zoonotic and originate from wildlife via direct or indirect contact. For example, a case of Ebola virus infection resulted from contact with an infected wild chimpanzee (Morell 1994; Le Guenno et al. 1995; Taylor et al. 2001). Chimpanzee caretakers and researchers are at risk of exposure to unknown chimpanzee infectious pathogens. In addition to basic hygiene, keepers and researchers need appropriate vaccinations and should wear masks, gloves, and protective clothing during quarantine periods.

Disease prevention management for wild chimpanzees

Our study provides important information for hygiene management in wild chimpanzee conservation by adding information about possible human–chimpanzee zoonotic diseases. Infectious agents newly identified by their antibodies as agents possibly transmittable to chimpanzees (ADV-1, 2, 3, 5, 6, 19, CVA-5, 7, and JEV) or previously detected pathogens might cause the next outbreak in wild chimpanzees, not only in primate institutes, but also zoological gardens. Furthermore, we should consider agents that were not seropositive in the chimpanzees because the chimpanzees do not possess immunity against them, or at least not antibodies.

For chimpanzees at KUPRI, unlike some groups of wild chimpanzees, a previous infection of RSV or hMPV did not cause a severe respiratory disease. Captive chimpanzees might have more opportunity to be exposed to human pathogens compared with wild populations, but their environment and diet might not be as harsh as in the wild, which could alter the incidence and effects of the same pathogens in captive and wild populations. In addition, captive chimpanzees are at risk of new infectious diseases to which they have never been exposed and against which they have not established immunity. Consequently, monitoring results should be analysed carefully at each research institute or zoo, and care should be taken with all pathogens, including those to which chimpanzees are highly susceptible and those for which only a few or no chimpanzees were seropositive. Ultimately, without direct surveillance of wild populations, we cannot elucidate the prevalence of human-borne infectious diseases in the wild, but our data may still be used as a model. Our data suggest that a means of detecting antibodies in faeces should be developed to facilitate further studies in the wild.

In recent years, a vaccination program to protect wild chimpanzees against Ebola virus has been planned and is in preparation (Walsh 2009) after vaccine challenge against polio virus in wild chimpanzees at Gombe and against measles virus in gorillas at Virunga (Whittier et al. 2001). Furthermore, its effectiveness has been reported in one case of intervention (Robbins et al. 2011). This study shows that groups of chimpanzees under captive conditions produced specific antibodies against human diseases and that the chimpanzees were probably protected by their acquired immunity. Therefore, pre-immunity probably effectively protects wild chimpanzees from the human infectious diseases that tourists or researchers unknowingly transmit. However, the vaccination campaign needs careful consideration in terms of negative side effects for wild chimpanzees and nature. Recently, Ryan and Walsh (2011) reviewed the positives and negatives of the intervention and described the available vaccines against human pathogens.

Conclusion

We conducted serological surveillance for human-borne zoonoses in chimpanzees, and revealed the possibility of disease transmission between humans and chimpanzees. To reduce the chance of transmitting disease to captive chimpanzees in research institutes and zoos and to prevent disease transmission among researchers, animal caretakers, and chimpanzees, it is necessary to evaluate the risk of disease transmission. The serology of captive chimpanzees provides important information for hygiene management in ecotourism involving wild chimpanzees and other great apes.

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A Hepatitis C Virus (HCV) Vaccine Comprising Envelope Glycoproteins gpE1/gpE2 Derived from a Single Isolate Elicits Broad Cross-Genotype Neutralizing Antibodies in Humans

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Abstract

Although a cure for HCV is on the near horizon, emerging drug cocktails will be expensive, associated with side-effects and resistance making a global vaccine an urgent priority given the estimated high incidence of infection around the world. Due to the highly heterogeneous nature of HCV, an effective HCV vaccine which could elicit broadly cross-neutralizing antibodies has represented a major challenge. In this study, we tested for the presence of cross-neutralizing antibodies in human volunteers who were immunized with recombinant glycoproteins gpE1/gpE2 derived from a single HCV strain (HCV1 of genotype 1a). Cross neutralization was tested in Huh-7.5 human hepatoma cell cultures using infectious recombinant HCV (HCVcc) expressing structural proteins of heterologous HCV strains from all known major genotypes, 1–7. Vaccination induced significant neutralizing antibodies against heterologous HCV genotype 1a virus which represents the most common genotype in North America. Of the 16 vaccinees tested, 3 were selected on the basis of strong 1a virus neutralization for testing of broad cross-neutralizing responses. At least 1 vaccinee was shown to elicit broad cross-neutralization against all HCV genotypes. Although observed in only a minority of vaccinees, our results prove the key concept that a vaccine derived from a single strain of HCV can elicit broad cross-neutralizing antibodies against all known major genotypes of HCV and provide considerable encouragement for the further development of a human vaccine against this common, global pathogen.

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Introduction

HCV is a major global health concern infecting 170 million people worldwide [1]. Replication of the HCV RNA genome is mediated by virus-encoded non-structural protein NS5B, an error prone RNA-dependent RNA polymerase, and the low fidelity of the enzyme has contributed to the high mutagenic rate and broad antigenic diversity of the hepacivirus genus creating a major challenge in developing a global vaccine. Historical therapy using a combination of interferon-alpha and ribavirin has had significant but limited success and while the recent addition of drugs inhibiting a viral protease have increased the overall therapeutic response, this combination exhibits substantial toxicity and more than 30% of patients are not cured [2]. New, highly promising drug cocktails are expected to be available over the next few years and while a complete cure can be envisaged for nearly all treated patients, the high expense and sophisticated clinical care required for these drug combinations makes the prospect of universal delivery very unlikely. Therefore, it remains imperative to develop a global HCV vaccine. However, there are 7 major genotypes of HCV and many hundreds of subtypes distributed globally, with genotype 1a being the most prominent virus in the North America and genotype 1b infecting the most people worldwide [3,4]. Among all genotypes, there is up to 31–33% nucleotide diversity [4]. Various genotypes of HCV have been shown to have differences in disease outcome and response to antiviral therapy [5,6]. A global vaccine will therefore have to be effective against

this vast diversity of HCV variants and has represented a major challenge.

A small fraction of individuals can spontaneously clear HCV infection leading to the belief that prevention of HCV is possible if a vaccine can elicit similar immune responses [7,8,9]. Cellular immunity has been shown to be important to control HCV infection. Depletion of CD4+ or CD8+ T cells has been shown to allow chronic, persistent infection in chimpanzees [10]. On the other hand, the role of antibodies to control HCV infection has been understudied, largely due to the lack of suitable assays for neutralizing and cross-neutralizing antibodies, until recently [11,12,13,14,15]. Cross-neutralizing antibodies can be isolated from chronically-infected patients [16,17,18] but only years after the original infection when virus-specific cellular immune responses are already blunted [17]. Despite the failure of these antibodies to eradicate chronic infection, there is evidence that they are actively driving evolution of the viral envelope glycoproteins suggesting they are partially controlling infection [19]. More recently, studies have demonstrated a correlation between the presence of neutralizing antibodies and the clearance of acute infection without the development of chronic, persistent infection [9,20,21]. Furthermore, cross-neutralizing antibodies have been shown to confer protection in passively-immunized SCID mice transplanted with human hepatocytes [16,22].

