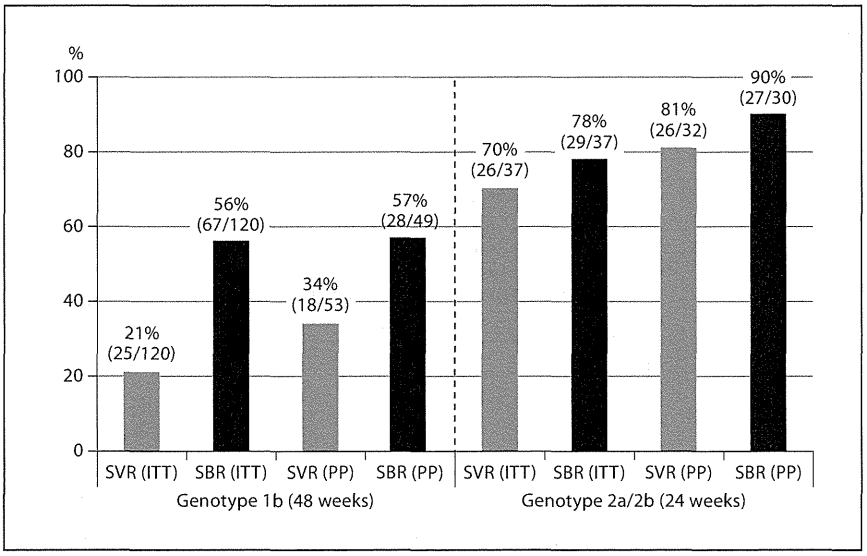

Fig. 2. In 157 patients with HCV-related compensated cirrhosis treatment efficacy with interferon plus ribavirin therapy was evaluated for 48 weeks of HCV genotype 1b or 24 weeks of genotype 2a/2b. In HCV genotype 1b, rates of sustained biochemical response (SBR) were significantly higher than those of sustained virological response (SVR; ITT analysis, $p < 0.001$, and PP analysis, $p = 0.028$).

COBAS TaqMan HCV test was 1.2 log IU/ml. The undetectable samples by HCV-RNA qualitative analysis or COBAS TaqMan HCV test were defined as negative HCV-RNA.

Follow-Up and Diagnosis of Hepatocellular Carcinoma

Clinical and laboratory assessments were performed at least once every month before, during, and after treatment. Adverse effects were monitored clinically by careful interviews and medical examination at least once every month. Patient compliance with treatment was evaluated with a questionnaire. Blood samples were also obtained at least once every month before, during, and after treatment, and were also analyzed for levels of serum alanine aminotransferase and HCV-RNA at various time points.

Patients were examined for hepatocellular carcinoma by abdominal ultrasonography every 3–6 months. If hepatocellular carcinoma was suspected based on ultrasonographic results, additional procedures, such as computed tomography, magnetic resonance imaging, abdominal angiography, and ultrasonography-guided tumor biopsy if necessary, were used to confirm the diagnosis.

Statistical Analysis

χ^2 test, Fisher's exact probability test, and Mann-Whitney's U test were used to compare the background characteristics between groups. Multiple comparisons were examined by the Bonferroni test. The cumulative hepatocarcinogenesis rates were calculated using the Kaplan-Meier technique, and differences between the curves were tested using the log-rank test. Statistical analysis of the hepatocarcinogenesis rates according to groups was calculated using the period from the end of the first course of interferon monotherapy until the appearance of hepatocellular carcinoma or until the last visit or until the start of the third course of interferon-based treatment. Stepwise Cox regression analysis was used to determine independent predictive factors that were associated with hepatocarcinogenesis. The hazard ratio (HR) and 95% confidence interval were also calculated. Potential predictive factors associated with hepatocarcinogenesis included the following 13 variables: age, sex, serum aspartate aminotransferase, serum alanine aminotransferase, platelet count, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, HCV genotype, levels of viremia, total duration of additional treatment, and group of additional treatment. Each variable was transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. All p values <0.05 and <0.1 by the two-tailed test were considered significant ($p < 0.05$) and marginally significant ($p < 0.1$), respectively. Variables that achieved statistical significance ($p < 0.05$) on univariate analysis were tested by multivariate Cox proportional hazard model to identify significant independent factors. Statistical comparisons were performed using the SPSS software (SPSS Inc., Chicago, Ill., USA).

Results

Efficacy of Ribavirin Combination Therapy (Study 1)

Treatment efficacy of a 48-week regimen of interferon plus ribavirin combination therapy in 120 patients infected with HCV-1b was evaluated. In ITT analysis, rates of sustained virological response and sustained biochemical response were 21% (25 of 120 patients) and 56% (67 of 120 patients), respectively. In the PP analysis, rates of sustained virological response and sustained biochemical response were 34% (18 of 53 patients) and 57% (28 of 49 patients), respectively (fig. 2). In both analyses, rates of sustained biochemical response were significantly higher than those of sustained virological response (ITT analysis, $p < 0.001$, and PP analysis, $p = 0.028$).

Table 2. Profile and laboratory data of 185 patients with HCV-related compensated cirrhosis according to additional treatment groups (study 2)

	No treatment	Interferon monotherapy (≥24 weeks)	Ribavirin combination therapy[1] (≥24 weeks)
Demographic data			
Patients, n	106	55	24
Sex (male/female), n	64/42	37/18	20/4
Age, years	56 (30–75)[a]	56 (35–76)[b]	51 (34–68)
Laboratory data			
Serum aspartate aminotransferase, IU/l	75 (26–285)	83 (35–213)	62 (30–160)
Serum alanine aminotransferase, IU/l	92 (17–400)	104 (30–316)	93 (36–250)
Platelet count, $\times 10^4/mm^3$	10.7 (2.5–18.2)[c]	10.8 (5.7–19.8)[d]	13.0 (5.2–23.5)
Total cholesterol, mg/dl	165 (103–273)[h]	152 (101–220)	160 (111–211)
High-density lipoprotein cholesterol, mg/dl	46 (25–93)	43 (21–65)	47 (28–56)
Low-density lipoprotein cholesterol, mg/dl	93 (38–168)	87 (45–139)	100 (34–135)
Triglycerides, mg/dl	96 (36–437)	80 (51–215)	108 (52–206)
HCV genotype (1b/2a or 2b), n	70/36	39/16	17/7
Levels of viremia (high viral load/low viral load), n	84/16	37/15[e]	24/0
Additional treatment			
Duration of additional treatment, weeks	–	44 (24–382)[f]	26 (24–48)
Sustained virological response (ITT), n	–	11 (20%)	7 (29%)
Sustained biochemical response (ITT), n	–	25 (45%)[g]	16 (67%)

Unless otherwise indicated, values represent median (range).

Demographic data and laboratory data, at the start of the first course of interferon monotherapy, are shown.

