure 2E) with GL-treatment. We found that GL did not affect the size of LDs in un-infected cells (Figure 2F right panel). On the other hand, the size of LDs increased in HCV-infected cells with GL-treatment (Figure 2F left panel).

HCVcc-infected cells (Figure 2G) and un-infected cells (Figure 2H), treated with GL, were prepared for EM analysis. In the cytoplasm of HCV-infected cells, we observed increased numbers of LDs in close proximity to endoplasmic reticulum (ER) and the electron-dense signals on ER attached to LD (Figure 2G upper panel), which are thought to act as platforms for the assembly of viral components [29]. Interestingly, in the cytoplasm of HCV-infected cells after treatment with GL, accumulated electron-dense particles were observed on ER attached to LD (Figure 2G lower panel). IEM experiments showed that anti-core antibody stained the membrane around LDs (Figure 2I lower panel). In naïve Huh7 cells, the close association of LDs with ER was rarely observed (Figure 2H).

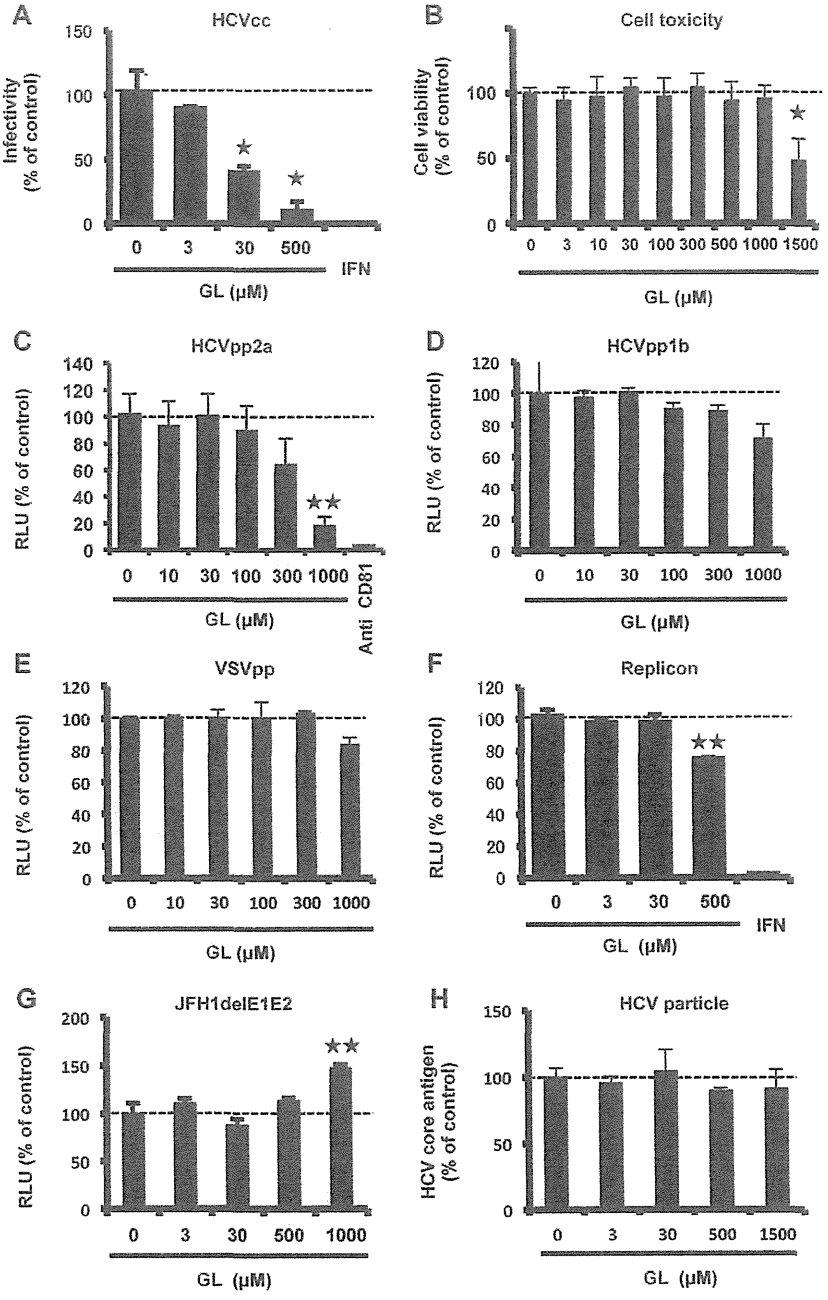

Figure 1. Anti-HCV effects of GL. (A) HCVcc-infected cells were treated with various concentrations of GL for 72 hours. Naïve Huh7 cells were inoculated with supernatant and cultured for 72 hours. Infectivity was determined by immunostaining. (B) Cell viability was assessed using Cell Titer-Glo Luminescent Cell Viability Assay. Huh7 cells were infected with HCVpp2a (C), HCVpp1b (D), and VSVpp (E) in various concentrations of GL for 24 hours, and then medium was replaced. Effects of GL on entry of HCVpp and VSVpp were determined by measuring the luciferase activity at 72 hours post-transfection. (F) Huh7 cells harboring the type-2a subgenomic replicon were treated with various concentrations of GL for 72 hours. Replication efficiency of the replicon was estimated by measuring the luciferase activity. (G) The effects of GL on HCV replication were tested by electroporation of HCV RNA lacking E1E2 (JFH1delE1E2). (H) HCV particles were treated with increasing concentrations (0 to 1500μM) of GL. The viral samples were then used to inoculate Huh7 cells with GL-containing medium. Several hours post-infection, medium was replaced with DMEM without GL. The levels of HCV core antigen of the medium were determined at 72 h postinfection (p.i.). IFN (300 IU/ml) was used as a positive control for reduced HCV replication. Anti-human CD81 antibody (10 μg/ml) was used as a positive control for reduced HCV entry to the cells. Results are expressed as the mean ± SD of the percent of the control from four independent experiments. *P < 0.05, **P < 0.005 versus control (0 μM treatment).

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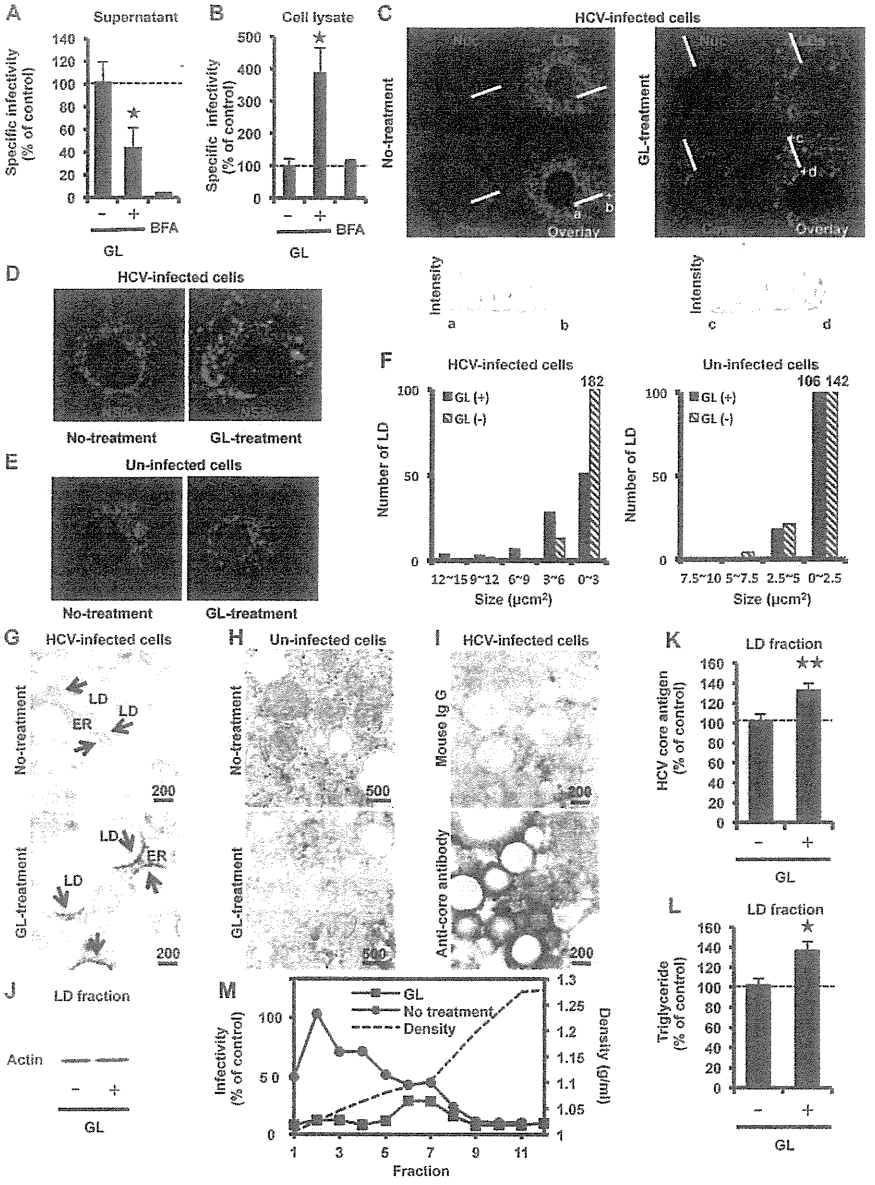

Figure 2. Effects of GL in release of infectious HCV particles. HCVcc-infected cells were treated with GL at a concentration of 500 μM for 72 hours. Untreated cells were used as controls. Extra- (A) and intracellular specific infectivity (B) were determined. Subcellular co-localization of HCV core (C) or NS5A (D) with LDs in HCVcc-infected cells with or without GL treatment. (E) Un-infected cells. LDs and nuclei were stained with BODYPI 493/503 (green) and DAPI (blue), respectively. (C) Points a and b, as well as c and d, define two line segments that each cross several structures. Intensity profiles along the line segments shown on the bottom of the images. (F) The size of LDs in un-ifected cells (right panel) and HCV-infected cells (left panel) were quantified. Transmission EM of LDs in infected cells (G) and un-infected cells (H) treated with GL at 500 μM. Arrows indicate electron-dense signals (G upper panel) and particles (G lower panel). (I) IEM using the LAB method of LDs in infected cells treated with GL at 500 μM. Mouse IgG (upper panel) or anti-core monoclonal antibody (lower panel) was used for primary antibody. (J) Immunoblotting with anti-actin antibody in the LD fraction. Quantification of HCV core antigen (K) and TG (L) in the LD fraction. The LD fraction was collected from cell lysates. The ratio of HCV core antigen level in the LD fraction to that in total cell lysate was determined. (M) HCVcc-infected cells were treated with GL at 500 μM for 72 hours. Untreated cells were used as controls. Supernatant was ultracentrifuged through a 10-60% sucrose gradient and the infectivity of each fraction was determined. Infectivity of fraction 2 of untreated cells was assigned the arbitrary value of 100%. The density of each fraction was measured by refractive index measurement. Brefeldin A (1 μM for 24 hours) was used as a positive control for reduced HCV release. Results are expressed as the mean ± SD of the percent of the control from four independent experiments. *P < 0.05, **P < 0.005 versus control (0 μM treatment). Scale bars, 200 and 500 nm.

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To confirm the accumulation of core antigen around LD, we purified the LD [23], and quantified HCV core antigen and TG in the LD fraction, followed by immunoblotting with anti-actin antibody (Figure 2J). Analysis of the levels of HCV core antigen and TG in the LD fraction of the total cell lysate showed that the amount in GL-treated cells was increased by 31% and 35% compared with controls, respectively (Figure 2K and 2L). Taken together, these results suggested that GL inhibits release, but not assembly and budding, of infectious HCV particles in cells.

To characterize the infectivity of HCV particles released from HCVcc-infected cells treated with GL, supernatant from cell cultures treated or not treated with GL was subjected to continuous 10-60% (w/v) sucrose density gradient centrifugation, and the infectivity titer of each fraction was measured. A reduction in infectivity by GL-treatment was observed in fractions 1-7 (Figure 2M). These results suggest that GL may decrease the amount of HCV infectious particles in the supernatant.

Role of PLA2 in HCV lifecycle

GL is known to have an inhibitory effect on PLA2 [8]. PLA2 is classified into several groups and their biological functions are not the same. It is unknown which group of PLA2 is targeted by GL. We analyzed the effect of GL on PLA2G1B and PLA2G2A, which were major groups of PLA2 family. To confirm the effects of GL on expression of PLA2G1B, cells, transfected with an expression plasmid for PLA2G1B, were treated with GL and OPC, which is a specific inhibitor for PLA2G1B. Treatment with GL effectively decreased the cellular level of PLA2G1B (Figure S2). To verify whether PLA2 has a role in viral entry and replication, we tested the effect of PLA2 inhibitors on HCVpp infection and the replicon system, respectively. OPC has no significant effect on virus entry and replication (Figure 3A and 3B). On the other hand, sPLA2IIA inhibitor I, which is a specific inhibitor for PLA2G2A, inhibited both HCVpp entry (Figure 3A) and subgenomic replicon replication (Figure 3B). There was no significant cytotoxicity seen after these treatments (data not shown).