All successful viral vaccines developed to date have been based on the induction of neutralizing antibodies [23,24] usually targeting the virion surface proteins. An important function of these proteins is to interact with cellular receptors to mediate cell entry and to fuse with host membranes during uncoating [25]. Neutralizing antibodies have been identified in natural HCV infections targeting these proteins [26]. Our earlier work has shown that a recombinant gpE1/gpE2 HCV vaccine is immunogenic in guinea pigs [27] and chimpanzees [28] and has been shown to induce protective immune responses in the latter model against experimental challenge with either homologous or heterologous genotype 1a HCV strains [28]. Vaccinated chimpanzees had a significantly reduced rate of chronicity following experimental challenge and some animals were even sterilized against homologous virus challenge [29,30,31]. The gpE1/gpE2 antigen was derived from strain HCV1 of genotype 1a, the first identified HCV genome [32]. A phase I dose-ranging clinical trial has been conducted to test the safety and immunogenicity of this vaccine in healthy volunteers [33]. All volunteers elicited antibodies against the glycoproteins gpE1/gpE2 as measured in EIA formats [33] and the vaccine was effective in inducing strong T-helper responses to the vaccine [33]. Further studies have shown that the vaccine induced antibodies targeting known neutralizing epitopes and the sera of selected vaccinees prevented *in vitro* infection by HCV derived from genotypes 1a and 2a [34,35]. Evidence for cross-neutralization of some diverse genotypes was derived from vaccinated animals [22,27,28].

In this study, antisera from the phase I clinical trial was assessed for cross neutralizing activity against representatives of all seven major genotypes of HCV that occur globally. Very broad cross-neutralization activity was evident but not all genotypes were neutralized with similar efficiencies. We conclude that this vaccine can elicit cross-neutralizing antibodies in human that target epitopes that are highly conserved among all major genotypes of HCV. When combined with the demonstrated efficacy of this vaccine in the chimpanzee model [30], the current findings strongly encourage the further development of this and related vaccine candidates.

Results

Recombinant gpE1/gpE2 vaccine elicits neutralizing antibodies

During the phase I dose-ranging clinical trial testing the recombinant gpE1/gpE2 vaccine in healthy volunteers, maximal anti-gpE1/gpE2 EIA antibody titers were observed at two weeks post-third immunization [33]. Accordingly, we examined the neutralizing activity of volunteers' sera collected at two weeks post third vaccination using the highest dose (100ug) of antigen in this study. Chimeric virus encoding core, gpE1, gpE2, p7 and NS2 genes from heterologous H77C (genotype 1a) in the backbone of JFH-1 genome has been produced to allow the study of genotype 1a specific entry [3]. This heterologous 1a chimeric virus was pre-incubated with dilutions of volunteers' sera then added to cultured hepatoma Huh7.5 cells and the subsequent level of infection was quantified 2 days post-infection. As shown in Figure 1A, post-vaccination sera showed significant neutralization of the heterologous 1a chimeric virus, with 5 of 13 sera being able to neutralize over 50% of the virus, two of which neutralized up to 80% of viral infectivity. Components in human sera, such as apolipoproteins, have been shown to have non-specific effects on virus entry and therefore may have contributed to the variable background in the pre-vaccination samples [36,37]. In order to control for individual differences in serum components, the neutralization activity of post-vaccination sera was normalized using the neutralization activity of the pre-vaccination sera from the same individual (Figure 1B). This analysis showed that 92% (12/13) individuals elicited significant neutralization activities, 5 of which (volunteers 1, 2, 5, 6 and 7) showed higher neutralizing activity compared to others within the group. These data showed the vaccine was capable of eliciting neutralizing antibodies against heterologous 1a virus infection. Importantly, volunteer 4 appeared to display somewhat less virus neutralization after vaccination which is consistent with an possible enhancement effect of the vaccine. Further work is needed to ascertain if this effect is truly due to vaccine enhancement in this individual or due to a high, variable background in this particular individual combined with a low level of neutralization elicited by the vaccine in volunteer 4.

Vaccine induced antibodies confer broad cross-genotype neutralization *in vitro*

We chose sera from volunteers 1, 5 and 7, due to their high neutralization activities among assayed volunteers, to test for cross-genotype neutralization activity using chimeric HCVcc encoding core, gpE1, gpE2, p7 and NS2 genes derived from representative strains of all 7 major genotypes that occur globally [3]. In Figure 2, volunteers 5 and 7 showed a broad range of neutralization activity against viruses of all 7 major genotypes. The profiles of broad cross neutralization activity are very similar using both sera but in both cases (and with volunteer 1), less cross-neutralization was observed against 2b, 3a and 7a viruses. This indicates that while there must be a neutralizing epitope(s) that is highly conserved across all clades of HCV, genotype-specific neutralizing epitopes may also be present.

Interestingly, all sera tested showed strong neutralization activity against the chimeric virus HK6a/JFH-1. There are two adaptive mutations in the glycoprotein region, F350 within gpE1 and N417 within gpE2. The N417 is a highly conserved residue among all genotypes and mutation of this site leads to elimination of a N-glycosylation site [3]. These mutations appear to confer higher sensitivity to neutralization (Figure 2). This is consistent with other data showing that various HCV antibodies neutralize the tissue culture-adapted HK6a efficiently despite showing low