[a] $p = 0.013$, [b] $p = 0.030$, [c] $p = 0.002$, [d] $p = 0.015$, [e] $p = 0.006$, [f] $p = 0.044$, [g] $p = 0.083$ compared with ribavirin combination therapy by Bonferroni test, Mann-Whitney U test, or χ^2 test. [h] $p = 0.039$ compared with interferon monotherapy by Bonferroni test.

[1] 24 of 157 patients with HCV-related compensated cirrhosis in study 1 were also included in study 2. They showed no sustained virological response following the first course of interferon monotherapy (≥24 weeks), and were additionally treated with ribavirin combination therapy (≥24 weeks).

Treatment efficacy of a 24-week regimen of interferon plus ribavirin combination therapy in 37 patients infected with HCV-2a or 2b was evaluated. In the ITT analysis, rates of sustained virological response and sustained biochemical response were 70% (26 of 37 patients) and 78% (29 of 37 patients), respectively. In the PP analysis, rates of sustained virological response and sustained biochemical response were 81% (26 of 32 patients) and 90% (27 of 30 patients), respectively (fig. 2). In both analyses, rates of the sustained biochemical response were not significantly higher than those of the sustained virological response.

Profile, Laboratory Data, and Efficacy according to Additional Treatment Groups (Study 2)

Profile and laboratory data, at the start of the first course of interferon monotherapy of 185 patients with HCV-related compensated cirrhosis, are summarized in table 2. The age of patients with ribavirin combination therapy was significantly lower than that of patients with no treatment ($p = 0.013$; Bonferroni test) and interferon monotherapy ($p = 0.030$; Bonferroni test). The platelet count of patients of ribavirin combination therapy was significantly higher than that of patients without treatment ($p = 0.002$; Bonferroni test) and interferon monotherapy ($p = 0.015$; Bonferroni test). The total cholesterol level of patients with interferon monotherapy was significantly lower than that of patients without treatment ($p = 0.039$; Bonferroni test). Low viral load rates of patients with interferon monotherapy were significantly higher than those of patients with ribavirin combination therapy ($p = 0.006$; Bonferroni test). There were no other significant differences in clinical features at the start of the first course of interferon monotherapy among the three groups.

Additional treatment duration of only 1 patient, who was diagnosed with hepatocellular carcinoma during additional treatment, was evaluated using the period from the start of the second course of interferon monotherapy

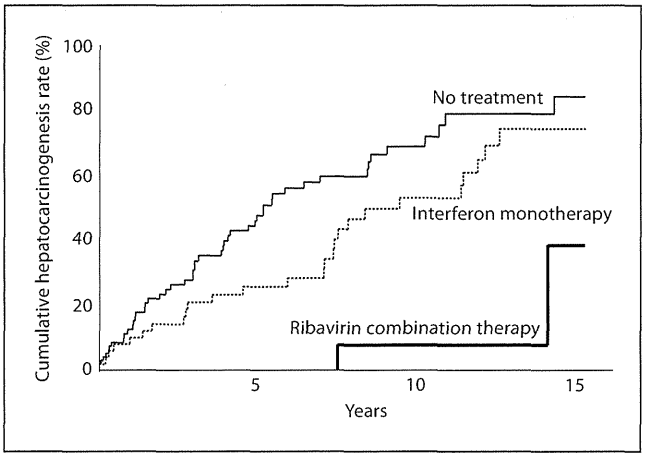

Fig. 3. Cumulative hepatocarcinogenesis rates in the three groups of additional treatment. The rates in no treatment were significantly higher than those in interferon monotherapy ($p = 0.047$; log-rank test) and ribavirin combination therapy ($p < 0.001$; log-rank test), and the rates in interferon monotherapy were significantly higher than those in ribavirin combination therapy ($p < 0.001$; log-rank test).

Table 3. Factors associated with hepatocarcinogenesis in 185 patients of HCV-related compensated cirrhosis identified by multivariate analysis (study 2): Cox proportional hazard model

Factors/category	Hazard ratio (95% confidence interval)	p
Additional treatment		
Ribavirin combination therapy	1	
Interferon monotherapy	4.47 (1.04–19.3)	0.045
No treatment	9.14 (2.19–38.2)	0.002
Age		
<55 years	1	
≥55 years	2.87 (1.76–4.67)	<0.001
Aspartate aminotransferase		
<58 IU/l	1	
≥58 IU/l	2.11 (1.20–3.74)	0.010

until the appearance of hepatocellular carcinoma. During additional treatment, the total duration of interferon monotherapy was significantly longer than that of ribavirin combination therapy ($p = 0.044$; Mann-Whitney U test). In ITT analysis, sustained virological response rates of ribavirin combination therapy (29%) were not different from those of interferon monotherapy (20%), but sustained biochemical response rates of ribavirin combination therapy (67%) tended to be higher than those of interferon monotherapy (45%; $p = 0.083$; χ^2 test) (table 2).

Predictive Factors Associated with Hepatocarcinogenesis by Multivariate Analysis

The data for the whole population sample were analyzed to determine those factors that could predict hepatocarcinogenesis. Hepatocarcinogenesis rates in older patients (≥55 years), in patients with higher levels of aspartate aminotransferase (≥58 IU/l), and lower levels of platelet count (<15.0 × 10^4/mm^3) were significantly higher than those in younger patients (<55 years), in patients with lower levels of aspartate aminotransferase (<58 IU/l), and higher levels of platelet count (≥15.0 × 10^4/mm^3), respectively ($p < 0.001$, $p = 0.006$, and $p = 0.017$; log-rank test). Furthermore, the rates in no treatment were significantly higher than those in interferon monotherapy ($p = 0.047$; log-rank test) and ribavirin combination therapy ($p < 0.001$; log-rank test), and the rates in interferon monotherapy were significantly higher than those in ribavirin combination therapy ($p < 0.001$; log-rank test) (fig. 3). Thus, univariate analysis identified four parameters that significantly correlated with hepatocarcinogenesis. These factors were entered into multivariate analysis, which then identified three parameters that significantly influenced hepatocarcinogenesis independently: additional treatment (no treatment; HR 9.14, $p = 0.002$), age (≥55 years; HR 2.87, $p < 0.001$), and levels of aspartate aminotransferase (≥58 IU/l; HR 2.11, $p = 0.010$) (table 3).