To evaluate the effects of PLA2 inhibitors on HCVcc infectivity, infected cells were treated with PLA2 inhibitors and extra- and intracellular specific infectivity were measured (Figure 3C and 3D). OPC slightly decreased specific infectivity of virus in the supernatant and significantly increased specific infectivity of virus in the cell lysate. On the other hand, sPLA2IIA inhibitor I significantly decreased the specific infectivity of virus in both the supernatant and cell lysate. To confirm the importance of PLA2G1B in HCV release, we silenced PLA2G1B with its specific siRNA and monitored its effect on HCV release. PLA2G1B siRNA decreased the cellular level of PLA2G1B (Figure S3). Suppression of PL2G1B reduced core protein level in the medium (Figure 3E left panel) and increased specific infectivity in the cells (Figure 3E right panel). We performed GL treatment with or without OPC and showed that GL and OPC had no additive effect when applied together (Figure 3F). There was no significant cytotoxicity seen after these treatments (data not shown). Taken together, these results suggest that the suppression of virus release by GL may be derived from its inhibitory effect on PLA2G1B. These results also suggested that PLA2G1B has a role in virus release.

Antiviral effects of IFN along with GL

We have demonstrated that the target causing the anti-HCV effect of GL differs from that of IFN. To analyze the antiviral effect of IFN combined with GL, HCVcc-infected cells were treated with 0.1 and 1.0 IU/ml of IFN in combination with various concentrations of GL. HCV core level in culture medium (Figure 4A) and in the cell (Figure 4B), specific infectivity in culture medium (Figure 4C) and in the cells (Figure 4D) were measured. Regardless to the IFN concentration, HCV core level and specific infectivity of the supernatant decreased in response to GL treatment in a dose dependent manner (Figure 4A and 4C). On the other hand, HCV core level and specific infectivity of the cell increased (Figure 4B and 4D), suggesting that GL inhibited HCV release. The results indicated that a combination therapy of IFN with GL could be an effective treatment for HCV.

Effect of GL on IFN induction and secretion proteins

The IFN-inducing ability of GL has also been previously reported [30]. We evaluated IFN stimulated gene induction by GL, but no effects were observed (Figure S4). PLA2 is known to be associated with various intracellular trafficking events and secretion of very low-density lipoprotein (VLDL) [31]. HCV particles are known to be secreted using the host membrane trafficking system [32]. There is now increasing evidence that VLDL participates in HCV assembly and release [33]. Therefore, we analyzed the level of albumin, an abundantly secreted protein from hepatocytes, and apolipoprotein E (ApoE), a component of lipoproteins, in the culture supernatants of Huh7 cells and found that they were not influenced by GL treatment (Figure S5).

Discussion

Recently, Ashfaq et al. found the inhibitory effect of GL on HCV production in patient serum infected Huh7 cells [34]. Their cell culture system does not produce HCV efficiently. Thus, it does not permit analysis of the complete viral life cycle. In this study, we observed distinct suppression of HCV release by GL, using the HCVcc system (Figure 1A). Anti-viral effects of GL on early steps in the viral lifecycle have been reported previously, for example the inhibition of endocytosis of influenza A virus (IAV), the direct fusion of HIV-1 [35], the penetration of the plasma membrane of HAV [11] and EBV [15], the virus entry of SARS [14], and infection by pseudorabies virus [36]. GL effectively inhibits the replication of VZV [10], HSV-1 [9], EBV [15] and HIV [13]. This is the first report that GL can suppress virus release, however, the detailed mechanisms of these remain elusive. It has also been reported that GL had a membrane stabilizing effect [37] and a reduction of membrane fluidity [35], [38]. HCV uses cellular membrane structure in its lifecycle [39], [40]. Thus, it is conceivable that membrane alterations may play a negative role in the HCV lifecycle.

We found core protein accumulation on LDs in GL-treated cell (Figure 2C, 2I and 2K). This inverse correlation between

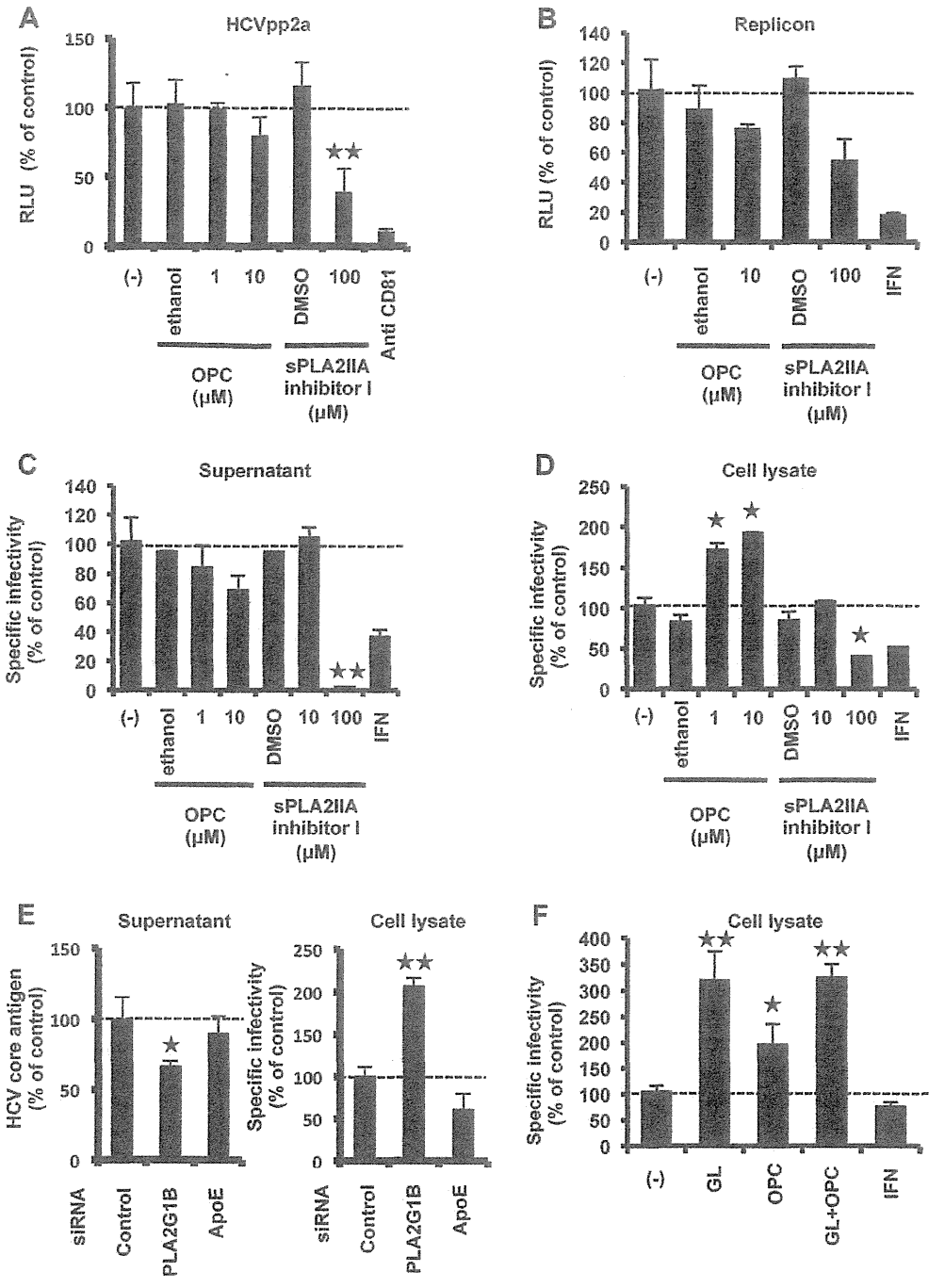

Figure 3. A role of PLA2 in HCV lifecycle. (A) Huh7 cells were infected with HCVpp in the presence and absence of OPC or sPLA2IIA inhibitor for 2 hours, then medium was replaced. Effects of PLA2 inhibitor on the entry of HCVpp were determined by measuring the luciferase activity at 72 hours post-infection. Anti-human CD81 antibody (10 μg/ml) was used as a positive control for reducing HCV entry to the cells. (B) Huh7 cells harboring the type-2a subgenomic replicon were treated with OPC or sPLA2IIA inhibitor for 72 hours. Replication efficiency of the replicon was estimated by measuring HCV RNA titer. HCVcc-infected cells were treated with PLA2 inhibitor for 72 hours. Specific infectivity of the supernatant (C) and cell lysate (D) were evaluated by quantifying the HCV core antigen in cells at 72 hours post-infection. (E) Effects of siRNA against PLA2G1B on core level in the medium (left panel) and specific infectivity in HCV-infected cells (right panel). ApoE siRNA was used as a positive control for reduced HCV infectivity. (F) HCVcc-infected cells were treated with GL (500 μM) with or without OPC (10 μM), and intracellular specific infectivity was measured. IFN (10 IU/ml) was used as a positive control. Results are expressed as the mean ± SD of the percent of the control from four independent experiments. *P < 0.05, **P < 0.005 versus control (0 μM treatment).

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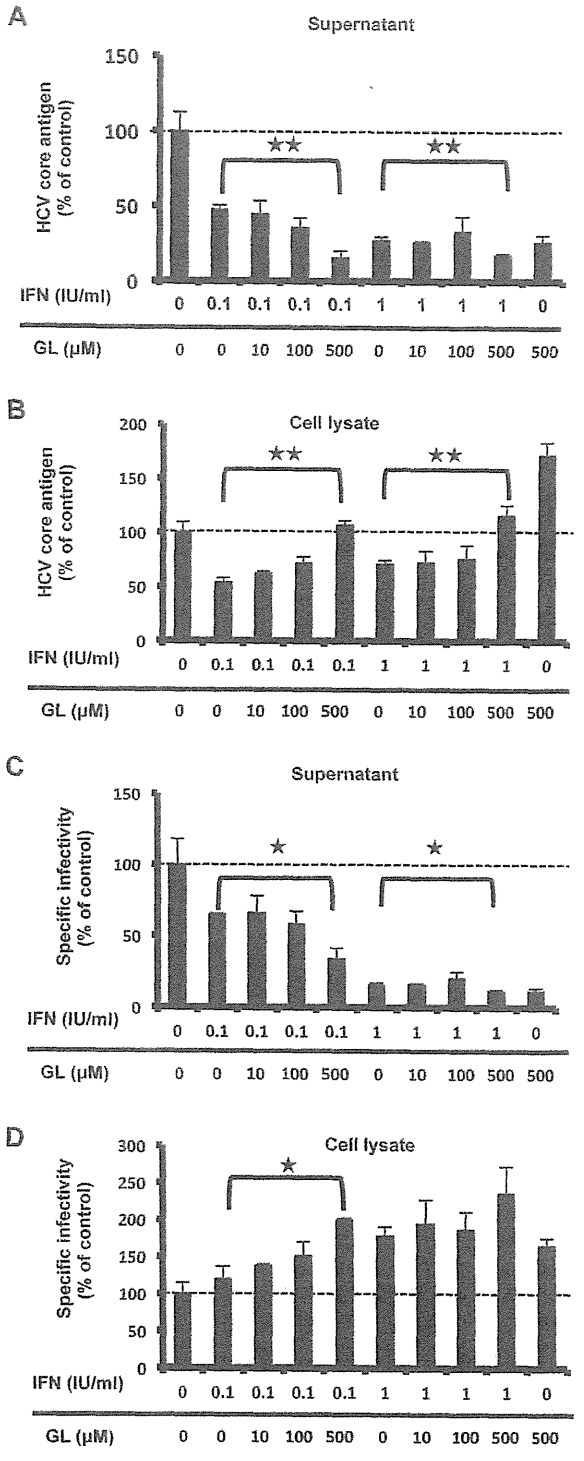

Figure 4. Anti-HCV effects IFN in combination with GL. HCVcc-infected cells were treated with IFN alone, or IFN with GL for 72 hours. HCV production was assessed by measuring the HCV core antigen in culture medium (A) and cell (B). Specific nfectivity in culture medium (C) and cell (D) were measured. Results are expressed as the mean ± SD of the percent of the control from four independent experiments. *P < 0.05, **P < 0.005 versus versus IFN mono-therapy.

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the efficiency of virus production and core protein accumulation on LDs was also observed that colocalization of HCV protein with LDs was low in cases of the chimera Jc1, supporting up to 1,000-fold higher infectivity titers compared with JFH1 [41], [29]. In this study, we demonstrated that GL did not affect the size of LDs in un-infected cells (Figure 2F right panel). On the other hand, the size of LDs increased in HCV-infected cells with GL-treatment (Figure 2F left panel), probably because accumulated-HCV enhanced the formation of LDs [29].