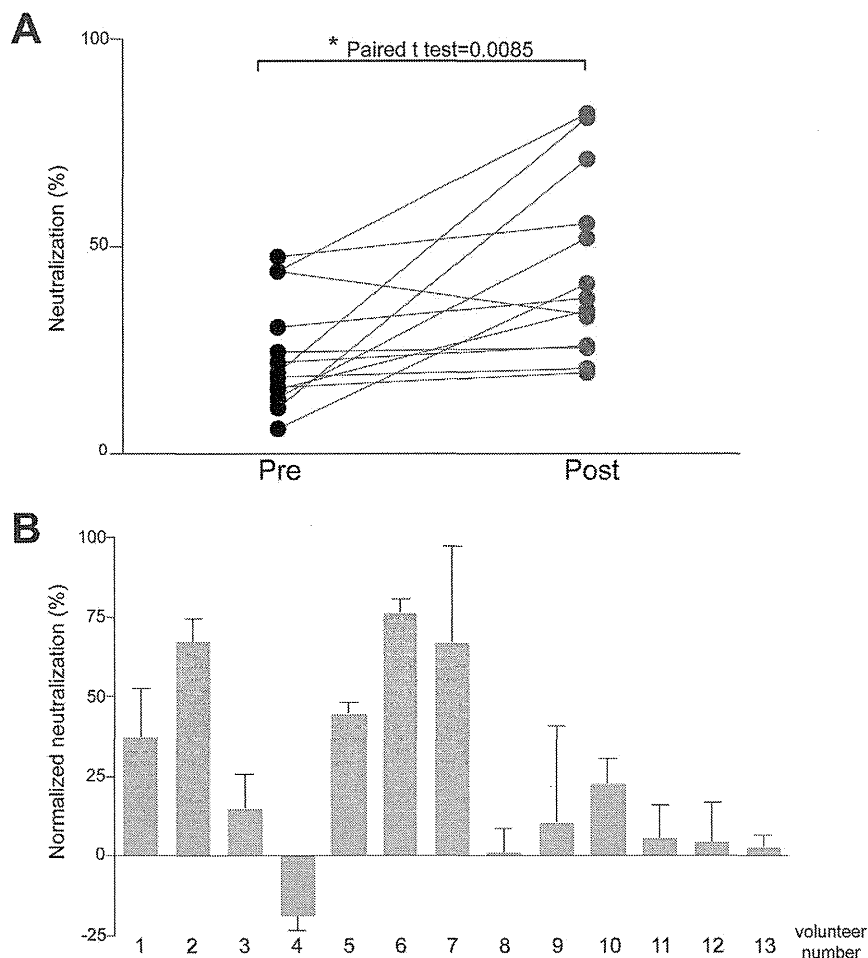

Figure 1. Human antisera neutralizes heterologous 1a infectivity in cell culture. Pre or post vaccination sera were incubated with chimeric H77C/JFH-1 HCVcc followed by infection of naïve huh7.5 cells. Representatives of two independent experiements performed in triplicates are shown. The number of infected cells was quantitated by immunostaining using anti-NS5A antibodies 48 hour post-infection. (A) Neutralization activity was calculated using a negative control lacking serum (0%) and anti-CD81 antibody as a positive control (%). "Pre" sera were collected prior to vaccination and "post" sera were collected 2 weeks post-third immunization [33]. Grey line connects the neutralization activity of pre- and post- vaccination of the same volunteer. The paired t test score of mean neutralization activity between pre- and post-groups is shown indicating a significant difference. (B) Neutralization activity of post-vaccination sera was normalized using pre-vaccination sera of the same individual. The neutralization activities of three volunteers' sera within this group were not shown due to inconsistent results from two independent experiments.
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neutralization activity against other genotypes of HCV (data not shown).

We observed a dose-dependent neutralization with increasing amount of sera (Figure 3). Increasing the antiserum concentration two-fold (to a 1:25 dilution) resulted in a significant enhancement of neutralization activity compared with the standard dilution used in the other figures of this paper (1 in 50 dilution). This enhancement was observed against infection of both genotype 1a and genotype 2a viruses.

The Neutralization activity of human antisera is mediated by immunoglobulin

The sera of the vaccinated volunteers was shown to have neutralization activity inhibiting HCV infection. We wanted to examine if this inhibition was antibody-mediated. Immunoglobulins were purified from antisera and tested for neutralization activity. The isolation achieved over 90% purity of immunoglobulin monitored by SDS-PAGE (Figure 4A). Subsequent neutralization assays revealed that the purified-immunoglobulin accounts for the majority of the neutralization activity, since the level of neutralization activity was comparable between serum and purified immunoglobulins. As expected, the neutralization activity of the purified immunoglobulin was diminished upon dilution of the amount of IgG added to the assay (Figure 4B).

Discussion

In this study, the neutralization activities of sera from human volunteers vaccinated with a recombinant HCV gpE1/gpE2 vaccine in a phase I clinical trial were evaluated. The vaccine can induce cross neutralizing activity against heterologous 1a challenge *in vitro* (Figure 1). The neutralization activity of sera was mediated by neutralizing antibodies (Figure 4) and 92% of post-vaccinated sera showed evidence of neutralization activity against the 1a genotype which predominates in Canada and the USA. Furthermore, two of the three tested sera showed broad cross-neutralizing activity against representative viruses from all 7 HCV major genotypes that are known to occur globally (Figure 2). This shows that despite previous concerns about HCV envelope glycoprotein

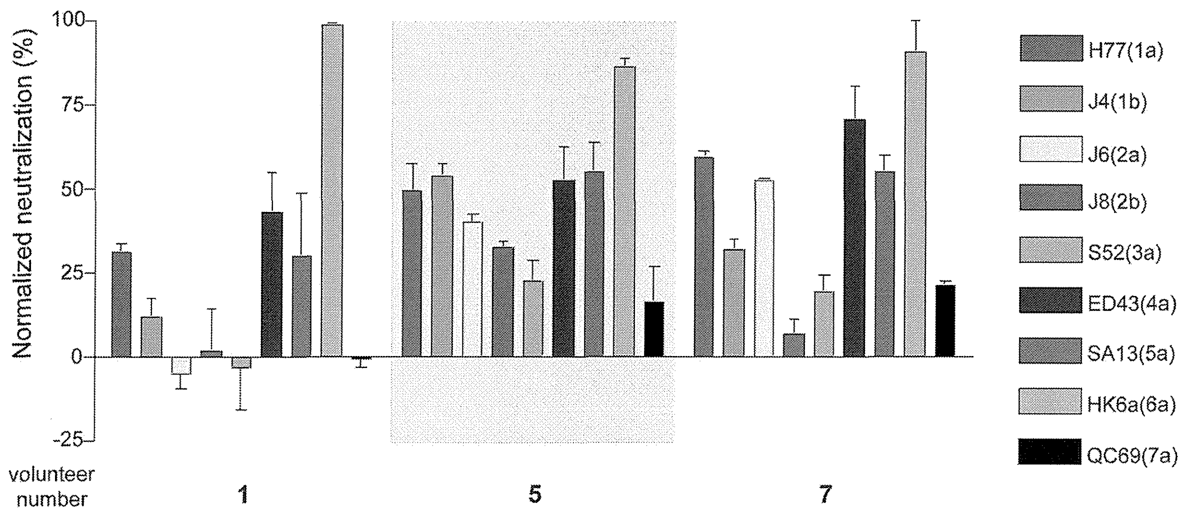

Figure 2. Human antisera cross-neutralizes all 7 major HCV genotypes. Sera of volunteers 1, 5 and 7 were tested for neutralization activity against chimeric 1a (H77C), 1b (J4), 2a (J6), 2b (J8), 3a (S52), 4a (ED43), 5a (SA13), 6a (HK6a) or 7a (QC69) HCVcc [3]. Virus neutralization assays were performed using pre- and post- vaccination sera at a concentration of 1 in 50. Levels of neutralization activity of post-vaccination sera were normalized with the activity of pre-vaccination sera. Representative of two independent experiments performed in triplicate are shown.
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vaccines being only able to elicit isolate-specific neutralization, a vaccine derived from envelope glycoproteins of a single genotype can elicit a broad, cross-genotype neutralizing response. The tested sera showed better neutralization activities against viruses of genotypes 1a/b, 2a, 4a, 5a and 6a, as compared with genotypes 2b, 3a and 7a, indicating that a cocktail of diverse antigens may constitute an optimal global vaccine, although it remains to be determined if antigens from a single strain can still confer adequate global protection.