The data for 167 patients, except for 18 patients who showed a sustained virological response following additional treatment, were also analyzed to determine those factors that could predict hepatocarcinogenesis. Hepatocarcinogenesis rates in older age (≥55 years) and higher levels of aspartate aminotransferase (≥58 IU/l) were significantly higher than those in younger age (<55 years) and lower levels of aspartate aminotransferase (<58 IU/l), respectively ($p < 0.001$ and $p = 0.007$; log-rank test). Furthermore, the rates in ribavirin combination therapy were significantly lower than those in interferon monotherapy ($p < 0.001$; log-rank test) and no treatment ($p < 0.001$; log-rank test) (fig. 4). Thus, univariate analysis identified three parameters that significantly correlated with hepatocarcinogenesis. These factors were entered into multivariate analysis, which then identified three parameters that significantly influenced hepatocarcinogenesis independently: additional treatment (no treatment; HR 7.87, $p = 0.005$), age (≥55 years; HR 2.52, $p < 0.001$), and levels of aspartate aminotransferase (≥58 IU/l; HR 2.13, $p = 0.010$) (table 4).

Discussion

One of our previous studies indicated that the cancer-suppressive activity of interferon monotherapy in patients with HCV-RNA eradication was similar to that in patients with alanine aminotransferase normalization without HCV-RNA elimination [9]. Other studies also indicated a higher incidence and more rapid development of hepatocellular carcinoma in HCV patients with high levels of alanine aminotransferase [13, 14]. Collectively, these results suggest that the carcinogenic process in patients with chronic HCV infection is enhanced by high levels and fluctuations of alanine aminotransferase, and indicate a close relationship between suppression of inflammatory necrosis of hepatocytes and a lower incidence of hepatocellular carcinoma in patients with HCV-associated chronic liver disease. Recent studies based on interferon plus ribavirin combination therapy also showed that the attainment of sustained virological response or lower levels of alanine aminotransferase after ribavirin combination therapy could reduce the rates of hepatocellular carcinoma [15, 16], but the small numbers of patients with compensated cirrhosis (5% or less of all patients) were recruited. The present study 1 based on the patients with compensated cirrhosis showed that rates of sustained virological response and sustained biochemical response in HCV-2a/2b were high rates of 70 and 78%, and that rates of sustained biochemical response (57%) were significantly higher than those of sustained virological response (34%) in HCV-1b. Furthermore, the present study 2 based on the patients with compensated cirrhosis, who showed no sustained virological response following the first course of interferon monotherapy, also showed that sustained biochemical response rates of ribavirin combination therapy (67%) tended to be higher than those of interferon monotherapy (45%). Thus, in ribavirin combination therapy for compensated cirrhosis, higher rates of sustained biochemical response might be associated with lower rates of hepatocarcinogenesis. One limitation is that the present study was performed based on the small numbers of patients who showed no sustained virological response with interferon monotherapy. In further prospective studies a larger number of patients need to be investigated to confirm this finding.

Previous studies have shown that gender, age, fibrosis stage, alanine aminotransferase, and interferon regimen are important pretreatment predictors of hepatocarcinogenesis [9, 10, 17]. In the present study 2 based on the patients with compensated cirrhosis, higher age and aspartate aminotransferase were associated with higher hepatocarcinogenesis rates in the whole population sample and in the sample which excluded patients who showed sustained virological response following additional treatment. Furthermore, as treatment-related factors, the hepatocarcinogenesis rates in ribavirin combination therapy were significantly lower than those in interferon monotherapy. Thus, in patients with compensated cirrhosis representing a high-risk group of hepatocarcino-

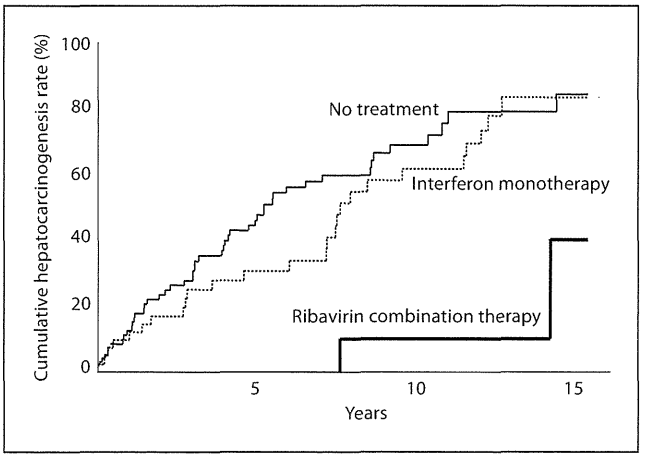

Fig. 4. Cumulative hepatocarcinogenesis rates in the three groups of additional treatment, except for patients who showed sustained virological response following additional treatment. The rates in ribavirin combination therapy were significantly lower than those in interferon monotherapy ($p < 0.001$; log-rank test) and no treatment ($p < 0.001$; log-rank test).

Table 4. Factors associated with hepatocarcinogenesis in 167 patients of HCV-related compensated cirrhosis, except for 18 patients who showed sustained virological response following additional treatment identified by multivariate analysis (study 2): Cox proportional hazard model

Factors/category	Hazard ratio (95% confidence interval)	p
Additional treatment		
Ribavirin combination therapy	1	
Interferon monotherapy	4.68 (1.08–20.3)	0.039
No treatment	7.87 (1.89–32.9)	0.005
Age		
<55 years	1	
≥55 years	2.52 (1.54–4.11)	<0.001
Aspartate aminotransferase		
<58 IU/l	1	
≥58 IU/l	2.13 (1.20–3.79)	0.010

genesis, ribavirin combination therapy might reduce the risk of hepatocellular carcinoma in comparison with interferon monotherapy. One reason for the higher anticarcinogenic activity by ribavirin combination therapy might be due to higher rates of sustained biochemical response. The other reason might be due to the difference in the background (lower age and higher levels of platelet count as an indicator of fibrosis stage) of patients with ribavirin combination therapy. Further studies of a larger number of patients matched for background, including age, sex, genotype, and platelet count, are required to investigate the rates of hepatocarcinogenesis and the mechanism of anticarcinogenic activity by ribavirin combination therapy for HCV-related compensated cirrhosis.

Two previous studies (PROVE1 and PROVE2) showed that the 12- and 24-week regimen of telaprevir/PEG-IFN/ribavirin could achieve sustained virological response rates of 35–60 and 61–69% in patients infected with HCV-1, respectively [18, 19]. However, a recent study (PROVE3) also showed that the sustained virological response rates were the lower rates of 39 and 38% with the 24- and 48-week regimen of triple therapy in previously nonresponding patients infected with HCV-1, who do not become HCV-RNA negative during or at the end of the initial PEG-IFN/ribavirin treatment, respectively [20]. Furthermore, the telaprevir-based regimen induces resistant variants [21–23] and has side effects including anemia and rash [18–20, 24]. Hence, patients, who do not achieve sustained virological response by triple therapy, need to be identified, in order to avoid unnecessary side effects and telaprevir-resistant variants. Recent studies identified amino acid substitutions at position 70 and/or 91 in the HCV-1b core region, advanced fibrosis stage, and higher levels of α-fetoprotein as pretreatment predictors of poor virological response to PEG-IFN/ribavirin combination therapy or triple therapy of telaprevir/PEG-IFN/ribavirin [23, 25–28], and these factors are also risk factors and surrogate markers of hepatocarcinogenesis [29–34]. Hence, ribavirin combination therapy for these patients might be an efficacious therapeutic regimen for sustained biochemical response and thus a reduction of the risk of hepatocarcinogenesis. Large-scale prospective studies should be conducted in the future to confirm this finding.