We demonstrated the importance of PLA2G1B in HCV release by PLA2G1B inhibitor and siRNA against PLA2G1B (Figure 3). The overexpression of PLA2G1B did not have any effect on HCV release (data not shown), probably because enough PLA2G1B existed in the cells. This result is generally observed in other host factors that involved in HCV lifecycle. For example, overexpression of the human homologue of the 33-kDa vesicle-associated membrane protein-associated protein (hVAP-33), which has a critical role in the formation of HCV replication complex, did not increase HCV replication [42]. PLA2 family proteins have been known as lipid-signaling molecules, inducing inflammation [43]. On the basis of the nucleotide sequence, the superfamily of PLA2 enzymes consists of 15 groups, comprising 4 main types: cytosolic PLA2 (cPLA2), calcium-independent PLA2, platelet activating factor acetyl hydrolase/oxidized lipid lipoprotein associated PLA2, and the secretory PLA2 (sPLA2) including PLA2G1B, 2A, and 4A [44]. In this study, we showed that GL, PLA2G1B inhibitor, and PLA2G1B siRNA inhibited HCV release and that GL and OPC had no additive effect when applied together, suggesting that suppression of HCV release by GL may be derived from its inhibitory effect on PLA2G1B. The role of PLA2G1B in the HCV lifecycle has not been reported. In this study, we also demonstrated that PLA2G2A inhibitor decreased entry, replication, and assembly of infectious HCV particles in cells (Figures 3A, 3B, 3C, and 3D). The role of PLA2G2A in the HCV lifecycle has not been reported. PLA2G2A is known to affect the secretion of VLDL (30). Therefore, PLA2G2A may contribute to HCV assembly. In the case of PLA2G4A, Menzel et al. showed that inhibition of PLA2G4A produces aberrant HCV particles [45]. These observations suggest that PLA2 has a role in several steps of the HCV lifecycle.

In this study, we showed that the EC_{50} of GL treatment for intracellular infectivity was 16.5 μM (Figure 1A). It has been reported that the maximum peripheral concentration of GL in normal patients is 145 μM [46]. The placebo-controlled phase I/II trial revealed no significant effect on viral titer [47]. In vivo, accumulated HCV in GL treated cells may cause lysis and apoptosis of the cells, leading to the release of infectious particles in the circulation. This may be a major limitation to use GL mono-therapy against HCV infection in patients. On the other hand, combination treatment with GL augmented the IFN-induced reduction in HCV core antigen levels (Figure 4A).

Although a number of natural compounds with anti-HCV activities were identified in recent years (Silymarin, EGCG, Ladanein, Naringenin, Quercetin, Luteolin, Honokiol, 3-hydroxy caruilignan C, and other things) [48], many aspects concerning their mechanisms of action remain unknown. In this study, GL is identified as a novel anti-HCV agent that targets the release

steps of infectious HCV particles. We found that the suppression of viral release by GL may be due to an inhibitory effect of PLA2G1B. These observations provide a basis for development of an improved IFN-based combination therapy against chronic hepatitis C.

Supporting Information

Figure S1. Anti-HCV effect of GL. HCVcc-infected cells were treated with various concentrations of GL for 72 hours. HCV production was assessed by measuring the level of HCV core antigen in culture medium. Results are expressed as the mean ± SD of the percent of the control from four independent experiments. IFN (10 IU/ml) was used as a positive control. *P < 0.05, **P < 0.005 versus control (0 μM treatment).
(TIF)

Figure S2. Effect of GL on expression of PLA2G1B. A human PLA2G1B cDNA was inserted into the EcoRI site of pCAGGS, yielding pCAGPLA2G1B. Since there was no effective antibody to detect endogenous expression of PLA2G1B, 293T cells transfected with the pCAGPLA2G1B plasmid were treated with GL (500 μM) for 72 hours and lysed in lysis buffer, followed by immunoblotting with anti-PLA2G1B and anti-actin antibodies. OPC (10 μM) was used as a positive control to reduce PLA2G1B protein in the cells.
(TIF)

Figure S3. Effect of PLA2G1B siRNA on expression of PLA2G1B. HCVcc infected-Huh7 cells in a 24-well plate were transfected with siRNAs targeted to PLA2G1B and scramble negative control siRNA, followed by immunoblotting with anti-PLA2G1B and anti-actin antibodies.
(TIF)

Figure S4. Effect of GL on IFN induction. The pISRE-Luc vector contains the firefly luciferase reporter gene, downstream of the IFN-Stimulated Response Element (ISRE) cis-acting enhancer element. The pRL-TK vector contains the renilla luciferase reporter downstream of the herpes simplex virus thymidine kinase (HSV-TK promoter), and was used as an internal control. Huh7 cells transfected with the pISRE-Luc vector and the pRL-TK vector were treated with various concentrations of GL for 72 hours, and luciferase activities were measured using the Dual-Luciferase Reporter Assay System. IFN (300 U/ml) was used as a positive control. Results are expressed as the mean ± SD percent of the controls (treatment with IFN).
(TIF)

Figure S5. Effect of GL on secretion of lipoprotein and the host proteins. Huh7 cells were treated or untreated with GL at 500 μM for 72 hours. ApoE and albumin in the culture supernatants were measured by immunoblotting and ELISA, respectively. Results are expressed as the mean ± SD of the percent of the control from four independent experiments.
(TIF)

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Author Contributions

Conceived and designed the experiments: YM NW RS SI TS T. Miyamura T. Matsuura TW SH K. Wake K. Watashi. Performed the experiments: YM H. Aoyagi H. Aizaki. Analyzed the data: YM H. Aoyagi H. Aizaki. Contributed reagents/materials/analysis tools: MM TD. Wrote the manuscript: YM H. Aoyagi.

References

1. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M et al. (2001) Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 358: 958-965. doi:10.1016/S0140-6736(01)06102-5. PubMed: 11583749.
2. Fujisawa Y, Sakamoto M, Matsushita M, Fujita T, Nishioka K (2000) Glycyrrhizin inhibits the lytic pathway of complement--possible mechanism of its anti-inflammatory effect on liver cells in viral hepatitis. Microbiol Immunol 44: 799-804. PubMed: 11092245.
3. Hibasami H, Iwase H, Yoshioka K, Takahashi H (2006) Glycyrrhetic acid (a metabolic substance and aglycon of glycyrrhizin) induces apoptosis in human hepatoma, promyelotic leukemia and stomach cancer cells. Int J Mol Med 17: 215-219. PubMed: 16391818.
4. Inoue H, Mori T, Shibata S, Saito H (1987) Pharmacological activities of glycyrrhetinic acid derivatives: analgesic and anti-type IV allergic effects. Chem Pharm Bull (Tokyo) 35: 3888-3893. doi:10.1248/cpb.35.3888. PubMed: 3435983.
5. Pompei R, Flore O, Marccialis MA, Pani A, Loddo B (1979) Glycyrrhizic acid inhibits virus growth and inactivates virus particles. Nature 281: 689-690. doi:10.1038/281689a0. PubMed: 233133.
6. Francischetti IM, Monteiro RQ, Guimarães JA (1997) Identification of glycyrrhizin as a thrombin inhibitor. Biochem Biophys Res Commun 235: 259-263. doi:10.1006/bbrc.1997.6735. PubMed: 9196073.
7. Ohuchi K, Kamada Y, Levine L, Tsurufuji S (1981) Glycyrrhizin inhibits prostaglandin E2 production by activated peritoneal macrophages from rats. Prostaglandins Med 7: 457-463. doi:10.1016/0161-4630(81)90033-1. PubMed: 6798590.
8. Ohtsuki K, Abe Y, Shimoyama Y, Furuya T, Munakata H et al. (1998) Separation of phospholipase A2 in Habu snake venom by glycyrrhizin (GL)-affinity column chromatography and identification of a GL-sensitive enzyme. Biol Pharm Bull 21: 574-578. doi:10.1248/bpb.21.574. PubMed: 9657040.
9. Sekizawa T, Yanagi K, Itoyama Y (2001) Glycyrrhizin increases survival of mice with herpes simplex encephalitis. Acta Virol 45: 51-54. PubMed: 11394578.
10. Baba M, Shigeta S (1987) Antiviral activity of glycyrrhizin against varicella-zoster virus in vitro. Antiviral Res 7: 99-107. doi:10.1016/0166-3542(87)90025-8. PubMed: 3034150.
11. Crance JM, Lévêque F, Biziagos E, van Cuyck-Gandré H, Jouan A et al. (1994) Studies on mechanism of action of glycyrrhizin against hepatitis A virus replication in vitro. Antiviral Res 23: 63-76. doi:10.1016/0166-3542(94)90176-7. PubMed: 8141593.
12. Takahara T, Watanabe A, Shiraki K (1994) Effects of glycyrrhizin on hepatitis B surface antigen: a biochemical and morphological study. J Hepatol 21: 601-609. doi:10.1016/S0168-8278(94)80108-8. PubMed: 7814808.