The recombinant gpE1/gpE2 vaccine has been shown earlier to elicit a significant level of cross neutralization antibodies in the chimpanzee model [28] and also to be efficacious at reducing the incidence of chronic infection following experimental challenge with either homologous or heterologous viral strains [30]. The breadth of neutralizing antibodies elicited in chimpanzees was similar to our finding reported here, with the vaccinated sera of chimpanzees being more effective at neutralizing virus of genotypes 1a, 4a 5a, 6a compared with genotype 2a and 3a (genotype 7a was not tested) [28]. Although human antisera only partially neutralized HCVcc *in vitro*, lower dilutions of serum resulted in greater neutralization (Figure 3). Cross-neutralizing antibodies isolated from chronically infected patients or by molecular cloning have been shown to protect humanized mice against heterologous viral infections [16,22]. It will be important to test if the vaccine-induced antibodies reported here exhibit a similar protective potency although this would seem feasible.

The vaccine was successful at inducing E1E2-reactive antibodies [33], but not all sera have strong neutralization activity as shown in this study and elsewhere [35]. Limited characterization of these vaccine antibodies has been reported [34]. Viral neutralization may be facilitated by a strong avidity of antibodies to previously identified neutralizing eptitopes [35], although new, unidentified neutralization epitopes may exist. We are performing research aimed at mapping the neutralization epitopes targeted by this vaccine candidate in humans. Since broad cross-neutralization has been observed, at least one cross-neutralizing epitope must be highly conserved throughout all genotypes of HCV despite the presence of considerable genetic heterogeneity elsewhere in the viral genome. Broad, cross-neutralizing monoclonal antibodies

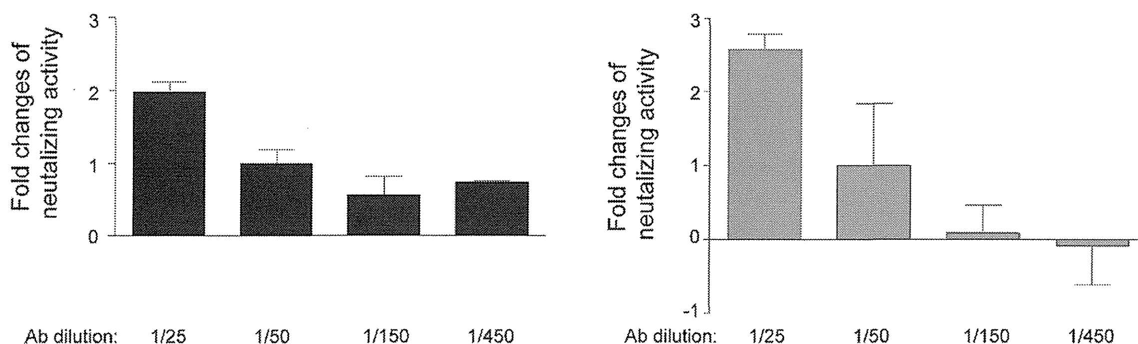

Figure 3. Human antisera neutralizes HCVcc in a dose-dependent fashion. Serum of volunteer 5 was tested for neutralization activity in various doses against 1a (H77C, blue) or 2a (J6, orange) chimeric HCVcc. Sera at indicated dilution were incubated with 100 TCID50 of HCVcc. The level of infection was monitored after staining using NS5A antibodies. Results of an experiment preformed in triplicates are shown. Fold changes of neutralization activity is shown compared to serum using at 1 in 50 dilution.
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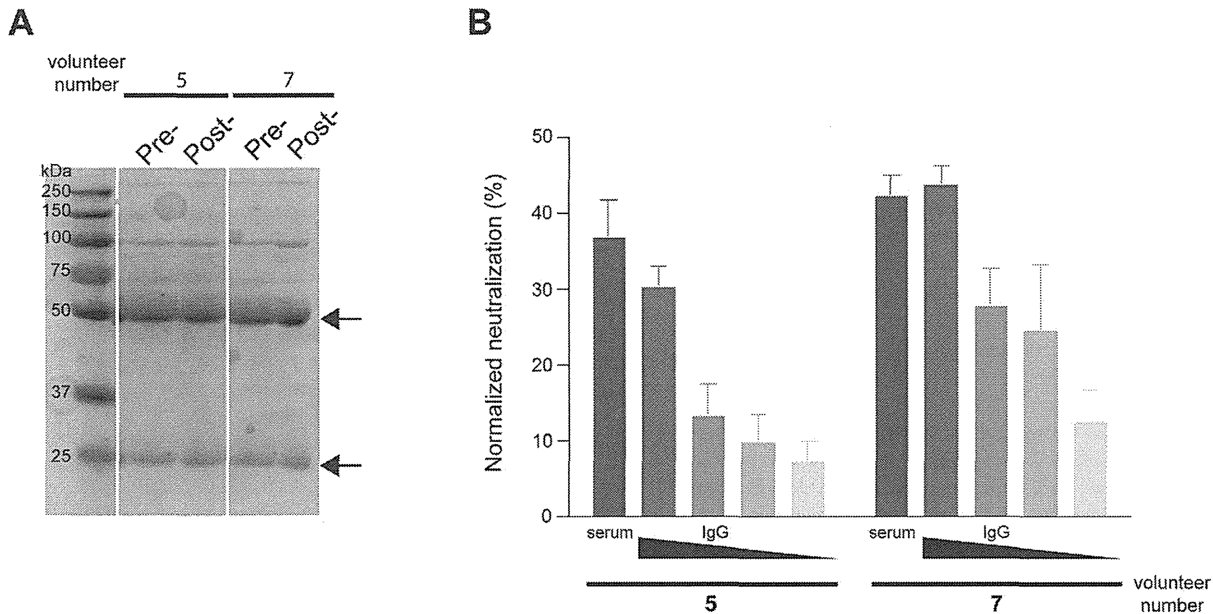

Figure 4. Neutralization of human antisera is mediated by immunoglobulin. Immunoglobulin was purified from volunteers' pre- or post-vaccinated sera. (A) 3 µg total protein of purified immunoglobulin of volunteers 5 and 7 were separated by SDS-PAGE followed by coomassie staining for visualization. Heavy and light chains of purified immunoglobulin are marked with arrows. (B) The starting concentration of purified immunoglobulin was used at 1 in 5 (by volume) followed by three 3-fold dilutions (i.e. 1 in 15, 1 in 45 and 1 in 135) in the neutralization assay using chimeric 1a H77C/JFH-1 HCVcc (grey). The neutralization activities of the post- vaccinated samples were normalized against pre-vaccinated samples of the same individual at the indicated dilution. As comparison, the neutralization activity of sera using at 1 in 50 dilution is shown (red). During the purification, the recovered immunoglobulin were diluted 10 times in the procedure, therefore, 10 times more in volume of purified immunoglobulin (1 in 5 dilution) was used compared with sera (1 in 50 dilution) (see materials and methods).
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have been isolated previously [26] and it remains to be determined which of these antibody epitopes are also targeted by this vaccine.