In conclusion, the present retrospective study indicated that ribavirin combination therapy for HCV-related compensated cirrhosis could reduce the risk of hepatocarcinogenesis in comparison with interferon monotherapy. Large-scale prospective studies need to be conducted in the future to confirm these findings.

Acknowledgments

This study was supported in part by a Grant-in-Aid from the Ministry of Health, Labor and Welfare, Japan.

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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.

Citation: Matsumoto Y, Matsuura T, Aoyagi H, Matsuda M, Hmwe SS, et al. (2013) Antiviral Activity of Glycyrrhizin against Hepatitis C Virus *In Vitro*. PLoS ONE 8(7): e68992. doi:10.1371/journal.pone.0068992

Editor: Stephen J Polyak, University of Washington, United States of America

Received March 28, 2013; **Accepted** June 3, 2013; **Published** July 18, 2013

Funding: This study was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, the Ministry of Health, Labour, and Welfare of Japan, the Ministry of Education, Culture, Sports, Science, and Technology, the National Institute of Biomedical Innovation, and Research on Health Sciences Focusing on Drug Innovation of the Japan Health Sciences Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: K. Wake is employed by Minophagen Pharmaceutical Co., Ltd. GL (20<beta>-carboxyl-11-oxo-30-norolean-12-en-<beta>-yl-2-O-<beta>-D-glucopyranuronosyl-<beta>-D-glucopyranosiduronic acid) was kindly provided by the Minophagen Pharmaceutical Co., Ltd. There are no further patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials, as detailed online in the guide for authors.

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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).

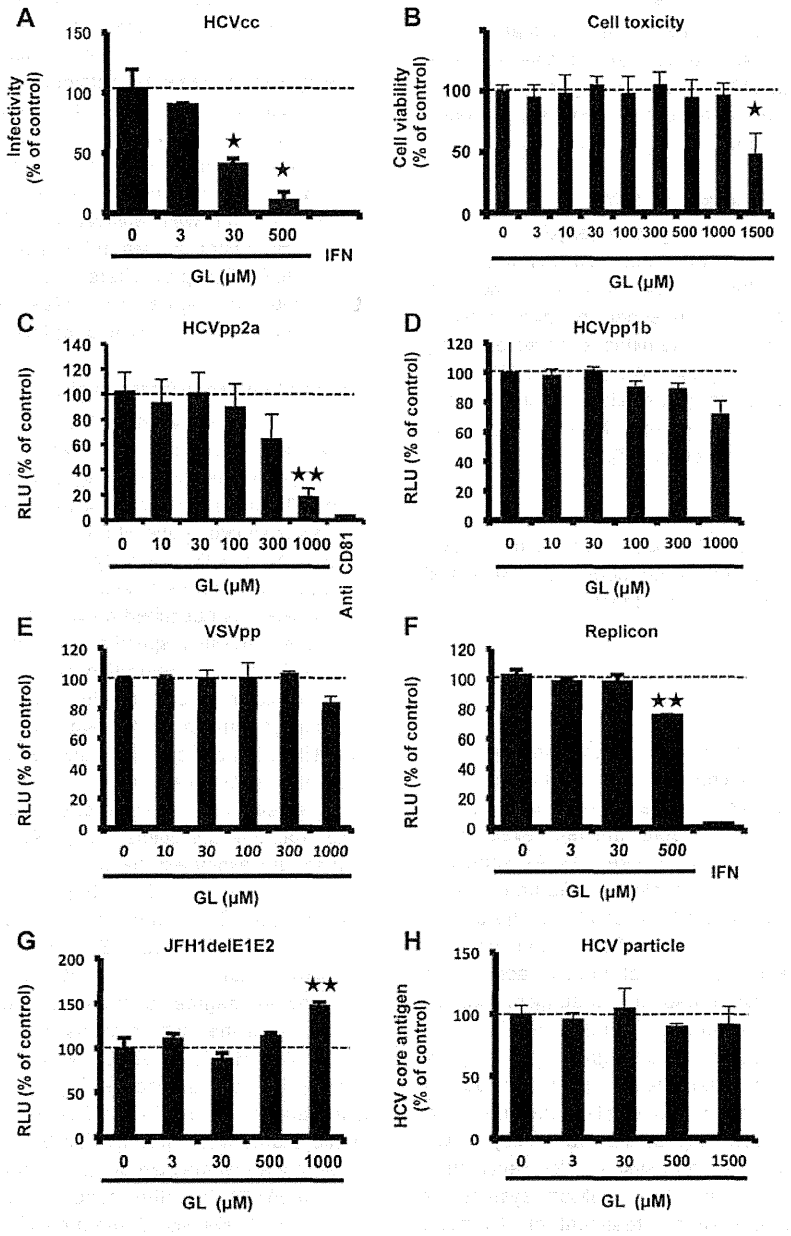

Figure 1. Anti-HCV effects of GL. (A) HCVcc-infected cells were treated with various concentrations of GL for 72 hours. Naïve Huh7 cells were inoculated with supernatant and cultured for 72 hours. Infectivity was determined by immunostaining. (B) Cell viability was assessed using Cell Titer-Glo Luminescent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