13. Kimoto M, Kawai R, Mitsui T, Harada Y, Sato A et al. (2005) Site-specific incorporation of fluorescent probes into RNA by specific transcription using unnatural base pairs. Nucleic Acids Symp Ser (Oxf): 287-288. PubMed: 17150746.
14. Hoever G, Baltina L, Michaelis M, Kondratenko R, Tolstikov GA et al. (2005) Antiviral activity of glycyrrhizic acid derivatives against SARS-coronavirus. J Med Chem 48: 1256-1259. doi:10.1021/jm0493008. PubMed: 15715493.
15. Lin JC (2003) Mechanism of action of glycyrrhizic acid in inhibition of Epstein-Barr virus replication in vitro. Antiviral Res 59: 41-47. doi: 10.1016/S0166-3542(03)00030-5. PubMed: 12834859.
16. Harada H, Suzu S, Ito T, Okada S (2005) Selective expansion and engraftment of human CD16+ NK cells in NOD/SCID mice. Eur J Immunol 35: 3599-3609. doi:10.1002/eji.200535125. PubMed: 16304638.
17. Utsunomiya T, Kobayashi M, Pollard RB, Suzuki F (1997) Glycyrrhizin, an active component of licorice roots, reduces morbidity and mortality of mice infected with lethal doses of influenza virus. Antimicrob Agents Chemother 41: 551-556. PubMed: 9055991.
18. Manns MP, Wedemeyer H, Singer A, Khomutjanskaja N, Dienes HP et al. (2012) Glycyrrhizin in patients who failed previous interferon alpha-based therapies: biochemical and histological effects after 52 weeks. J Viral Hepat 19: 537-546. doi:10.1111/j.1365-2893.2011.01579.x. PubMed: 22762137.
19. Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T et al. (2005) Robust hepatitis C virus infection in vitro. Proc Natl Acad Sci U S A 102: 9294-9299. doi:10.1073/pnas.0503596102. PubMed: 15939869.
20. Goto K, Watashi K, Murata T, Hishiki T, Hijikata M et al. (2006) Evaluation of the anti-hepatitis C virus effects of cyclophilin inhibitors, cyclosporin A, and NIM811. Biochem Biophys Res Commun 343: 879-884. doi:10.1016/j.bbrc.2006.03.059. PubMed: 16564500.
21. Miyamoto M, Kato T, Date T, Mizokami M, Wakita T (2006) Comparison between subgenomic replicons of hepatitis C virus genotypes 2a (JFH-1) and 1b (Con1 NK5.1). Intervirology 49: 37-43. doi:10.1159/000087261. PubMed: 16166787.
22. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M et al. (2005) Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 11: 791-796. doi.10.1038/nm1268. PubMed: 15951748.
23. Sato S, Fukasawa M, Yamakawa Y, Natsume T, Suzuki T et al. (2006) Proteomic profiling of lipid droplet proteins in hepatoma cell lines expressing hepatitis C virus core protein. J Biochem 139: 921-930. doi: 10.1093/jb/mvj104. PubMed: 16751600.
24. Akazawa D, Date T, Morikawa K, Murayama A, Miyamoto M et al. (2007) CD81 expression is important for the permissiveness of Huh7 cell clones for heterogeneous hepatitis C virus infection. J Virol 81: 5036-5045. doi:10.1128/JVI.01573-06. PubMed: 17329343.
25. Takeuchi T, Katsume A, Tanaka T, Abe A, Inoue K et al. (1999) Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology 116: 636-642. doi:10.1016/S0016-5085(99)70185-X. PubMed: 10029622.
26. Koutsoudakis G, Herrmann E, Kallis S, Bartenschlager R, Pietschmann T (2007) The level of CD81 cell surface expression is a key determinant for productive entry of hepatitis C virus into host cells. J Virol 81: 588-598. doi:10.1128/JVI.01534-06. PubMed: 17079281.
27. Miyamoto Y, Kitamura N, Nakamura Y, Futamura M, Miyamoto T et al. (2011) Possible existence of lysosome-like organella within mitochondria and its role in mitochondrial quality control. PLOS ONE 6: e16054. doi:10.1371/journal.pone.0016054. PubMed: 21264221.
28. Kato T, Choi Y, Elmowalid G, Sapp RK, Barth H et al. (2008) Hepatitis C virus JFH-1 strain infection in chimpanzees is associated with low pathogenicity and emergence of an adaptive mutation. Hepatology 48: 732-740. doi:10.1002/hep.22422. PubMed: 18712792.
29. Miyanari Y, Atsuzawa K, Usuda N, Watashi K, Hishiki T et al. (2007) The lipid droplet is an important organelle for hepatitis C virus production. Nat Cell Biol 9: 1089-1097. doi:10.1038/ncb1631. PubMed: 17721513.
30. Abe N, Ebina T, Ishida N (1982) Interferon induction by glycyrrhizin and glycyrrhetinic acid in mice. Microbiol Immunol 26: 535-539. PubMed: 6290851.
31. Brown WJ, Chambers K, Doody A (2003) Phospholipase A2 (PLA2) enzymes in membrane trafficking: mediators of membrane shape and function. Traffic 4: 214-221. doi:10.1034/j.1600-0854.2003.00078.x. PubMed: 12694560.
32. Coller KE, Heaton NS, Berger KL, Cooper JD, Saunders JL et al. (2012) Molecular determinants and dynamics of hepatitis C virus secretion. PLOS Pathog 8: e1002466. PubMed: 22241992.
33. McLauchlan J (2009) Hepatitis C virus: viral proteins on the move. Biochem Soc Trans 37: 986-990. doi:10.1042/BST0370986. PubMed: 19754437.
34. Ashfaq UA, Masoud MS, Nawaz Z, Riazuddin S (2011) Glycyrrhizin as antiviral agent against Hepatitis C Virus. J Transl Med 9: 112. doi: 10.1186/1479-5876-9-112. PubMed: 21762538.
35. Harada S (2005) The broad anti-viral agent glycyrrhizin directly modulates the fluidity of plasma membrane and HIV-1 envelope. Biochem J 392: 191-199. doi:10.1042/BJ20051069. PubMed: 16053446.
36. Sui X, Yin J, Ren X (2010) Antiviral effect of diammonium glycyrrhizinate and lithium chloride on cell infection by pseudorabies herpesvirus. Antiviral Res 85: 346-353. doi:10.1016/j.antiviral.2009.10.014. PubMed: 19879899.
37. Shiki Y, Shirai K, Saito Y, Yoshida S, Mori Y et al. (1992) Effect of glycyrrhizin on lysis of hepatocyte membranes induced by anti-liver cell membrane antibody. J Gastroenterol Hepatol 7: 12-16. doi:10.1111/j.1440-1746.1992.tb00927.x. PubMed: 1543863.
38. Wolkerstorfer A, Kurz H, Bachhofner N, Szolar OH (2009) Glycyrrhizin inhibits influenza A virus uptake into the cell. Antiviral Res 83: 171-178. doi:10.1016/j.antiviral.2009.04.012. PubMed: 19416738.
39. Aizaki H, Lee KJ, Sung VM, Ishiko H, Lai MM (2004) Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. Virology 324: 450-461. doi:10.1016/j.virol.2004.03.034. PubMed: 15207630.
40. Aizaki H, Morikawa K, Fukasawa M, Hara H, Inoue Y et al. (2008) Critical role of virion-associated cholesterol and sphingolipid in hepatitis C virus infection. J Virol 82: 5715-5724. doi:10.1128/JVI.02530-07. PubMed: 18367533.
41. Pietschmann T, Kaul A, Koutsoudakis G, Shavinskaya A, Kallis S et al. (2006) Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. Proc Natl Acad Sci U S A 103: 7408-7413. doi:10.1073/pnas.0504877103. PubMed: 16651538.
42. Gao L, Aizaki H, He JW, Lai MM (2004) Interactions between viral nonstructural proteins and host protein hVAP-33 mediate the formation of hepatitis C virus RNA replication complex on lipid raft. J Virol 78: 3480-3488. doi:10.1128/JVI.78.7.3480-3488.2004. PubMed: 15016871.
43. Funk CD (2001) Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 294: 1871-1875. doi:10.1126/science.294.5548.1871. PubMed: 11729303.
44. Burke JE, Dennis EA (2009) Phospholipase A2 structure/function, mechanism, and signaling. J Lipid Res 50 Suppl: S237-S242. PubMed: 19011112.
45. Menzel N, Fischl W, Hueging K, Bankwitz D, Frentzen A et al. (2012) MAP-Kinase Regulated Cytosolic Phospholipase A2 Activity Is Essential for Production of Infectious Hepatitis C Virus Particles. PLOS Pathog 8: e1002829. PubMed: 22911431.
46. van Rossum TG, Vulto AG, Hop WC, Schalm SW (1999) Pharmacokinetics of intravenous glycyrrhizin after single and multiple doses in patients with chronic hepatitis C infection. Clin Ther 21: 2080-2090. doi:10.1016/S0149-2918(00)87239-2. PubMed: 10645755.
47. van Rossum TG, Vulto AG, Hop WC, Schalm SW (2001) Glycyrrhizin-induced reduction of ALT in European patients with chronic hepatitis C. Am J Gastroenterol 96: 2432-2437. doi:10.1016/S0002-9270(01)02612-0. PubMed: 11513186.
48. Calland N, Dubuisson J, Rouillé Y, Séron K (2012) Hepatitis C virus and natural compounds: a new antiviral approach? Viruses 4: 2197-2217. doi:10.3390/v4102197. PubMed: 23202460.

BASIC AND TRANSLATIONAL—LIVER

Neutralizing Antibodies Induced by Cell Culture–Derived Hepatitis C Virus Protect Against Infection in Mice

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BACKGROUND & AIMS: Hepatitis C virus (HCV) infection is a major cause of liver cancer, so strategies to prevent infection are needed. A system for cell culture of infectious HCV particles (HCVcc) has recently been established; the inactivated HCVcc particles might be used as antigens in vaccine development. We aimed to confirm the potential of HCVcc as an HCV particle vaccine. **METHODS:** HCVcc derived from the J6/JFH-1 chimeric genome was purified from cultured cells by ultrafiltration and ultracentrifugation purification steps. Purified HCV particles were inactivated and injected into female BALB/c mice with adjuvant. Sera from immunized mice were collected and their ability to neutralize HCV was examined in naive Huh7.5.1 cells and urokinase-type plasminogen activator–severe combined immunodeficiency mice ($uPA^{+/+}$-SCID mice) given transplants of human hepatocytes (humanized livers). **RESULTS:** Antibodies against HCV envelope proteins were detected in the sera of immunized mice; these sera inhibited infection of cultured cells with HCV genotypes 1a, 1b, and 2a. Immunoglobulin G purified from the sera of HCV-particle-immunized mice (iHCV-IgG) inhibited HCV infection of cultured cells. Injection of IgG from the immunized mice into $uPA^{+/+}$-SCID mice with humanized livers prevented infection with the minimum infectious dose of HCV. **CONCLUSIONS: Inactivated HCV particles derived from cultured cells protect chimeric liver $uPA^{+/+}$-SCID mice against HCV infection, and might be used in the development of a prophylactic vaccine.**

Keywords: HCV Particle; HCV Vaccine; Immunization; Virology.

Hepatitis C virus (HCV), an enveloped virus that belongs to the *Hepacivirus* genus of the *Flaviviridae* family, is a human pathogen that is a major cause of chronic hepatitis, cirrhosis, and hepatic carcinoma. HCV therapy mainly involves treatment with pegylated interferon and ribavirin. However, these agents are not very effective for patients with high titer HCV-RNA and genotype 1. Thus, it is necessary to develop new, more effective therapies and preventive care treatments for HCV infection.

A cell culture-derived HCV (HCVcc) was developed using the genome of the genotype 2a JFH-1 strain. HCVcc was shown to be infectious in vitro and in vivo,[1-3] and its analysis has made it possible to understand the HCV lifecycle. Thus, HCVcc can be considered as a useful model for the screening of new anti-HCV drugs. However, the fact that only human beings and chimpanzees are susceptible to HCV has greatly slowed the in vivo study. As a tool for circumventing this problem, a human liver chimeric albumin enhancer/promoter urokinase plasminogen activator-severe combined immunodeficiency (uPA-SCID) mouse was established.[4] Thus, the HCV cell culture system and the human liver chimeric mouse are potent tools for pharmaceutical research concerning HCV.

Prevention of HCV infection is important for control of the pathogenesis of hepatitis C. However, there is no commercial vaccine for HCV at present, although vaccines are now being developed using HCV components, peptides, DNA, and viral vectors.[5] Because HCVcc is a structural mimic of the native HCV particle, it has the potential to function as an immunogen for the development of an anti-HCV vaccine. In this study, to use HCVcc as an HCV-particle vaccine, we purified HCVcc and determined the neutralizing effects of the HCV-particle vaccine against HCV infection in mice.

Materials and Methods

Cell Culture

Huh7 and Huh7.5.1 cells[2] (a generous gift from Dr Francis V. Chisari) were cultured in 5% CO_2 at 37°C in Dulbecco's modified

Abbreviations used in this paper: Cont, control; DMEM, Dulbecco's modified Eagle medium; Frac, fraction; HCV, hepatitis C virus; HCVcc, cell-cultured hepatitis C virus; HCVpp, hepatitis C virus pseudoparticle; IC50, 50% inhibitory concentration; iHCV, vaccine immunized hepatitis C virus; rE1, recombinant E1; rE2, recombinant E2; RTD-PCR, real-time detection reverse-transcription polymerase chain reaction; SCID, severe combined immunodeficiency; uPA, albumin enhancer/promoter urokinase plasminogen activator.

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Eagle medium (DMEM) containing 10% fetal bovine serum (DMEM-10) as previously reported.[1]

Plasmids

pJ6/JFH1 was generated by replacing the JFH-1 structural region with that of the J6CF strain as previously reported.[6]

HCV Particle Purification

J6/JFH-1 chimeric HCVcc was purified by ultrafiltration and ultracentrifugation. The methods for HCVcc production and purification were as described in the Supplementary Materials and Methods section.

Infectivity Titration

Huh7.5.1 cells were used to determine the viral infectivity titer using end-point dilution and immunofluorescence methods as described previously.[7] The titer was expressed as focus-forming units per milliliter.

HCV Pseudo-Particle Production

Murine leukemia virus pseudotypes were generated according to methods described previously.[8] HCV pseudo-particle (HCVpp) harboring the envelope of the genotype 1a (H77[9]), 1b (TH[10]), or 2a (J6CF[11]) strain were generated as described in the Supplementary Materials and Methods section.

Preparation of Recombinant Proteins

HCV-E1 and HCV-E2 recombinant proteins were generated as described in the Supplementary Materials and Methods section.

Immunization of Mice With the HCV Vaccine

Mice were immunized as described in the Supplementary Materials and Methods section.

Enzyme Immunoassay

Anti-E1 and anti-E2 antibodies were detected by enzyme immunoassay for stabilized recombinant J6E1/FLAG and J6E2/FLAG protein, respectively, as described in the Supplementary Materials and Methods section.

HCV Inhibition Assay

Inhibition of HCV infection in cultured cells was assayed using HCVpp and HCVcc for infection as described in the Supplementary Materials and Methods section.

IgG Purification

Mouse IgG was purified using KAPTIV-GY resin (Technogen, Tehran, Iran) as described in the Supplementary Materials and Methods section.

Anti-HCV Envelope IgG Adsorption

Anti-HCV envelope antibodies were absorbed using recombinant FLAG-tagged HCV-E1 and HCV-E2 proteins. Briefly, 1 mg/mL IgG from HCV-particle-immunized mice (iHCV-IgG) was precleared with anti-FLAG M2 agarose, and then mixed with 50 μg each of J6E1/FLAG and J6E2/FLAG protein. The IgG recombinant protein mixture was incubated at 4°C overnight, and anti-FLAG M2 agarose then was added into the mixture, which was centrifuged at 3000 $\times$ *g* for 2 minutes and the supernatant was collected as anti-envelope IgG-adsorbed IgG.

Inhibition of HCV Infection in Chimeric Mice in Which the Liver Was Repopulated With Human Hepatocytes

SCID mice that were transgenic for the urokinase-type plasminogen activator gene and in which the liver was repopulated with human hepatocytes (chimeric mice) were purchased from Phoenix Bio Co, Ltd (Hiroshima, Japan). Only mice showing levels of human albumin greater than 7 mg/mL were used in the experiments. The mice were injected intraperitoneally with 100 μg of control (Cont)-IgG or iHCV-IgG at 1 week and at day -1 before virus challenge. J6/JFH-1 HCVcc particles (10^3, 10^4, and 10^5 RNA copies/mouse) were mixed with 50 μg/mL of Cont-IgG or iHCV-IgG, and used to challenge the mice. Blood samples were collected within 1 week, and HCV RNA was determined using real-time detection reverse-transcription polymerase chain reaction (RTD-PCR).

Western Blot Analysis

Purified HCV particle samples were resolved on 12% sodium dodecyl sulfate-polyacrylamide gels and transferred to an Immobilon-P membrane (Millipore, Billerica, MA). Western blot was performed using an anti-E1 or an anti-E2 antibody as described in the Supplementary Materials and Methods section.

Statistical Analysis

The Dunnett test was used to compare the statistical significance of differences between groups of data. A *P* value less than .05 was considered significant. Data are reported as means ± SEM as indicated.