In this study, we have used chimeric viruses derived in cell culture to identify cross-neutralizing antibodies. It has been shown that the physical properties of cell culture-derived virus are different from animal-derived virus due to differences in lipid composition [38]. The term "lipo-viro particle" has been used to reflect the close association of HCV virus with apolipoproteins which may affect the cell entry process [39,40]. Further studies are needed to directly examine the neutralization activity of human vaccinee antisera against virus derived from infected chimpanzees and humans. Dreux *et al* have reported a negative impact of human serum components on the activity of neutralizing antibodies against HCV pseudoparticles [41]. However, we have observed similar neutralization activity when immunoglobin was purified with high efficiency from our human antisera and tested at amounts equivalent to the original volume of serum. This would indicate that in human vaccinees, no such inhibition of neutralization of HCVcc is detectable. It was also of interest to detect very effective neutralizing antibodies against the chimeric virus bearing genotype 6a envelope glycoproteins since this particular chimeric virus is the only one containing adaptive mutations within the envelope glycoprotein coding region [3]. It remains to be determined which of these mutations mediates this enhanced sensitivity to neutralization.

The data shown in this work indicates that a vaccine derived from a single strain of HCV is capable of eliciting broad cross-neutralizing antibodies implying that there must be a highly-conserved neutralization epitope(s) within the highly variable gpE1/gpE2 envelope glycoproteins. The cross-neutralization titers have so far been detected in only a minority of vaccinees and tended to be low and although it is unknown what titers actually mediate vaccine efficacy, it will be important in future to attempt to enhance the immunogenicity of the vaccine by further modifications to the antigens, adjuvant and formulation (work in progress). When combined with previous data demonstrating the protective efficacy of this vaccine in the chimpanzee model, these data offer considerable encouragement for the eventual production of an efficacious global vaccine to prevent the development of chronic, persistent infection and associated disease in exposed individuals.

Materials and Methods

Cells and viruses

Huh7.5 cells were cultured in DMEM supplemented with 10% FBS, 0.1 mM NEAA and 100 µg each of penicillin and streptomycin as described [14].

Cell culture derived HCV (HCVcc) are produced using previously described protocol [14]. Cells were washed twice with ice cold PBS and subsequently resuspended to 1.5×10^7 cell/ml. 400 µl of the cell suspension were mixed with 5 µg *in vitro* transcribed RNA encoding HCV genome in 2 mm gap electroporation curvettes. 5 pulses of 860 V (99 µs, 1.1 s interval) were delivered using the ElectroSquare Porator ECM 830 (BTX, Holliston, MA). Post-electroporation, cells were incubated at room temperature for 10 minutes before plating. Pre-cleared media was collected as virus stocks either 3 or 4 day post-electroporation. Virus titer (50% tissue culture infectious dose (TCID50)) was determined by limited dilution as described [14].

Volunteers' sera and neutralization assay

All sera were acquired from a completed phase I randomized, double-blinded, placebo-controlled study assessed the safety and

immunogenicity of HCV E1E2/MF59C.1 (DMID01–012), approved by the Saint Louis University Institutional Review Board (IRB #15719) [33]. All volunteers' sera of pre- and post-vaccination were heat inactivated at 56°C for 1 hour.

1×10^4 huh 7.5 cells per well were seeded on poly-lysine coated 96 well plates, 1 day prior to infection. For infection, 100 TCID50 HCVcc were premixed with heat inactivated sera diluted at 1 in 50 (by volume), for 1 hour at 37°C followed by adding to cells. 12 hour post-infection, the antibody-virus inoculum was replaced with fresh culture media. Cells were then fixed 48 hours post-infection with methanol using previously described methods [14]. The amount of infection was quantitated by counting the number of NS5A-positive foci detected using mouse monoclonal NS5A antibody (9E10) [14]. The foci were detected and counted using a CTL S6 immunospot analyzer (CTL, Cleveland Oh) as described [42]. The percentage of neutralization was reported by comparison with no serum control or normalized with pre-vaccination serum as described in text. The neutralization activity was calculated using the following formula: % neutralization = (pre-post)/pre $\times$100% where pre/post represent the number of NS5A-positive foci done after incubating with either the pre- or post-vaccination sera as described in text.

In vitro RNA transcription

Plasmids encoding chimeric HCV genomes representing all major genotypes (1–7) have been described [3]. DNA templates were generated by linearizing plasmids using *Xba* I and infectious RNA were generated using T7 RiboMAX large scale RNA production system (Promega, Madison, WS). RNA was subsequently purified using the RNeasy mini kit (QIagen, Hilden Germany).

Immunoglobulin purification

Immunoglobulin was purified using the Melon gel IgG spin purification kit (Thermo Scientific, Rockford IL). The purified immunoglobulin was diluted 10 times in volume compared to the starting material, i.e. 500 µl of purified immunoglobulin was recovered at the end by starting with 50 µl of serum. Therefore, ten times more (by volume) of purified immunoglobulin (as compared with the original serum) was used to compare the neutralization activity between the purified immunoglobulin and the serum. The purity of isolated immunoglobulin was monitored by SDS-PAGE followed by coomassie blue staining. The purity of immunoglobulin using this method was higher than 90%.

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Author Contributions

Conceived and designed the experiments: JL CC JW DH DMS MH. Performed the experiments: JL CC JW DH DMS. Analyzed the data: JL CC JW DH DMS MH. Contributed reagents/materials/analysis tools: SEF RBB TW JB CTJ CMR SA DLT. Wrote the paper: JL DMS MH.

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Antiviral Activity of Glycyrrhizin against Hepatitis C Virus *In Vitro*

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Abstract

Glycyrrhizin (GL) has been used in Japan to treat patients with chronic viral hepatitis, as an anti-inflammatory drug to reduce serum alanine aminotransferase levels. GL is also known to exhibit various biological activities, including anti-viral effects, but the anti-hepatitis C virus (HCV) effect of GL remains to be clarified. In this study, we demonstrated that GL treatment of HCV-infected Huh7 cells caused a reduction of infectious HCV production using cell culture-produced HCV (HCVcc). To determine the target step in the HCV lifecycle of GL, we used HCV pseudoparticles (HCVpp), replicon, and HCVcc systems. Significant suppressions of viral entry and replication steps were not observed. Interestingly, extracellular infectivity was decreased, and intracellular infectivity was increased. By immunofluorescence and electron microscopic analysis of GL treated cells, HCV core antigens and electron-dense particles had accumulated on endoplasmic reticulum attached to lipid droplet (LD), respectively, which is thought to act as platforms for HCV assembly. Furthermore, the amount of HCV core antigen in LD fraction increased. Taken together, these results suggest that GL inhibits release of infectious HCV particles. GL is known to have an inhibitory effect on phospholipase A2 (PLA2). We found that group 1B PLA2 (PLA2G1B) inhibitor also decreased HCV release, suggesting that suppression of virus release by GL treatment may be due to its inhibitory effect on PLA2G1B. Finally, we demonstrated that combination treatment with GL augmented IFN-induced reduction of virus in the HCVcc system. GL is identified as a novel anti-HCV agent that targets infectious virus particle release.