doi: 10.1371/journal.pone.0068992.g001

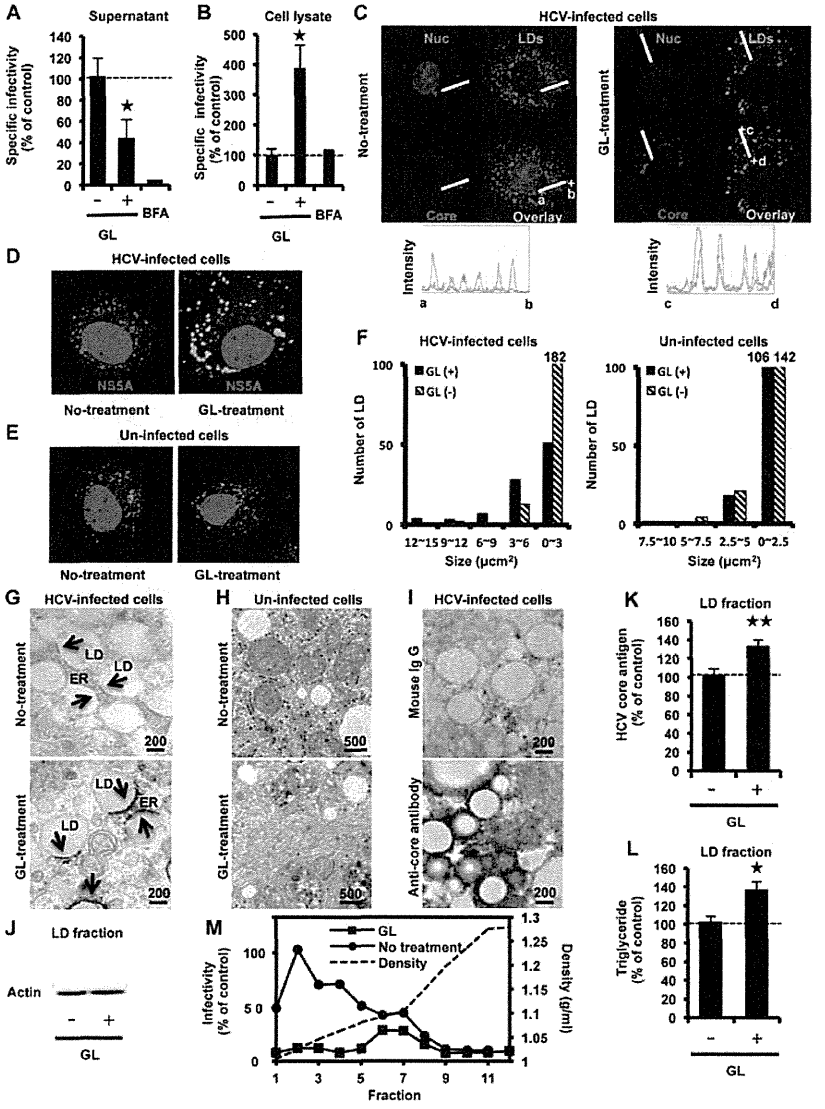

Figure 2. Effects of GL in release of infectious HCV particles. HCVcc-infected cells were treated with GL at a concentration of 500 μM for 72 hours. Untreated cells were used as controls. Extra- (A) and intracellular specific infectivity (B) were determined. Subcellular co-localization of HCV core (C)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

doi: 10.1371/journal.pone.0068992.g002

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

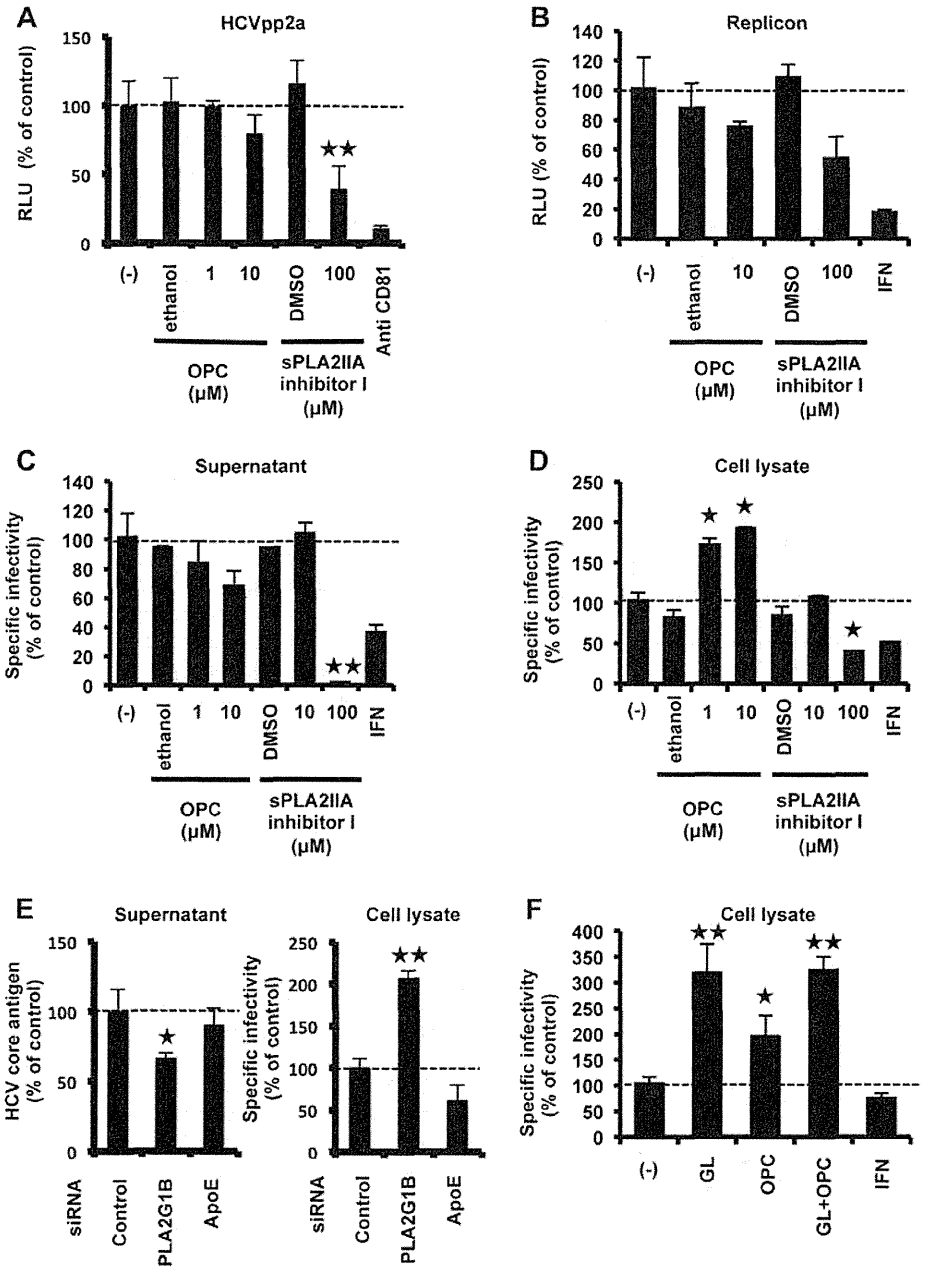

Figure 3. A role of PLA2 in HCV lifecycle. (A) Huh7 cells were infected with HCVpp in the presence and absence of OPC or sPLA2IIA inhibitor for 2 hours, then medium was replaced. Effects of PLA2 inhibitor on the entry of HCVpp were determined by measuring the luciferase activity at 72 hours post-infection.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