Results

HCV Particle Purification

HCV particles for mouse immunization were obtained using the cell culture system described in the Materials and Methods section. J6/JFH-1 chimeric HCV was secreted into culture medium supplemented with 2% fetal bovine serum, which was collected and centrifuged; the supernatant was concentrated by ultrafiltration using a 500-kilodalton cut-off membrane. The concentrated culture fluid was diafiltered using phosphate-buffered saline. These purification procedures removed approximately 90% of the contaminating proteins in the solution of virus particles. Sucrose cushion or gradient centrifugation then was performed to purify the virus particles further. After each procedure, the fractionated viruses were concentrated by ultrafiltration and the amount of HCV core and RNA, the concentration of total protein, and viral infectivity were determined (Supplementary Table 1). As shown in Figure 1, HCV particles, which had 2 distinct characteristics, were obtained in different fractions after sucrose gradient fractionation. One fraction (Fraction [Frac] 6) had low infectivity and a relatively high level of HCV core and RNA, and the other fraction (Frac 8) had high infectivity and a lower level of HCV core and RNA. Thus, the yield of HCV particles was highest in sucrose gradient Frac 6 (Supplementary Table 1), whereas Frac 8 displayed

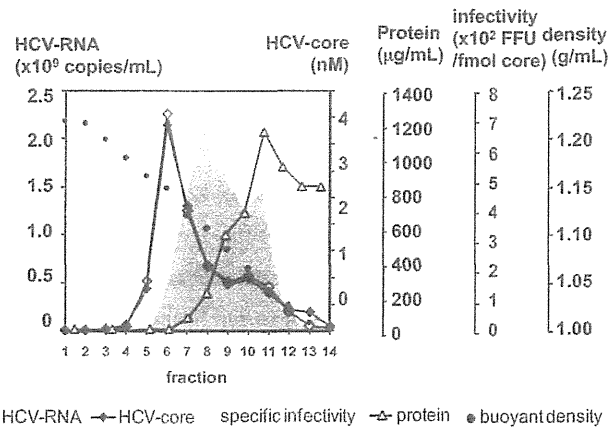

Figure 1. HCV-particle purification from cell cultures. Huh7 cells were inoculated with J6/JFH-1 chimeric HCVcc (multiplicity of infection, 0.2) and infected cells were passaged into CellSTACK (Corning, Corning, NY). The cell supernatant was collected and concentrated by ultrafiltration using a hollow fiber UFP-500-C-3MA (GE Healthcare, Little Chalfont, United Kingdom). Concentrated virus then was diafiltered using phosphate-buffered saline, and the viral fluid was layered on top of a preformed continuous 10%–60% sucrose gradient in 10 mmol/L Tris, 150 mmol/L NaCl, 0.1 mmol/L EDTA. The gradients were centrifuged using an SW28 rotor (Beckman Coulter, Fullerton, CA) at 28,000 rpm for 4 hours at 4°C, and 1-mL fractions were collected from the bottom of the tube. The level of the HCV core protein, HCV RNA, infectivity toward naive Huh7.5.1 cells, total protein, and buoyant density were determined for each fraction. Infectivity is indicated as specific infectivity, which was calculated as the infectivity titer (focus-forming unit [FFU]) divided by the HCV core protein (fmol).

approximately 7-fold higher infectivity than Frac 6 (Table 1). After sucrose cushion purification, the total protein level of the solution of purified virus particles was reduced to less than 0.03% of that of the initial cell culture supernatant (Supplementary Table 1).

Immunization of Mice With HCV Particles

We next immunized BALB/c mice with 2 or 5 pmol of HCV particles that were purified through a sucrose cushion (termed *cushion-2* and *cushion-5*, respectively) or with the HCV particles (2 pmol HCV core) from the earlier-described sucrose gradient fractions 6 or 8, and confirmed the immunogenicity of these particles. Before immunization, the purified HCV particles were inactivated by ultraviolet irradiation and conjugated with monophosphoryl lipid A plus trehalose-6,6-dimycolate. The HCV particles then were injected intraperitoneally into female BALB/c mice and blood samples were collected. Immunization was repeated 4 times at 2-week intervals, and the presence of anti-E1 and anti-E2 antibodies in immunized mouse serum was analyzed using an enzyme immunoassay. Both anti-E1 and anti-E2 antibodies were induced in mice immunized with the HCV particles and the efficiency of antibody induction was the same regardless of the nature of the antigen (Figure 2*A* and *B*).

To confirm that the sera from these immunized mice could inhibit HCV infection, an HCVpp inhibition assay was performed. In this assay, sera from HCV-particle-immunized mice inhibited HCVpp infection of naive Huh7.5.1 cells by HCV strains H77 (1a), TH (1b), and J6CF (2a) (Figure 2*C–E*). Furthermore, the inhibitory effects of different amounts (2 or 5 pmol) of HCV core purified by the sucrose cushion method or of HCV particles from different fractions obtained by sucrose gradient purification were not significantly different. Inhibitory effects of the serum from immunized mice were detected against TH/JFH-1 (genotype 1b) and J6/JFH-1 (genotype 2a) HCVcc infection (Supplementary Figure 1). The 50% inhibitory dilution titer of Frac 6 vaccine-immunized mouse serum was 1/181 and 1/614, and that of Frac 8 vaccine-immunized mouse serum was 1/432 and 1/766 for TH/JFH-1 and J6/JFH-1 HCVcc, respectively. These results indicate that the neutralizing effects of the serum from immunized mice were intergenotypic, and that 2 pmol of the HCV core was a sufficient dose for HCV-particle immunization. In contrast, immunization with saline or bovine serum albumin and adjuvant did not induce any neutralizing effects in mice (Figure 2*C–E*). We used bovine serum albumin as a control because albumin was assumed to be the main contaminating protein in the purified HCV particles (Supplementary Figure 2).

The HCV-Particle Vaccine Induced Anti-HCV Antibody More Efficiently Than an HCV-Component Vaccine

For comparative study of HCV-component and HCV-particle vaccines, recombinant E1 and E2 proteins were produced and used to immunize mice using the same adjuvant as that used earlier for the HCV-particle vaccine. These recombinant FLAG-tagged J6E1 and J6E2 proteins (J6E1/FLAG and J6E2/FLAG) were expressed and secreted into the culture supernatant of COS-1 cells, and were purified by affinity chromatography using M2-agarose. Before injection into mice, the purified recombinant proteins, as well as the inactivated HCV particles obtained from the sucrose gradient fractionation described earlier, were analyzed by Western blot using anti-E1 and anti-E2 antibodies, and the amount of E1 or E2 protein present in the HCV particles was determined. Densitometric analysis of the immunoblot shown in Figure 3 indicated that an aliquot of HCV particles that contained 1 pmol of HCV core protein included approximately 10 ng each of

Table 1. Composition of Purified HCV Particles After Ultracentrifugation

	Core/RNA, fmol/copy	Infectivity/core, FFU/fmol	Infectivity/RNA, FFU/copy	Core/protein, fmol/μg
Frac 6	6.54×10^{-7}	129.8	8.50×10^{-5}	113.8
Frac 8	5.38×10^{-7}	987.9	5.32×10^{-4}	19.3
Ratio (Frac 8/Frac 6)	0.82	7.61	6.26	0.17

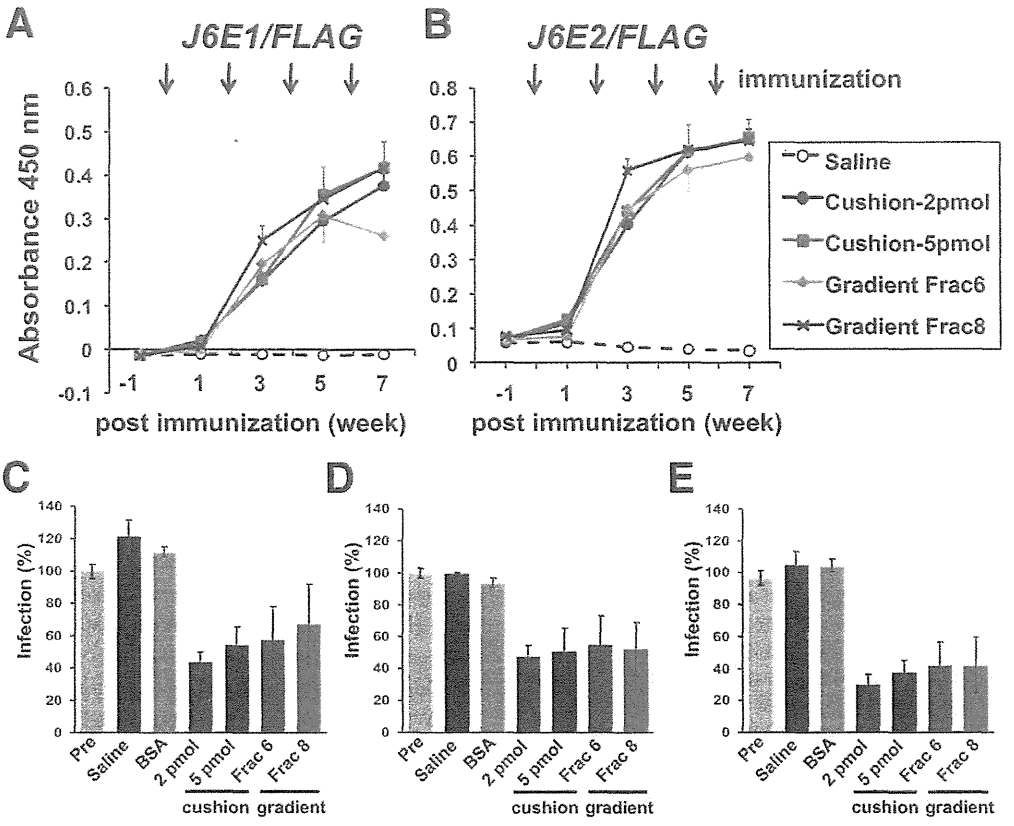

Figure 2. Cross-genotypic neutralizing effect of the sera from HCV-particle–immunized mice. HCV particles that were purified using a sucrose cushion (2 or 5 pmol HCV core) or sucrose gradient fractionation (Frac 6 and Frac 8, 2 pmol HCV core each) were inactivated by ultraviolet irradiation. The inactivated HCV -particles were conjugated with Sigma Adjuvant System (Sigma, St Louis, MO), and were injected intraperitoneally into BALB/c mice (5 weeks old, female, n = 3). Saline or bovine serum albumin (150 μg) with adjuvant was injected into mice as a control. Immunization was repeated 4 times at 2-week intervals (at weeks 0, 2, 4, and 6). Blood was collected at weeks 1, 3, 5, and 7 after immunization. Pre-immune sera and immunized mouse sera were prepared from these blood samples and were heat-inactivated at 56°C for 1 hour. The heat-inactivated sera were diluted (1000-fold) and their reactivity with (*A*) J6E1/FLAG and (*B*) J6E2/FLAG recombinant proteins was analyzed using an enzyme immunoassay. Assays were performed in triplicate. The *y*-axis indicates absorbance at 450 nm. Mean values ± SEM are shown. Mouse serum-mediated inhibition of HCV infection was assayed using HCVpp genotypes (*C*) 1a (H77), (*D*) 1b (TH), and (*E*) 2a (J6CF). The sera were diluted (100-fold) and HCVpp infection in naive Huh7.5.1 cells was examined. Assays were performed in triplicate and infection rates are expressed as mean ± SEM.

the E1 and E2 proteins. Thus, HCV particles that contained 2 pmol of HCV core contained approximately 20 ng of each envelope protein.

Recombinant J6E2/FLAG was inoculated into mice at a concentration of 20, 200, or 2000 ng/mouse or was inoculated together with the same dose of J6E1/FLAG (20 or 200 ng/mouse). Inactivated HCV particles from Frac 6 or Frac 8 were injected into mice using 2 pmol (20 ng each of E1 or E2) of HCV core protein per mouse. Sera from immunized mice were collected and the presence of anti-E2 antibodies was analyzed using an enzyme immunoassay. Anti-E2 antibodies were induced in all immunized mice, and the highest level of anti-E2 antibodies was induced in the group of mice that were immunized with 200 or 2000 ng of the J6E2 protein per mouse. On the other hand, the level of anti-E2 antibodies that was induced by immunization with 20 ng of the inactivated HCV particles was higher than that induced by the same amount of recombinant envelope proteins (Figure 4*A*). To analyze the inhibitory effects of the sera from immunized mice against HCV infection, HCVpp was incubated with heat-inactivated mouse sera, and then was used to inoculate Huh7.5.1 cells. Sera from HCV-particle–immunized mice were able to inhibit HCVpp infection; however, sera from mice immunized with 20 or 200 ng of the recombinant E2 or E2 plus E1 proteins did not show significant neutralization activity. Although sera from mice immunized with 2 mg of the recombinant E2 protein could neutralize HCVpp infection, the sera from mice immunized with only 20 ng of the HCV particles from sucrose gradient Frac 6 or Frac 8 also were able to induce similar levels of anti-envelope antibody and HCV neutralization (Figure 4*B*). These results suggest that HCV particles have a greater potential as vaccine candidates than recombinant envelope proteins in terms of their potency for antibody induction.