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Introduction

Hepatitis C virus (HCV) infection is a major public health problem since most cases cause chronic hepatitis, hepatic cirrhosis and hepatocellular carcinoma. Current treatment of chronic hepatitis C is based on the combination of pegylated interferon-alpha (IFN-α) and ribavirin. However, approximately 50% of treated patients infected with genotype 1 do not respond, or show only a partial or transient response, and therapy causes significant side effects [1]. In Japan, glycyrrhizin (GL) preparations (stronger neo-minophagen C (SNMC)) have been used for more than 20 years as a treatment for chronic hepatitis patients who do not respond to IFN therapy.

GL is the major component of licorice root extract, and is composed of glycyrrhetinic acid. GL has been shown to possess several beneficial pharmacological activities, including anti-inflammatory activity [2], anti-tumor activity [3], anti-allergic activities [4], and anti-viral activities [5]. Several mechanisms of the GL-induced anti-inflammatory effect are reported, such as inhibition of thrombin-induced platelet aggregation [6], inhibition

of prostaglandin E2 production [7] and inhibition of phospholipase A2 (PLA2) [8].

Many anti-viral effects of GL have been reported previously, for example, against herpes simplex type 1 (HSV-1) [9], varicella-zoster virus (VZV) [10], hepatitis A (HAV) [11] and B virus (HBV) [12], human immunodeficiency virus (HIV) [13], severe acute respiratory syndrome (SARS) and coronavirus [14], Epstein–Barr virus (EBV) [15], human cytomegalovirus [16] and influenza virus [17]. GL has been considered as a potential treatment for patients with chronic hepatitis C, and long term administration of GL to patients is effective in suppressing serum alanine aminotransferase (ALT) levels and histological change [18]. However, a direct anti-viral effect of GL against HCV has never been reported.

In this study, we evaluated the anti-HCV effects of GL, and demonstrated that GL targeted the release step of infectious HCV particles from infected cells. We found that the suppression of virus release by GL may be derived from its inhibitory effect on group 1B PLA2 (PLA2G1B). These findings suggest possible novel roles for GL in the treatment of patients with chronic hepatitis C.

Materials and Methods

Cell culture and reagents

The human hepatoma cell line, Huh7, and its derivative cell line, Huh7.5.1, provided by Francis Chisari (Scripps Research Institute, La Jolla, CA), were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) [19]. Huh7 cells harboring the subgenomic replicon [20] [21] were maintained in complete DMEM supplemented with 0.5 mg/ml G418 (Geneticin, Life Technologies Japan Ltd., Tokyo, Japan). GL (20β-carboxyl-11-oxo-30-norolean-12-en-3β-yl-2-O-β-D-glucopyranuronosyl-β-D-glucopyranosiduronic acid) and IFN-α were kindly provided by the Minophagen Pharmaceutical Co., Ltd., (Tokyo, Japan) and MSD K.K., (Tokyo, Japan) respectively. Oleyloxyethyl phosphorylcholine (OPC) (Cayman Chemical Company, Ann Arbor, MI), sPLA2IIA Inhibitor I (MERCK, Darmstadt, Germany), anti-Actin (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-Human CD81 (BD Pharmingen, San Jose, CA) antibodies were purchased. The solvents were distilled water (GL), ethanol (OPC), and DMSO (sPLA2IIA inhibitor).

Quantification of HCV core antigen and cell viability

The production of cell culture-produced HCV (HCVcc) has been previously reported [22]. Purification of LD has been previously reported [23]. The concentration of HCV core antigen in filtered culture medium, in cell lysates and in LD fraction of infected cells was determined using the Lumipulse Ortho HCV antigen kit (Ortho Clinical Diagnostics, Tokyo, Japan). Cell viability was analyzed by using Cell Titer-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) according to the manufacturers' protocol.

Electroporation of HCV RNA lacking E1 and E2

In vitro synthesis of HCV RNA JFH1 lacking E1 and E2 (JFH1delE1E2), and electroporation were performed as described previously [22].

HCV pseudoparticle (HCVpp) assay

HCVpp harboring E1 and E2 glycoproteins of the JFH-1 clone (genotype 2a) (HCVpp2a) and the TH clone (genotype 1b) (HCVpp1b) were produced as previously described [24]. Pseudotype virus with VSV G glycoprotein (VSVpp) were also generated [24]. Huh7 or Huh7.5.1 cells were seeded into 48-well plates, incubated overnight at 37°C, and then infected with the HCVpp in the presence of various concentration of GL. Several hours post-infection, medium was replaced with DMEM with 10% FBS, and the cells were harvested 48 hours later to determine intracellular luciferase activity (Luciferase Assay System, Promega).

HCV subgenomic replicon assay

The assay for the genotype 1b and 2a subgenomic reporter replicon has been previously reported [20] [21]. After 72 hours of treatment with GL, the replicon-transfected cells were harvested for either measurement of luciferase activity (Promega) or HCV RNA titer, as described previously [25]. The replication efficiency of HCV in each preparation was calculated as the percentage of luciferase activity or HCV RNA titer compared with that of cells subjected to the control treatment.

Extra- and intracellular infectivity

To determine extracellular HCV infectivity, naïve Huh7 cells were inoculated with cell culture supernatant medium containing HCVcc. After 3 hours of incubation, the medium was replaced with DMEM containing 10% FBS, and the cells were cultured for an additional 72 hours. The infectious HCV titer in the culture medium was determined by quantification using the Lumipulse Ortho HCV antigen kit or by immunostaining of the HCV core antigen. Using an immunoassay that also provided results indicative of HCV infectivity [26], we confirmed a good correlation between the levels of core antigen and infectious titers (data not shown). To estimate intracellular infectivity, cells in the culture plates filled with DMEM containing 10% FBS were subjected to four cycles of freezing and thawing, using dry ice and a 37°C water bath. Cells in the culture plates were centrifuged at 1,200 rpm for 5 min at 4°C to remove cell debris, and the supernatants were collected to evaluate infectivity as above.

RNA interference

The siRNA targeted to PLA2G1B, 5'-GCUGGACAGCUGUAAAUUUTT-3', and scramble negative control siRNA to PLA2G1B were purchased from Sigma (Tokyo, Japan). Cells in a 24-well plate were transfected with siRNA using HiPerFect transfection reagent (Qiagen, Tokyo, Japan) following the manufacturer's instructions.

Quantification of triglyceride

Triglyceride (TG) was measured with a Triglyceride kit (Wako, Tokyo, Japan) according to the manufacturer's instructions.

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).