doi: 10.1371/journal.pone.0068992.g003

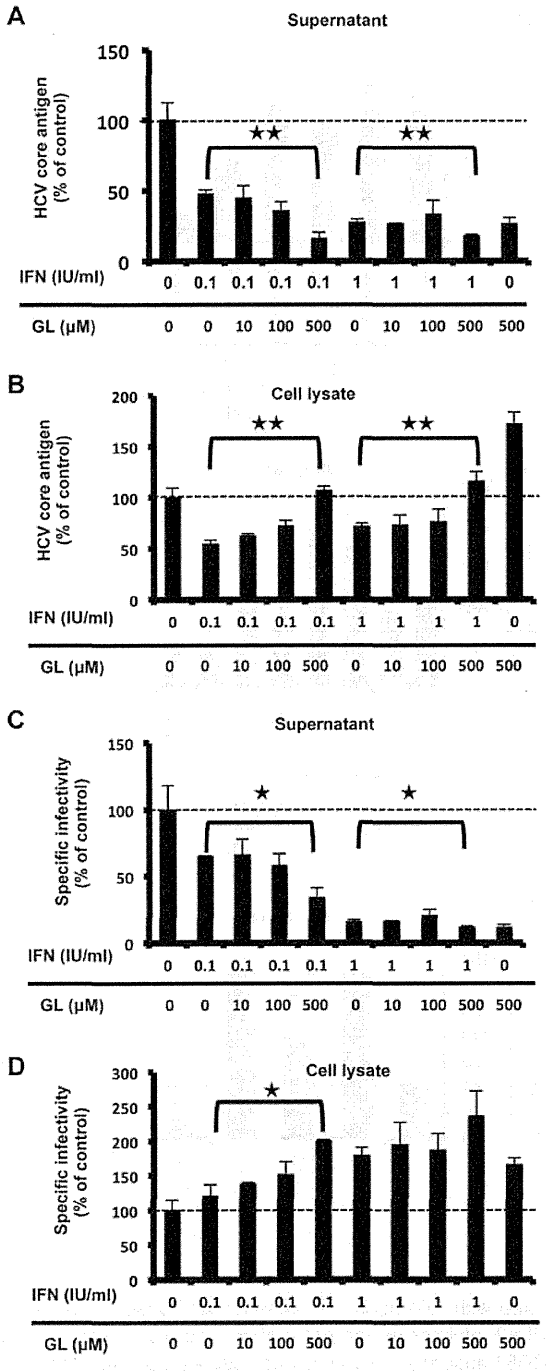

Figure 4. Anti-HCV effects IFN in combination with GL. HCVcc-infected cells were treated with IFN alone, or IFN with GL for 72 hours. HCV production was assessed by measuring the HCV core antigen in culture medium (A) and cell (B). Specific nfectivity in culture medium (C) and cell (D) were measured.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

doi: 10.1371/journal.pone.0068992.g004

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)

Acknowledgements

We are grateful to Francis V. Chisari (Scripps Research Institute) for Huh7.5.1 cells. We thank M. Sasaki for technical assistance and T. Mizoguchi for secretarial assistance.

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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Residual risk of transfusion-transmitted hepatitis B virus (HBV) infection caused by blood components derived from donors with occult HBV infection in Japan

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BACKGROUND: Nucleic acid amplification testing (NAT) for hepatitis B virus (HBV) during blood screening has helped to prevent transfusion-transmitted HBV infection (TT-HBV) in Japan. Nevertheless, 4 to 13 TT-HBV infections arise annually.

STUDY DESIGN AND METHODS: The Japanese Red Cross (JRC) analyzed repository samples of donated blood for TT-HBV that was suspected through hemovigilance. Blood donations implicated in TT-HBV infections were categorized as either window period (WP) or occult HBV infection (OBI) related. In addition, we analyzed blood from 4742 donors with low antibody to hepatitis B core antigen (anti-HBc) and antibody to hepatitis B surface antigen (anti-HBs) titers using individual-donation NAT (ID-NAT) to investigate the relationship between anti-HBc titer and proportion of viremic donors.

RESULTS: Introduction of a more sensitive NAT method for screening minipools of 20 donations increased the OBI detection rate from 3.9 to 15.2 per million, while also the confirmed OBI transmission rate increased from 0.67 to 1.49 per million. By contrast the WP transmission rate decreased from 0.92 to 0.46 per million. Testing repository samples of donations missed by minipools of 20 donations NAT showed that 75 and 85% of TT-HBV that arose from WP and OBI donations, respectively, would have been interdicted by ID-NAT. The ID-NAT trial revealed that 1.94% of donations with low anti-HBc and anti-HBs titers were viremic and that anti-HBc titers and the frequency of viremia did not correlate.

CONCLUSIONS: The JRC has elected to achieve maximal safety by discarding all units with low anti-HBc and anti-HBs titers that account for 1.3% of the total donations.

The prevalence of hepatitis B virus (HBV) surface antigen (HBsAg) in Japan is slightly higher than the average for developed countries. A recent screening of blood donors, local residents, and school pupils found an estimated national prevalence of HBsAg of 0.71%.[1] However, the prevalence was higher during the 1990s, being 1.5% among first-time blood donors aged in their 40s.[2] Taking into account horizontal transmission and a birth cohort effect, a relatively large cohort with historical HBV infection might persist among older individuals in Japan.

To prevent transfusion-transmitted HBV (TT-HBV) infection, Japanese Red Cross (JRC) blood centers introduced HBsAg screening for all blood donations in 1972. In 1989, antibody to hepatitis B core antigen (anti-HBc) testing was introduced to exclude donations by people with prior HBV infection. Because total elimination of anti-HBc–reactive donations might have seriously reduced the blood supply, donations with high antibody to hepatitis B surface antigen (anti-HBs) titers and those

ABBREVIATIONS: CLEIA(s) = chemiluminescence enzyme immunoassay(s); ID = individual donation; JRC = Japanese Red Cross; LOD = limit of detection; OBI = occult hepatitis B virus infection; PC(s) = platelet concentrate(s); S/CO = signal-to-cutoff ratio; TT-HBV = transfusion-transmitted hepatitis B virus infection; TTI = transfusion-transmitted infection; WP = window period.

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Received for publication December 28, 2011; revision received August 13, 2012, and accepted August 14, 2012.

doi: 10.1111/j.1537-2995.2012.03909.x

TRANSFUSION 2013;53:1393-1404.

with low anti-HBc titers have been accepted, and only donations with low anti-HBs and high anti-HBc titers were excluded.

In addition to this serologic screening algorithm, the JRC implemented multiplex nucleic acid amplification testing (NAT) for HBV, hepatitis C virus (HCV), and human immunodeficiency virus Type 1 (HIV-1) in 1999.[3] Although NAT has greatly reinforced blood safety regarding TT-HBV infection, 4 to 13 TT-HBV infections continue to arise annually. While some occur as a result of transfusion with blood components obtained during the window period (WP), others arise due to components being derived from donors with occult HBV infection (OBI) defined as detectable HBV DNA in peripheral blood but no detectable HBsAg.[4,5] Although donations from donors with OBI have helped to maintain an adequate blood supply, such donations have also raised a concern about the risk of TT-HBV. Here, we describe the current status of TT-HBV under the NAT screening system as well as problems inherent in the current HBV screening algorithm, especially with regard to OBI-derived blood donations. We also discuss the feasibility of strategies that could increase HBV safety in countries such as Japan with a slightly elevated prevalence of HBV.