Vaccination With Cell-Cultured HCV Induces Anti-HCV IgG

As described earlier, inactivated HCV particles can induce an anti-HCV effect in the sera of immunized mice. To confirm that this anti-HCV effect was dependent on HCV-specific antibodies, IgG was purified from the sera of Saline- or HCV-particle-immunize mice, respectively, and was assayed in an HCV inhibition assay. The purity of the

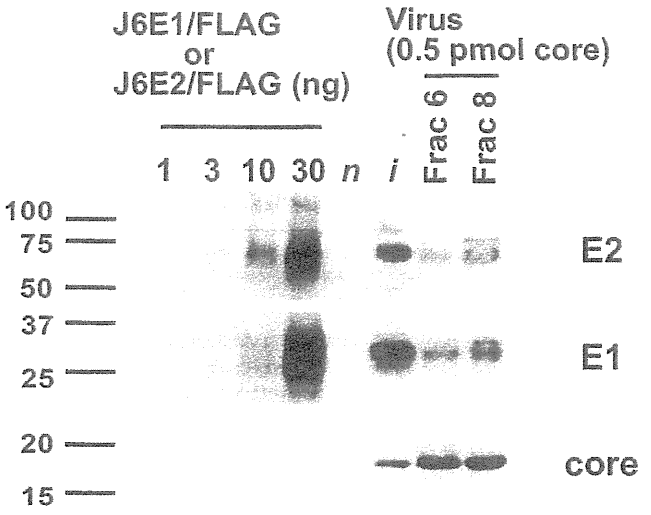

Figure 3. Western blot analysis of envelope proteins in purified HCV particles. The HCV core (0.5 pmol) of purified HCV particles (Frac 6 and Frac 8), as well as 1–30 ng of recombinant envelope proteins (J6E1/FLAG or J6E2/FLAG) were resolved on 12% sodium dodecyl sulfate–polyacrylamide gels and transferred to a polyvinylidene difluoride membrane. Naive Huh7 cells and J6/JFH-1–infected Huh7 cells were lysed using Passive Lysis Buffer (Promega, Madison, WI), and were assayed similarly as negative (*n*) and positive (*i*) controls, respectively. The membrane was blocked and probed with primary antibodies against the virus core (2H9; 3 μg/mL), E1 (B7567; 10 μg/mL), and E2 (AP33; 3 μg/mL) proteins. The membrane then was probed with horseradish-peroxidase–conjugated secondary antibody, and bound antibody was detected using ECL-plus (GE Healthcare, Little Chalfont, UK).

purified IgG was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis (Figure 5*A*). Purified IgGs were pre-incubated with J6/JFH-1 HCVcc and this mixture subsequently was used to inoculate Huh7.5.1 cells. The IgG from HCV-particle-immunized mice (iHCV-IgG) inhibited HCV infection in an iHCV-IgG dose-dependent manner, whereas the IgG from saline-immunized mice (Cont-IgG) did not show any neutralizing effects on this in vitro assay (Figure 5*B*). The 50% inhibitory concentration (IC50) of iHCV-IgG against HCVcc infection was 16.1 μg/mL. The iHCV-IgG from Frac 6– and Frac 8–immunized mice showed a neutralizing effect on TH (genotype 1b) and J6CF (genotype 2a) HCVpp infections (Supplementary Figure 3); the IC50 values were 18.0 (TH) and 30.4 (J6CF) μg/mL for Frac 6–IgG, and 15.1 (TH) and 33.1 (J6CF) μg/mL for Frac 8–IgG. Similarly, IgG from Frac 6– and Frac 8–immunized mice showed a neutralizing effect on HCVcc infection (Supplementary Figure 4). Thus, both fractions of the HCV particles appear to have similar immunogenicity. As a control, mice were immunized with naive Huh7-cultured media that was processed similar to the method used for the purification of HCV particles or saline together with adjuvant. IgG was purified from the mice sera, and HCVcc inhibition assay was performed. The purified IgG did not show a significant inhibitory effect for HCV infection compared with saline-immunized mice (Supplementary Figure 5). However, iHCV-IgG significantly inhibited the HCV infection at a concentration of 30 μg/mL. The effect of adsorption of the anti-E1 and anti-E2 antibodies in iHCV-IgG by incubation with recombinant J6E1 and J6E2/FLAG proteins on the inhibitory effect of iHCV-IgG then was examined. The HCV neutralizing effect of iHCV-IgG was reduced by adsorption of the anti-envelope antibodies, and the IC50 value was increased to 52.9 μg/mL (Figure 5*C*). These data indicate that anti-HCV envelope neutralizing antibodies were induced in mice by injection of the HCV-particle vaccine.

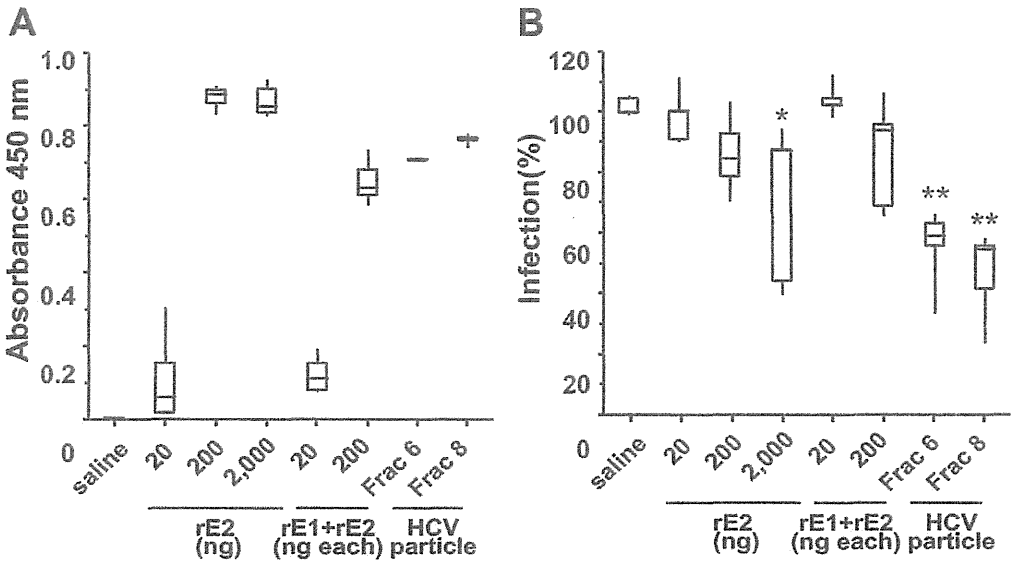

Figure 4. Neutralizing effects of sera from HCV-component and HCV-particle vaccine-immunized mice. BALB/c mice (n = 5) were immunized with recombinant proteins (20, 200, or 2000 ng of FLAG-tagged E2, 20 or 200 ng each of FLAG-tagged E1 and FLAG-tagged E2), or with purified HCV particles (2 pmol HCV core of Frac 6 or Frac 8) together with adjuvant. (*A*) The presence of anti-E2 antibodies in sera from immunized mice was analyzed using an enzyme immunoassay and the (*B*) ability of these sera to inhibit J6CF HCVpp infection of cultured Huh7.5.1 cells also was assayed. Each assay was performed in triplicate, and the data are presented as box plots. The statistical significance of differences between groups was analyzed using the Dunnett test (*$P < .05$, **$P < .001$ vs saline).

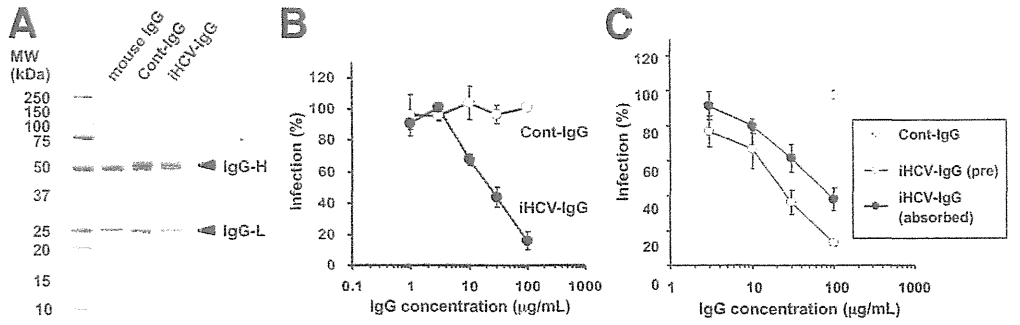

Figure 5. IgG purified from the sera of HCV-particle–immunized mice had a neutralizing effect against HCV infection in vitro. (*A*) IgG from the sera of mice at 7 weeks after immunization (iHCV-IgG) was purified using a KAPTIV-GY resin. Control mouse IgG was prepared similarly from saline-injected mice (Cont-IgG). Each IgG was analyzed for purity by SDS–polyacrylamide gel electrophoresis and Coomassie brilliant blue staining. As a control, a purchased mouse IgG was used. (*B*) Serially diluted Cont-IgG or iHCV-IgG was mixed with J6/JFH-1 HCVcc (multiplicity of infection, 0.1) and this mixture was inoculated into naive Huh7.5.1 cells. HCV infection was determined by analysis of the intracellular concentration of the HCV core protein. Infectivity is expressed as the percentage of cells that were infected. The IC50 value of iHCV-IgG was 16.1 μg/mL. (*C*) Anti-E1 and anti-E2 antibodies in iHCV-IgG were adsorbed by recombinant J6E1/FLAG and J6E2/FLAG protein, and the neutralizing effect of the E1E2-adsorbed IgG on HCV infection of Huh7 cells was assessed using the HCVcc inhibition assay as described for panel *B*. The IC50 of the E1E2-adsorbed IgG shifted to 52.9 μg/mL.

HCV Vaccine Induced Anti-HCV Infection In Vivo

As shown earlier, anti-HCV envelope neutralizing antibodies were induced in HCV-particle–immunized mice. To confirm the effect of the HCV-particle vaccine in vivo, we next examined its ability to neutralize an HCV challenge to human liver chimeric uPA-SCID mice. Purified IgG (100 μg) from HCV-particle–immunized mice was injected intraperitoneally into these chimeric mice 1 week before HCV infection. The mice then were challenged with multiple doses of 10^3, 10^4, and 10^5 RNA copies of J6/JFH-1 HCVcc at 2-week intervals. Blood samples were collected and HCV RNA was measured using RTD-PCR. HCV infection was inhibited (no infection) in all 6 mice in the vaccine-treated mouse group (iHCV-IgG) when 10^3 copies of HCV were inoculated (minimal infectious dose), whereas infection was observed in 4 of the 6 mice in the Cont-IgG mouse group. However, the neutralizing effect of iHCV-IgG vaccination was not detected when 10^4 or 10^5 copies of HCV were used for infection (Table 2). These data indicated that an inactivated HCV-particle vaccine potentially could be used as a prophylactic vaccine for HCV.

Discussion

There is no vaccine available for clinical use against HCV infection. Development of an HCV cell culture system has facilitated an understanding of the HCV life cycle, and the production of HCV prophylactic and therapeutic drugs. HCVcc mimics the native HCV particle, and thus has potential for use as an HCV-particle vaccine. In the present study, we established an efficient method for HCV particle production and purification, which allowed evaluation of the HCV particle as a vaccine candidate. HCV particles were purified from cell-cultured HCV of genotype 2a J6/JFH-1. The HCVcc in the culture supernatant was concentrated and partially purified by ultrafiltration using a 500-kilodalton cut-off membrane. Subsequently, HCVcc was purified by sucrose gradient ultracentrifugation. As reported previously,[12,13] the virus particles present in Frac 6 and Frac 8 after this sucrose gradient centrifugation displayed different characteristics. The virus in Frac 6 displayed low infectivity, but had a high concentration of viral components, whereas that in Frac 8 displayed high infectivity, but a low concentration of viral components. It is thought that the content of lipids or lipoproteins in these HCV particle fractions is different.[12,13] Finally, each viral fraction was purified and concentrated by ultrafiltration. When these HCV particles were inactivated, conjugated with a monophosphoryl lipid A plus trehalose-6,6-dimycolate adjuvant, and injected into mice, anti-E1 and anti-E2 antibodies were induced in the immunized mice, and the sera from these mice had a neutralizing effect against HCV infection in vitro. Because these sera displayed cross-reactivity for HCV strains 1a (H77), 1b (TH), and 2a (J6CF), it was considered that the HCV-particle vaccine had a broad neutralizing effect against HCV infection.

Western blot analysis of the envelope proteins in these HCV particles (Figure 3) indicated that the Frac 6 HCV particles contained approximately 10 ng each of the E1 and E2 proteins per 1 pmol HCV core protein. Because the molecular weight of the core, E1, and E2 proteins is approximately 20, 35, and 70 kilodaltons, respectively,[14] the molar ratio of the structural proteins in the purified HCV particles (core:E1:E2) was calculated to be 5:1:1 from Western blot analysis. Western blotting analysis also suggested that the amount of the E1 and E2 proteins in the Frac 8 HCV particle was likely to be higher than that

Table 2. Neutralizing Effect of IgG Purified From the Sera of HCV-Particle–Immunized Mice Against HCV Infection in Human Liver Chimeric uPA-SCID Mice

	Infected mice	
Virus challenge, HCV RNA copies	Cont-IgG	iHCV-IgG
10^3	4/6	0/6
10^4	5/6	6/6
10^5	6/6	6/6

in the Frac 6 HCV particle. Indeed, densitometric analysis of the blot indicated that the level of both E1 and E2 proteins in Frac 8 was 1.5-fold higher than that in Frac 6. This higher level of E1 and E2 proteins in Frac 8 may be the reason why the Frac 8 HCV particles had higher infectivity. Interestingly, the expression level of the core protein relative to that of the envelope proteins was different in infected cells and in purified HCV particles (Figure 3). These data suggest that only a limited amount of the envelope protein was incorporated into HCV particles.