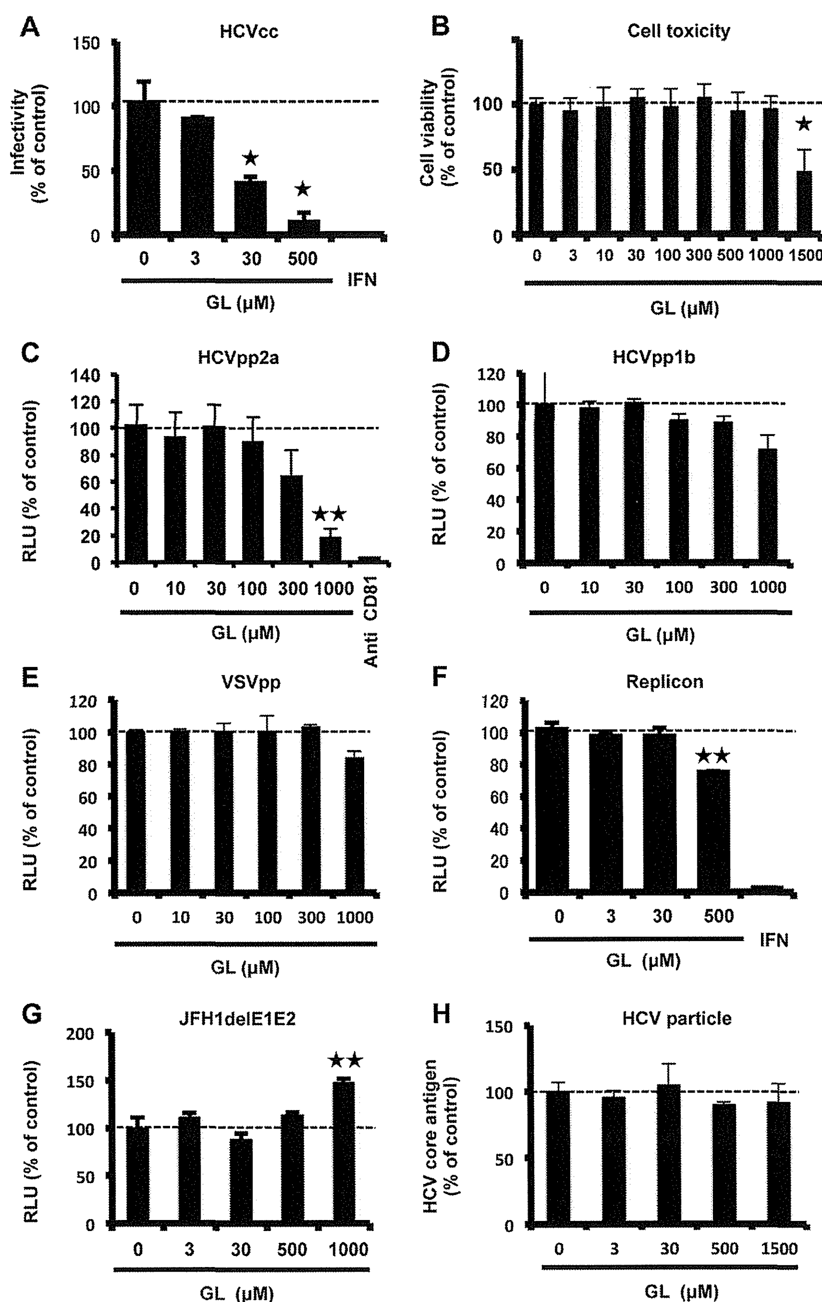

Figure 1. Anti-HCV effects of GL. (A) HCVcc-infected cells were treated with various concentrations of GL for 72 hours. Naïve Huh7 cells were inoculated with supernatant and cultured for 72 hours. Infectivity was determined by immunostaining. (B) Cell viability was assessed using Cell Titer-Glo Luminescent 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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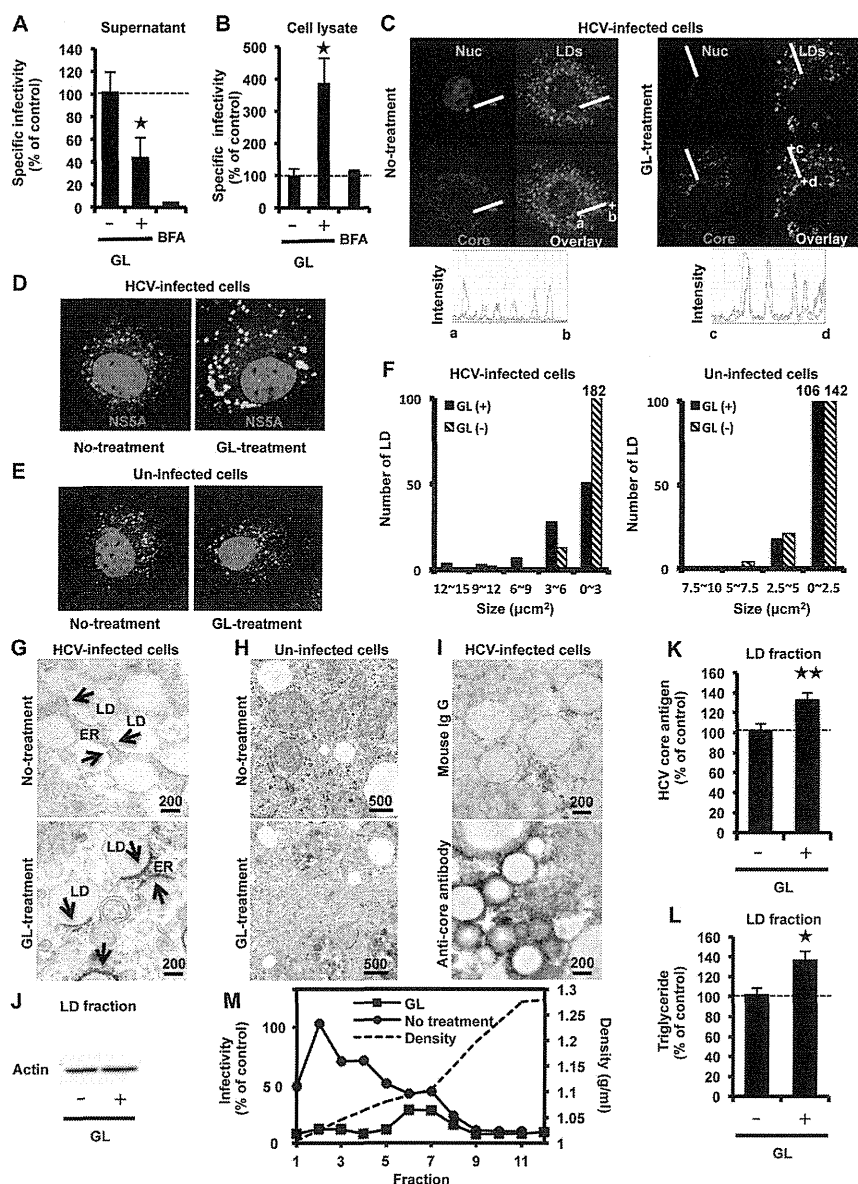

Figure 2. Effects of GL in release of infectious HCV particles. HCVcc-infected cells were treated with GL at a concentration of 500 μM for 72 hours. Untreated cells were used as controls. Extra- (A) and intracellular specific infectivity (B) were determined. Subcellular co-localization of HCV core (C) 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 un-treated 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