MATERIALS AND METHODS

Screening donated blood at JRC blood centers

The JRC blood centers are the only facilities authorized to handle blood collection, processing, testing, and delivery in Japan. Donated blood is screened at these centers for HBsAg, anti-HCV, anti-HIV-1 and -2, anti-human T-lymphotropic virus type 1, anti-*Treponema pallidum*, and human parvovirus B19 antigen. Whereas HBsAg-positive blood is rejected, HBsAg-negative samples are further tested for anti-HBc and anti-HBs (Table 1). Blood with a high anti-HBs titer (≥200 IU/L) is accepted irrespective of the anti-HBc titer and that with a low anti-HBc titer is also accepted irrespective of the anti-HBs titer. Blood with high anti-HBc and low anti-HBs titers (<200 IU/L) is disqualified. All blood had been serologically tested before 2008 using the agglutination method with the initial cutoff for a high anti-HBc titer being a dilution factor of 2^6, which was later revised to 2^5. All agglutination tests were replaced with chemiluminescence enzyme immunoassays (CLEIAs, CL4800 testing system, Fujirebio, Tokyo, Japan) in 2008 and the threshold for anti-HBc positivity is currently a signal-to-cutoff ratio (S/CO) of 12.0. This value was validated as being essentially equivalent to an agglutination titer of 2^5. Blood donations with elevated serum alanine aminotransferase (>60 IU/mL) are also rejected.

TABLE 1. HBV screening algorithm applied at JRC blood centers*

	Anti-HBc titer	
Anti-HBc reactive 4.9% 261,000, *49,000*	Low <2^6(2^5) or S/CO≥1.0 but <12.0	High ≥2^6(2^5) or S/CO ≥12.0
Anti-HBs ≥ 200 IU/L	Accepted 2.04% 108,000, *20,000*	Accepted 1.38% 73,000, *14,000*
Anti-HBs < 200 IU/L	Accepted 1.31% 69,000, *13,000*	Rejected 0.19% 10,000, *2,000*

* HBsAg-negative donations are tested for both anti-HBc and anti-HBs. Dilution factors for anti-HBc titers were applied for agglutination testing. Dilution factors in parentheses were applied between 1997 and 2007. The S/CO ranges are currently used for CLEIA. Ratios (%) of donations for each category are shown (2010 data). Observed number and number per million (italics) of donations are also included.

NAT

Samples that were qualified by the testing algorithm for anti-HBc and anti-HBs described above as well as by HBsAg testing are then screened using NAT. The JRC started NAT in 1999 using a real-time multiplex polymerase chain reaction system with a minipool format that originally comprised 500 samples (Ampli-NAT MPX system, Roche, Indianapolis, IN).[6] The pool size was decreased to 50 in 2000 and to 20 in 2004. The JRC implemented the Roche TaqScreen MPX system for NAT in 2008 with a pool size of 20, but with an approximately threefold increase in sensitivity because of the increased sample volume required for nucleic acid extraction and improvements in reagents. The screening sensitivity of HBV is 650, 260, and 76 copies/mL (50% limit of detection [LOD]; JRC data) for 50- (50p) and 20- (20p) sample pools using AmpliNAT and 20p using TaqScreen, respectively.

A trial screening using individual-donation NAT (ID-NAT) proceeded at the Tokyo Blood Center between December 2010 and May 2011 to verify the distribution of the rate of donations containing HBV DNA relative to anti-HBc titers and the residual TT-HBV risk that could arise from transfusion with blood donations that have low anti-HBc and anti-HBs titers. All available donations with both an anti-HBc titer between 1.0 and 12.0 S/CO and an anti-HBs titer of less than 200 IU/L were screened by ID-NAT using the Roche TaqScreen MPX system with a 50% LOD of 3.8 copies/mL (JRC data). The sensitivity of ID-NAT used in lookback studies (described below) was 13 copies/mL (50% LOD) using AmpliNAT until July 2008 and 3.8 copies/mL (50% LOD) using TaqScreen from August 2008.

Hemovigilance system

The JRC established a hemovigilance system in 1993 and has since collected reports on adverse effects caused by blood transfusion. Through blood screening the JRC obtains information about repeat donors who have

recently acquired infection.[7] Their previous donations are evaluated for transfusion-transmitted infection (TTI) risk by considering donation timing and performing ID-NAT on repository samples (lookback studies). If they are judged as harboring a TTI risk, the JRC notifies the relevant facilities that used the component at risk and requests that physicians investigate whether any patient who received a transfusion of the component has acquired the corresponding infection.

The JRC also obtains information about TTI in transfused patients through voluntary reports by physicians who are involved in blood transfusion at medical facilities.[7] Upon receiving such information, the JRC analyzes repository blood samples obtained from implicated donations using ID-NAT. The TTI risk of cocomponents derived from the implicated and previous donations provided by implicated donors is assessed. The JRC notifies the relevant medical facilities of the findings. Implicated blood components are interdicted if they have not yet been used for transfusion.

The JRC headquarters and central laboratory determine the causal relationship between the implicated donation and posttransfusion infection considering patient clinical course, results of virologic analysis including ID-NAT and sequence analysis, serologic viral markers, and donation timing. Even if all repository samples implicated for TTI are verified as being ID-NAT-negative, implicated donors are followed up for repeat donation thereafter for sero- or NAT conversion, because the possibility that the index donation was provided during the ID-NAT WP persists. All processes for lookback studies are defined in national guidelines[8] that describe in detail the test items and timing of testing for donated blood and at-risk patients in addition to the roles of the relevant physicians, blood centers, and blood authority.

Sequence analysis

The HBV genome sequence identity is assessed between implicated repository blood samples and patient samples by sequencing 1550 bp of the alpha region within the HBV pre-S and S regions using a genetic analyzer (ABI 3130XL, Life Technologies Japan, Tokyo, Japan). When the viral load is too low to sequence, viral nucleic acid is further extracted from larger plasma volumes if the accompanying plasma bag is available. When findings are ambiguous, HBV obtained from donor or patient samples is cloned, amplified, and sequenced.

Estimation of current risk of TT-HBV

Although universal pre- and posttransfusion testing of patient samples for TTI has been recommended, the likelihood that all transfused patients undergo this evaluation is low. Moreover, the JRC hemovigilance system described above is voluntary. Therefore, TTI might be underreported to JRC blood centers. The exact amount of TT-HBV infections that could occur under the current screening system must be defined to assess novel TT-HBV–mitigating strategies. This study therefore reevaluated the current risk of TT-HBV infection based on data obtained under current system.