We compared the potential of the purified HCV particles to function as a vaccine with that of HCV-envelope components. For this purpose, recombinant HCV E1 and E2 proteins were prepared and different doses of recombinant E2 (rE2), or recombinant E1 (rE1) plus rE2, were injected into mice with adjuvant. Other groups of mice were immunized with the HCV-particle vaccine (Frac 6 or Frac 8) using a dose of 2 pmol of HCV core per mouse. Because it was calculated that HCV particles that included 2 pmol of HCV core protein contained approximately 20 ng of recombinant E1 and E2, we used 20–2000 ng of rE2 or 20 or 200 ng each of rE1 plus rE2 proteins for comparative purposes. Although the sera from all immunized mice contained anti-E2 antibodies, and immunization with 200 or 2000 ng of recombinant E2 protein induced antibody production with the highest efficiency, HCV-particle immunization displayed the highest virus-neutralizing effect. Based on these results, it was concluded that purified HCV particles may be a more potent prophylactic HCV vaccine candidate than a vaccine that is composed of recombinant viral components. There are several possible explanations for the observed differences between these 2 types of vaccines. First, HCV envelope proteins that are a part of HCV particles are properly folded, which would maintain their function, whereas the recombinant envelope proteins may not be properly folded, and thus may lose their antigenicity. Second, other factors associated with the HCV particles may be important for inducing neutralizing antibodies. To address these possibilities, we adsorbed the anti-envelope antibodies in iHCV-IgG with recombinant envelope proteins. This procedure reduced, but did not completely abolish, the neutralizing effect of iHCV-IgG toward HCVcc infection. It is possible that if the recombinant envelope proteins were not folded properly as mentioned earlier, then not all of the anti-envelope antibodies were removed in this adsorption process. However, this possibility is considered unlikely because virus-neutralizing antibodies were induced by both immunization with the HCV particles and with the recombinant envelope proteins. This result suggests that HCV particles may include other factors, such as lipoproteins, that are important for HCV infection.[15–17] It also is necessary to compare the immunogenic activity of the inactivated virus vaccine with the potential immunogenic activities of recombinant HCV-like particles and E1-E2 heterodimers. Furthermore, attention needs to be paid as to whether the inactivated purified vaccine may induce autoantibodies against human proteins associated with the viral particles.

To confirm the in vivo efficacy of HCV-particle–induced neutralizing antibodies, the ability of iHCV-IgG to protect infected animals against HCV challenge was examined using human liver chimeric uPA-SCID mice. Interestingly, iHCV-IgG could protect against HCV challenge, at least at the lower virus challenge dose, although the mice challenged with the higher virus doses became infected with HCV. This result indicated that the HCV-particle vaccine could exert a prophylactic anti-HCV effect in vivo by induction of virus-neutralizing antibodies. However, the therapeutic effect of this vaccine is not yet clear and an understanding of its effect will require detailed study of its effect on cellular immune responses such as the cytotoxic T lymphocyte response. In addition, the prophylactic effect was observed only partially in the in vivo experiment. The reason for this insufficient protection is unclear; however, induction with a higher titer of neutralizing antibody may be needed, using adjuvants or more effective forms of the vaccine such as recombinant HCV-like particles. It also may be important to induce protective cellular immune responses in addition to humoral immune responses; effective cytotoxic T-lymphocyte responses may be necessary for a prophylactic effect against infection at higher virus doses.

Prophylactic and therapeutic HCV vaccines have been developed previously and some of these vaccines have been tested in clinical trials.[5,18,19] An HCV vaccine should be able to induce humoral and cellular immune response with specific anti-HCV effects. Previous vaccine candidates that have been tested have included recombinant proteins,[20–24] peptides,[25–29] DNA,[30] and viral vectors[31,32] with or without appropriate adjuvants. Recombinant E1 and E2 proteins with an MF59 adjuvant were developed by Chiron/Novartis (Basel, Switzerland) as a prophylactic vaccine, and the immunogenicity of this vaccine was confirmed in mice and guinea pigs.[33] The recombinant protein vaccine was well tolerated in a phase I clinical study, and induction of anti-envelope antibodies and an envelope-specific T-cell response was observed.[24] In another study, Fab fragments of anti-E2 antibodies that were obtained using a phage display library generated from an HCV-infected donor showed a broad HCV-neutralizing effect in human liver chimeric uPA-SCID mice.[34] A separate study showed that retroviral pseudotype-derived HCV-like particles (HCVLPs) also induced broad neutralizing antibodies in mice and macaques.[35] This result indicated that a prophylactic vaccine may be achievable by activation of the humoral immune response. Moreover, it was reported that insect cell-derived virus-like particles containing HCV structural proteins induced a humoral immune response[36] and stimulated dendritic cells[37] or a cytotoxic T-lymphocyte response.[38] Thus, it is possible that an HCVcc-derived HCV-particle vaccine also has the capacity to function as a therapeutic vaccine.

The HCV vaccine described in this study could be used for protection against HCV infection in high-risk patient groups. Given that the HCV particles are used as the vaccine, it will be necessary to improve the production and

purification of these particles before they can be used. We recently established a serum-free HCV culture system for efficient production and purification of virus particles that may be useful for this purpose.[39] It also will be necessary to examine other cell types for the production of HCV particles because the Huh7 cells used in this study are cells of a hepatoma cell line. Once HCV particle production using an industrial method is established and its safety is evaluated and confirmed, then it is expected that a purified HCV-particle vaccine will be of prophylactic use. Alternatively, a novel vaccine composed of HCV components should be developed using recombinant E1 and E2 proteins that can induce viral neutralizing antibodies with similar or even higher efficiency as HCV particles.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2013.05.007.

References

1. Wakita T, Pietschmann T, Kato T, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791-796.
2. Zhong J, Gastaminza P, Cheng G, et al. Robust hepatitis C virus infection in vitro. Proc Natl Acad Sci U S A 2005;102:9294-9299.
3. Lindenbach BD, Evans MJ, Syder AJ, et al. Complete replication of hepatitis C virus in cell culture. Science 2005;309:623-626.
4. Mercer DF, Schiller DE, Elliott JF, et al. Hepatitis C virus replication in mice with chimeric human livers. Nat Med 2001;7:927-933.
5. Halliday J, Klenerman P, Barnes E. Vaccination for hepatitis C virus: closing in on an evasive target. Expert Rev Vaccines 2011; 10:659-672.
6. Murayama A, Date T, Morikawa K, et al. The NS3 helicase and NS5B-to-3'X regions are important for efficient hepatitis C virus strain JFH-1 replication in Huh7 cells. J Virol 2007;81:8030-8040.
7. Akazawa D, Date T, Morikawa K, et al. Characterization of infectious hepatitis C virus from liver-derived cell lines. Biochem Biophys Res Commun 2008;377:747-751.
8. Bartosch B, Dubuisson J, Cosset FL. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complex. J Exp Med 2003;197:633-642.
9. Kolykhalov AA, Agapov EV, Blight KJ, et al. Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. Science 1997; 277:570-574.
10. Wakita T, Wands JR. Specific inhibition of hepatitis C virus expression by antisense oligodeoxynucleotides. J Biol Chem 1994; 269:14205-14210.
11. Yanagi M, Purcell RH, Emerson SU, et al. Hepatitis C virus: an infectious molecular clone of a second major genotype (2a) and lack of viability of intertypic 1a and 2a chimeras. Virology 1999;262:250-263.
12. Miyanari Y, Atsuzawa K, Usuda N, et al. The lipid droplet is an important organelle for hepatitis C virus production. Nat Cell Biol 2007;9:1089-1097.
13. Aizaki H, Morikawa K, Fukasawa M, et al. Critical role of virion-associated cholesterol and sphingolipid in hepatitis C virus infection. J Virol 2008;82:5715-5724.
14. Hijikata M, Kato N, Ootsuyama Y, et al. Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. Proc Natl Acad Sci U S A 1991;88:5547-5551.
15. Chang KS, Jiang J, Cai Z, et al. Human apolipoprotein e is required for infectivity and production of hepatitis C virus in cell culture. J Virol 2007;81:13783-13793.
16. Jiang J, Luo G. Apolipoprotein E but not B is required for the formation of infectious hepatitis C virus particles. J Virol 2009; 83:12680-12691.
17. Benga WJ, Krieger SE, Dimitrova M, et al. Apolipoprotein E interacts with hepatitis C virus nonstructural protein 5A and determines assembly of infectious particles. Hepatology 2010; 51:43-53.
18. Houghton M, Abrignani S. Prospects for a vaccine against the hepatitis C virus. Nature 2005;436:961-966.
19. Torresi J, Johnson D, Wedemeyer H. Progress in the development of preventive and therapeutic vaccines for hepatitis C virus. J Hepatol 2011;54:1273-1285.
20. Nevens F, Roskams T, Van Vlierberghe H, et al. A pilot study of therapeutic vaccination with envelope protein E1 in 35 patients with chronic hepatitis C. Hepatology 2003;38:1289-1296.
21. Leroux-Roels G, Depla E, Hulstaert F, et al. A candidate vaccine based on the hepatitis C E1 protein: tolerability and immunogenicity in healthy volunteers. Vaccine 2004;22:3080-3086.
22. Haller AA, Lauer GM, King TH, et al. Whole recombinant yeast-based immunotherapy induces potent T cell responses targeting HCV NS3 and core proteins. Vaccine 2007;25:1452-1463.
23. Drane D, Maraskovsky E, Gibson R, et al. Priming of CD4+ and CD8+ T cell responses using a HCV core ISCOMATRIX vaccine: a phase I study in healthy volunteers. Hum Vaccin 2009;5:151-157.
24. Frey SE, Houghton M, Coates S, et al. Safety and immunogenicity of HCV E1E2 vaccine adjuvanted with MF59 administered to healthy adults. Vaccine 2010;28:6367-6373.
25. Klade CS, Wedemeyer H, Berg T, et al. Therapeutic vaccination of chronic hepatitis C nonresponder patients with the peptide vaccine IC41. Gastroenterology 2008;134:1385-1395.
26. Wedemeyer H, Schuller E, Schlaphoff V, et al. Therapeutic vaccine IC41 as late add-on to standard treatment in patients with chronic hepatitis C. Vaccine 2009;27:5142-5151.
27. Yutani S, Komatsu N, Shichijo S, et al. Phase I clinical study of a peptide vaccination for hepatitis C virus-infected patients with different human leukocyte antigen-class I-A alleles. Cancer Sci 2009; 100:1935-1942.
28. Firbas C, Boehm T, Buerger V, et al. Immunogenicity and safety of different injection routes and schedules of IC41, a hepatitis C virus (HCV) peptide vaccine. Vaccine 2010;28:2397-2407.
29. Gowans EJ, Roberts S, Jones K, et al. A phase I clinical trial of dendritic cell immunotherapy in HCV-infected individuals. J Hepatol 2010;53:599-603.
30. Alvarez-Lajonchere L, Shoukry NH, Grá B, et al. Immunogenicity of CIGB-230, a therapeutic DNA vaccine preparation, in HCV-chronically infected individuals in a phase I clinical trial. J Viral Hepat 2009; 16:156-167.
31. Barnes E, Antonella F, Aston S, et al. Phase I trial of a highly immunogenic T cell vaccine for hepatitis c virus based on novel adenoviral vectors from rare serotypes. Hepatology 2009;50(Suppl 4):105A.
32. Honnet G, Veron L, Olivier D, et al. Phase I clinical trial with a novel HCV therapeutic vaccine tg4040: interim results of biomarker and immunomonitoring analyzes. Hepatology 2009;50(Suppl 4):413A.
33. Stamataki Z, Coates S, Evans MJ, et al. Hepatitis C virus envelope glycoprotein immunization of rodents elicits cross-reactive neutralizing antibodies. Vaccine 2007;25:7773-7784.
34. Law M, Maruyama T, Lewis J, et al. Broadly neutralizing antibodies protect against hepatitis C virus quasispecies challenge. Nat Med 2008;14:25-27.
35. Garrone P, Fluckiger AC, Mangeot PE, et al. A prime-boost strategy using virus-like particles pseudotyped for HCV proteins triggers broadly neutralizing antibodies in macaques. Sci Transl Med 2011; 3:94ra71.
36. Steinmann D, Barth H, Gissler B, et al. Inhibition of hepatitis C virus-like particle binding to target cells by antiviral antibodies in acute and chronic hepatitis C. J Virol 2004;78:9030-9040.
37. Barth H, Ulsenheimer A, Pape GR, et al. Uptake and presentation of hepatitis C virus-like particles by human dendritic cells. Blood 2005; 105:3605-3614.