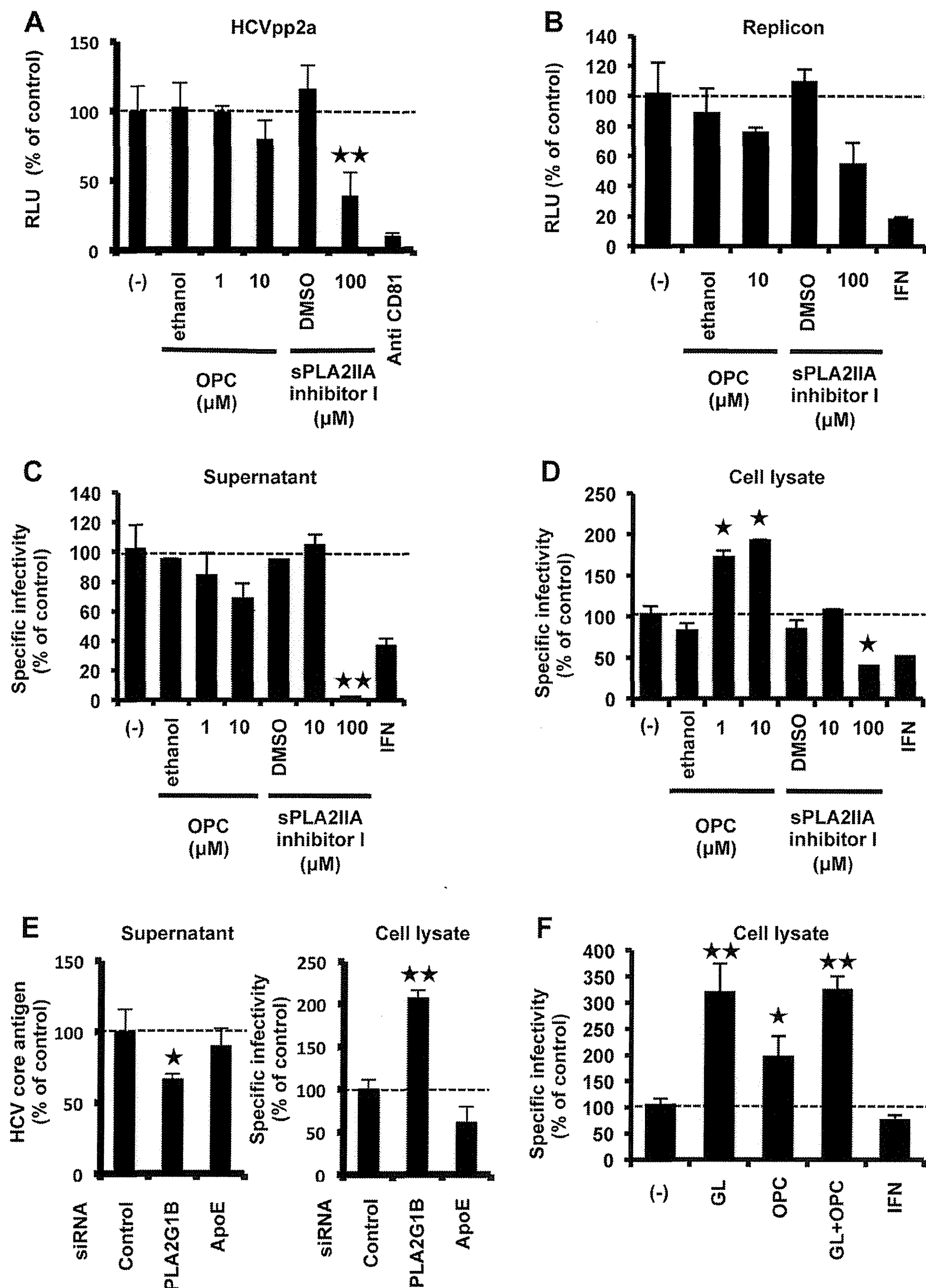

Figure 3. A role of PLA2 in HCV lifecycle. (A) Huh7 cells were infected with HCVpp in the presence and absence of OPC or sPLA2IIA inhibitor for 2 hours, then medium was replaced. Effects of PLA2 inhibitor on the entry of HCVpp were determined by measuring the luciferase activity at 72 hours post-infection. 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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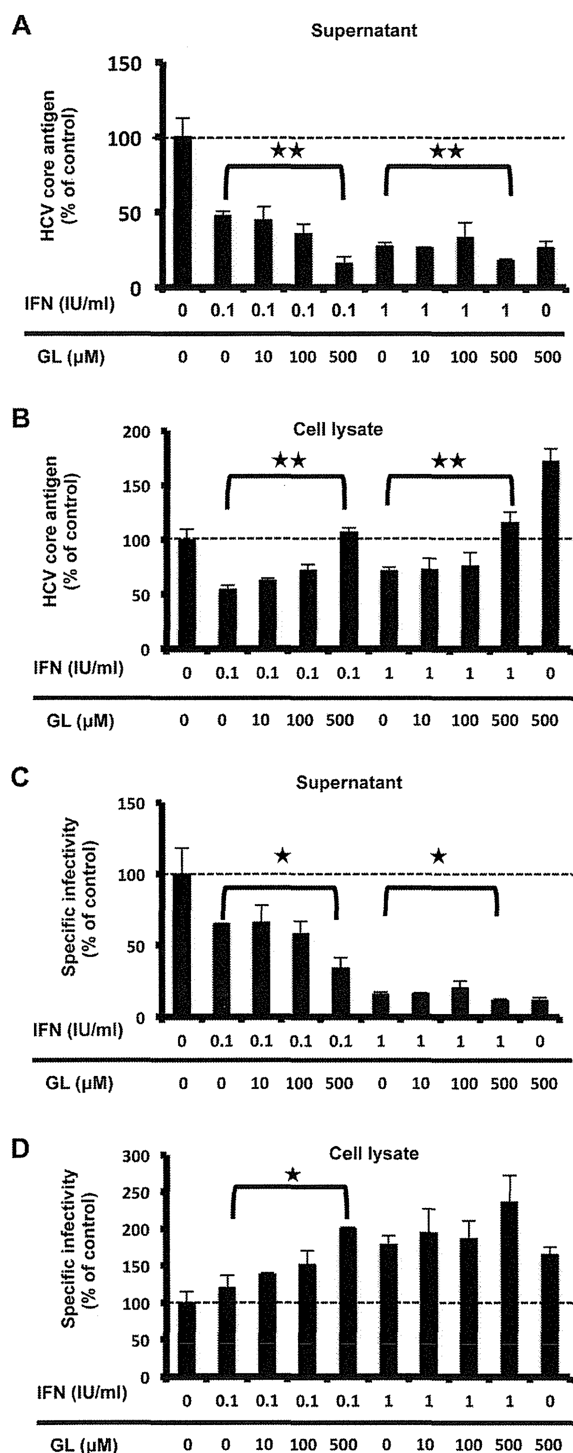

Figure 4. Anti-HCV effects IFN in combination with GL. HCVcc-infected cells were treated with IFN alone, or IFN with GL for 72 hours. HCV production was assessed by measuring the HCV core antigen in culture medium (A) and cell (B). Specific nfectivity in culture medium (C) and cell (D) were measured. 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.

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