The projected number of ID-NAT–positive donations derived from OBI donors was calculated using the ID-NAT positivity rate obtained in the ID-NAT trial screening described above and the number of donations with low anti-HBc and anti-HBs titers. The additional WP yield in donations determined by ID-NAT was calculated based on rates of detection of recently infected donors.[9,10] Assuming that the frequency of donation is constant at any time during the presymptomatic phase of acute infection, the yields by tests for an infection marker are in direct proportion to the length of time during which each test gives a yield. The potential ID-NAT yield (screening NAT negative) was calculated herein by multiplying the screening NAT yield by the ratio of the interval between ID-NAT detection and 20p-NAT detection (11.2 days) to that between 20p-NAT detection and HBsAg detection (9.7 days). The interval covered by each NAT strategy (11.2 and 9.7 days) was calculated using the value for the detection limit of each test (3.8, 76, and 1000 copies/mL for ID-NAT, 20p-NAT, and CLEIA detection, respectively) and the doubling time of HBV in human peripheral blood (2.6 days).[9,11] The number of donations that could appear in the ID-NAT–negative WP was similarly calculated separately for each component type taking into account both the interval between 1 copy/bag and ID-NAT detection deduced from the mean plasma volume of each component type and the number of each component issued to hospitals.

We estimated the number of TT-HBV infections with reference to our previous systematic lookback study.[7] The infectivity of ID-NAT–positive and screening NAT-negative components was calculated in that study as being 3% (95% confidence interval [CI], 0%-17.2%, n = 33) and 50% (95% CI, 28.2%-71.8%, n = 22) for OBI- and WP-derived components, respectively. The incidence rate for TT-HBV infections was thus obtained by multiplying the number of estimated at-risk donations deduced using the above method by the infection rates (0.03 or 0.5).

Statistical analysis

Data were statistically analyzed using computer software (SSRI for Windows, Excel Statistics Version 8, Social Survey Research Information Co. Ltd, Tokyo, Japan). Significance was determined using the chi-square test except for associations between total viral load in the components and alanine aminotransferase (ALT) levels in patients that were evaluated using the Mann-Whitney U test.

RESULTS

Reports of possible TT-HBV infections

The JRC blood centers received 789 reports of possible TT-HBV infections between 2001 and 2010 (Fig. 1). The number of such reports obviously increased in 2004 and 2005 because a nationwide systematic retrospective study started in 2004 that also identified patients with TT-HBV infection that would have previously been unrecognized. Causality was investigated in all but two of these possible TT-HBV infections. The possibility of TT-HBV infection was precluded in 97 (12.3%) of the 789 reported patients without testing repository samples based on evaluation of the patient's clinical course and the transfusion setting for each. For all of the remaining patients, repository samples were tested serologically and by ID-NAT to detect the HBV genome. Of the 789 initial reports, 98 (12.4%) were determined to be TT-HBV infections after the introduction of 50p-NAT. The HBV sequence identity was established between donor and recipient in 88 of these cases, and TT-HBV was determined considering other HBV markers and the clinical setting in the remaining 10. An HBV genome was not detected in repository samples for 587 (74.4%) potential TT-HBV infections. Although HBV was detected in four repository samples, HBV sequence identity was not confirmed between donors and recipients. Forty-two (43%) of the established TT-HBV infections were discovered through lookback studies that were started based on risk information provided by JRC blood centers. The remaining 56 (57%) were initially recognized by physicians at medical facilities. The number of established TT-HBV infections ranged from 4 to 13 per year between 2006 and 2010.

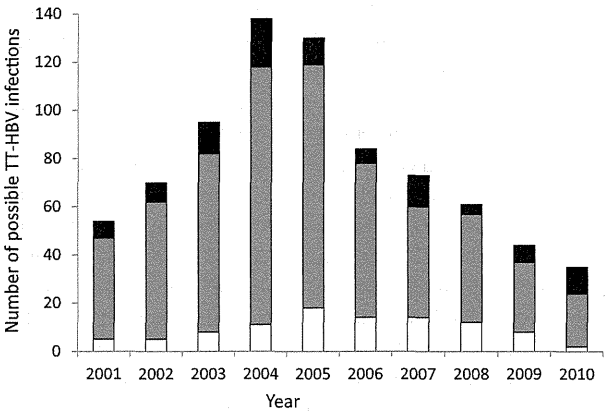

Fig. 1. Annual number of potential TT-HBV infections. (□) Patients in which possibility of TT-HBV was excluded (n = 97, 12.3%) without testing repository samples. (■, ▨) Patients in whom HBV DNA was identified or not, respectively, in the repository samples corresponding to a donation from the donor of the implicated blood components. Four patients are not included as HBV DNA sequence identity was not established.

Infection status of donors implicated in TT-HBV infection

The sensitivity of NAT screening improved through the three phases described above (50p-AmpliNAT, 20p-AmpliNAT, and 20p-TaqScreen). With the increased sensitivity of 20p-TaqScreen, the NAT yield of OBI donations increased from 3.9/million to 15.2/million, whereas the yield of WP donation decreased from 13.2/million to 5.7/million (Table 2). This was caused by the simultaneous introduction of CLEIA in 2008 for serologic screening including HBsAg detection, which effectively shortened the period that could be covered by 20p-NAT.

The established TT-HBV infections that occurred during each period were categorized based on the presence or absence of the HBV genome in the implicated component (that is, ID-NAT positive or negative) and the infection status of the donation (WP related [anti-HBc nonreactive] or OBI related [anti-HBc reactive]). Table 2 also shows the numbers of established TT-HBV infections associated with each group during each period. Figure 2 shows the incidence (per million donations) of estab-

TABLE 2. NAT yield and number of TT-HBV infections relative to three phases of screening NAT*

Screening system	50p-AmpliNAT	20p-AmpliNAT	20p-TaqScreen
Duration of screening period	Feb. 2000–Jul. 2004 (4.5 year)	Aug. 2004–Jul. 2008 (4.0 year)	Aug. 2008–Mar. 2010 (1.67 year)
Sensitivity of screening NAT (copies/mL)†	650	260	76
Sensitivity of ID-NAT used for lookback study (copies/mL)†	13	13	3.8
Number of donations tested	24,702,784	19,513,054	8,746,037
Confirmed WP donations (/million)	473 (19.1)	258 (13.2)	50 (5.7)
Confirmed OBI donations (/million)		76 (3.9)	133 (15.2)
Number of donations causing established HBV transmission			
ID-NAT–negative WP	5	6	1
ID-NAT–positive WP	28	12	3
ID-NAT–negative OBI	4 (1)‡	1 (0)‡	2 (1)‡
ID-NAT–positive OBI	13 (1)‡	12 (1)‡	11 (5)‡

* Yields by ID-NAT trial conducted from December 2010 are not included in the table.
† 50% LOD.
‡ Numbers of donations with anti-HBs of greater than 10 mIU/mL.