38. Elmowalid GA, Qiao M, Jeong SH, et al. Immunization with hepatitis C virus-like particles results in control of hepatitis C virus infection in chimpanzees. Proc Natl Acad Sci U S A 2007;104: 8427–8432.
39. Akazawa D, Morikawa K, Omi N, et al. Production and characterization of HCV particles from serum-free culture. Vaccine 2011; 29:4821–4828.

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

RNA Synthesis

RNA synthesis was performed as described previously.[1] Briefly, the pJ6/JFH1 plasmid was digested with *Xba* I and then treated with Mung Bean Nuclease (New England Biolabs, Beverly, MA). The digested plasmid DNA fragment then was purified and used as a template for RNA synthesis. HCV RNA was synthesized in vitro using a MEGAscript T7 kit (Ambion, Austin, TX). The synthesized RNA was treated with DNase I, followed by acid phenol extraction to remove any remaining template DNA.

RNA Transfection

RNA transfection was performed as described previously.[1] Briefly, trypsinized Huh7 cells were washed with Opti-MEM I reduced-serum medium (Invitrogen, Carlsberg, CA) and were resuspended at a density of 7.5×10^6 cells/mL in Cytomix buffer.[2] RNA (10 μg) that was synthesized from pJ6/JFH1 was mixed with 400 μL of the cell suspension and the mixture then was transferred into an electroporation cuvette (Precision Universal Cuvettes; Thermo Hybrid, Middlesex, UK). The cells then were pulsed at 260 V and 950 μF using the Gene Pulser II apparatus (Bio-Rad, Hercules, CA). Transfected cells were transferred immediately to a 10-cm dish containing 8 mL of culture medium. Cell culture supernatants were collected 72 hours after transfection and were passed through a 0.45-μm filter. The filtered culture media then was pooled and concentrated using an Amicon Ultra-15 (100,000 molecular weight cut-off; Millipore, Billerica, MA). The viral infectious titer was determined by immunofluorescence detection of infected foci using naive Huh7.5.1 cells, as described later.

HCV Particle Purification

Huh7 cells were infected with J6/JFH-1 chimeric HCVcc (multiplicity of infection, 0.2). After the infected cells were passaged into CellSTACK (Corning, Corning, NY) and cultured in 2% fetal bovine serum–supplemented medium, the cell supernatant was collected and passed through a 0.45-μm filter. The supernatant was concentrated by ultrafiltration using a hollow fiber UFP-500-C-3MA (GE Healthcare, Little Chalfont, UK) and then was diafiltered with 5 times the volume of phosphate-buffered saline. The concentrated and diafiltered cell supernatants were layered on top of a preformed continuous 10%–60% sucrose gradient or a 20%/60% sucrose cushion in 10 mmol/L Tris, 150 mmol/L NaCl, 0.1 mmol/L EDTA. The gradients were centrifuged using an SW28 rotor (Beckman Coulter, Fullerton, CA) at 28,000 rpm (141,000 $\times$ *g*) for 4 hours at 4°C, and 1-mL fractions were collected from the bottom of the tube. The density of each fraction was estimated by weighing a 100-μL decrease from fractions of a gradient run. The HCV core protein and HCV RNA in each fraction were quantified as described later. Purified HCV was prepared by collecting the peaks of HCV core and HCV RNA, which then were concentrated using an Amicon Ultra-15 (100,000 molecular weight cut-off; Millipore).

Quantification of HCV Core Protein and RNA

The concentration of HCV core proteins in sample aliquots was measured using the HCV Core enzyme-linked immunosorbent assay kit (Ortho Clinical Diagnostics, Tokyo, Japan). Viral RNA was isolated from harvested culture media or from sucrose density gradient fractions using the QiaAmp Viral RNA Extraction Kit (Qiagen, Tokyo, Japan). Copy numbers of HCV RNA were determined by RTD-PCR using an ABI Prism 7500 fast sequence detector system (Applied Biosystems, Tokyo, Japan).[3]

HCV Pseudo-Particle Production

Murine leukemia virus pseudotypes were generated according to methods described previously.[4] Briefly, DNA corresponding to the Gag-Pol packaging construct (3.1 μg), the transfer vector construct (3.1 μg), and the HCV glycoprotein-expressing construct (1 μg) was transfected into 2.5×10^6 293T cells, which had been seeded the day before in 10-cm dishes using the FuGENE6 transfection reagent (Roche Diagnostics, Tokyo, Japan). For construction of a vector that expresses the HCV glycoprotein, the coding region corresponding to aa 132-746 (H77 strain), 132-747 (TH strain), or 132-750 (J6CF strain) was cloned into the pcDNA3.1 vector (Invitrogen). For the negative control, all constructs except the glycoprotein-expressing construct were transfected. The medium (8 mL/dish) was replaced 6 hours after transfection. Supernatants containing the pseudotypes were collected 48 hours later, and were passed through a 0.45-μm filter. The supernatants were stored at -80°C until use.

Preparation of Recombinant Proteins

Each E1 (aa 192-353) and E2 (aa 384-720) coding region of the J6CF strain was cloned into the p3×FLAG-CMV-13 (Sigma, St Louis, MO) vector. Each expression vector then was transfected into COS-1 cells using the Diethylaminoethanol (DEAE)-dextran method. The culture supernatant of the transfected cells was collected and FLAG-tagged E1 (J6E1/FLAG) and FLAG-tagged E2 (J6E2/FLAG) proteins were purified using an anti-FLAG M2-agarose (Sigma) column under native conditions. The buffer containing the purified proteins was changed and the proteins were concentrated by ultrafiltration using Amicon Ultra-15 (Millipore).

Immunization of Mice With the HCV Vaccine

All animal procedures were approved by the Committees on Biosafety and Animal Handling Regulations of the National Institute of Infectious Diseases (Japan). Animal research was performed in compliance with the "Fundamental guidelines for proper conduct of animal experiment and related activities in institutions under jurisdiction (June 2006)" issued by the Ministry of Health, Labour and Welfare. Our animal work also adhered to the principles stated in the guidelines. BALB/c mice (4 weeks old, female) were purchased from SLC Japan (Shizuoka, Japan). Mice were tamed for 1 week before the start of experiments. HCV particles in virus-containing fluid were inactivated with ultraviolet irradiation and were conjugated with the same volume

of a solution containing monophosphoryl lipid A and trehalose-6,6-dimycolate using the Sigma Adjuvant System (Sigma). The conjugate (0.2 mL) was injected intraperitoneally into 5-week-old female BALB/c mice, and blood samples were collected. The same volume of saline was injected as a control. Mice were injected at weeks 0, 2, 4, and 6, and blood was collected at weeks 1, 3, 5, and 7 after injection. Blood samples were collected in a BD Vacutainer SST II (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 1200 × *g* for 10 minutes at room temperature. The supernatants were collected as sera and were heat-inactivated at 56°C for 1 hour. The heat-inactivated sera was used in viral inhibition assay experiments and for IgG purification.

HCV Inhibition Assay

Inhibition of HCV infection of cultured cells was assayed using HCVpp and HCVcc for infection. Naive Huh7.5.1 cells (2×10^4) were seeded into a 48-well plate. HCVpp or HCVcc was mixed with serum that was collected from vaccine-immunized mice and the mixtures then were incubated for 30 minutes at room temperature. Naive Huh7.5.1 cells were inoculated with the virus-antibody mixtures, and after 3 hours the mixtures were removed and the cells were washed once with phosphate-buffered saline. DMEM-10 was added to each well, and the cells were cultured for 72 hours. For the HCVpp assay, cells were washed once with phosphate-buffered saline and lysed with 40 μL/well of Cell Culture Lysis Reagent (Promega, Madison, WI), and luciferase activity was quantified using a Luciferase Assay System (Promega) as described previously.[5] For the HCVcc assay, cells were washed once with phosphate-buffered saline and lysed with 100 μL/well of Passive Lysis Buffer (Promega), and HCV core protein content was quantified using the HCV core enzyme-linked immunosorbent assay kit (Ortho Clinical Diagnostics). Assays were performed in triplicate and infectivity was calculated from the value of the luciferase activity or the HCV core content.

Enzyme Immunoassay

Recombinant J6E1/FLAG or J6E2/FLAG protein (50 ng) was coated onto a 96-well plate (Nunc, Roskilde, Denmark) at 4°C overnight. Each well was blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) for 1 hour at room temperature, and then was washed with phosphate-buffered saline. Sera from immunized mice were diluted 1000-fold in PBS, added to each well, and the plate was incubated for 1–2 hours at room temperature. The sera was discarded and each well was washed twice with phosphate-buffered saline. Horseradish-peroxidase–conjugated anti-mouse IgG antibody (GE Healthcare) was diluted 3000-fold with Blocking One, added into each well, and the plate then was incubated for 1 hour at room temperature. Nonbound antibody was discarded, and peroxidase activity of the horseradish-peroxidase–bound antibody was detected using a peroxidase detection kit (Sumitomo Bakelite, Tokyo, Japan).

HCV Inhibition Assay

Inhibition of HCV infection of cultured cells was assayed using HCVpp and HCVcc for infection. HCVpp or HCVcc was mixed with serum or immunoglobulin that was collected from vaccine-immunized mice and the mixtures then were incubated for 30 minutes at room temperature. Naive Huh7.5.1 cells were inoculated with the virus-antibody mixtures, and after 3 hours the mixtures were removed and the cells were washed once with phosphate-buffered saline. DMEM-10 was added to each well, and the cells were cultured for 72 hours. The cells were lysed and the lysates were assayed as described later.

Western Blot Analysis

The naive Huh7 cells that were used as a negative control and the J6/JFH-1–infected Huh7 cells that were used as a positive control were lysed using Passive Lysis Buffer (Promega), and then centrifuged to remove debris. Protein concentration was quantified using the BCA Protein Assay Kit (Pierce, Rockford, IL). The HCV core of purified HCV particles (0.5 pmol each of Frac 6 and Frac 8) and cell lysate samples (5 μg protein) then were denatured by boiling, resolved on 12% sodium dodecyl sulfate–polyacrylamide gels, and transferred to an Immobilon-P membrane (Millipore, Billerica, MA). Recombinant FLAG-tagged E1 (J6E1/FLAG) and FLAG-tagged E2 (J6E2/FLAG) proteins also were analyzed using 1, 3, 10, and 30 ng protein/lane. The membrane was blocked with BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan), and probed with primary antibodies against HCV core (2H9, 3 μg/mL), E1 (B7567[6], 10 μg/mL), and E2 (AP33, 3 μg/mL; a generous gift from Genentech, Inc, South San Francisco, CA) proteins in Tris-buffered saline containing Tween-20 (20 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 0.1% [vol/vol] Tween-20 [polyoxyethylene sorbitan monolaurate]) containing 10% (vol/vol) BlockAce. After several washes with Tris-buffered saline containing Tween-20, the membrane was probed with horseradish-peroxidase–conjugated secondary antibody (GE Healthcare, 1:5000 dilution), washed repeatedly, and bound antibodies were detected using ECL-plus (GE Healthcare) and visualized using LAS3000 (Fujifilm, Tokyo, Japan). After antigen detection, the antibody on the membrane was stripped using WB stripping solution (Nacalai Tesque) and the membrane was re-probed with another antibody.

IgG Purification

Mouse IgG was purified using the KAPTIV-GY resin (Technogen). Briefly, approximately 0.5 mL of the KAPTIV-GY resin was loaded onto an empty column (Bio-Rad) and was washed with loading buffer (50 mmol/L bis-Tris, pH 6.8). Sera from mice at 7 weeks after immunization were applied onto the column and the column was washed with loading buffer. IgG then was eluted with glycine-HCl (pH 3.0) and the eluates were neutralized with Tris-HCl (pH 9.0). The eluted IgG (iHCV-IgG) was concentrated using an Amicon Ultra-15 (molecular weight cut-off 30,000) and IgG purity was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subsequent Coomassie brilliant blue staining. Control mouse IgG was prepared similarly from saline-injected mice (Cont-IgG).

References

1. Kato T, Date T, Murayama A, et al. Cell culture and infection system for hepatitis C virus. Nat Protoc 2006;1:2334-2339.
2. van den Hoff MJ, Moorman AF, Lamers WH. Electroporation in 'intracellular' buffer increases cell survival. Nucleic Acids Res 1992; 20:2902.
3. Takeuchi T, Katsume A, Tanaka T, et al. Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology 1999;116:636-642.
4. Bartosch B, Dubuisson J, Cosset FL. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complex. J Exp Med 2003;197:633-642.
5. Akazawa D, Date T, Morikawa K, et al. CD81 expression is important for the permissiveness of Huh7 cell clones for heterogeneous hepatitis C virus infection. J Virol 2007;81:5036-5045.
6. Murakami K, Ishii K, Ishihara Y, et al. Production of infectious hepatitis C virus particles in three-dimensional cultures of the cell line carrying the genome-length dicistronic viral RNA of genotype 1b. Virology 2006;351:381-392.