厚生労働科学研究費補助金

肝炎等克服緊急対策研究事業

マイクロ RNA を標的とした新規抗 C 型肝炎ウイルス治療戦略の開発

平成２５年度　総括研究報告書

研究代表者　　渡士　幸一

平成２６（２０１４）年　３月

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I. 総括研究報告

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マイクロRNAを標的とした新規抗C型肝炎ウイルス治療戦略の開発

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研究要旨：本研究では、マイクロRNA（miRNA）等宿主因子を標的とした新規抗C型肝炎ウイルス（HCV）治療法の開発を目的とした。そのために（1）miRNA阻害剤TPFによるHCV複製抑制効果、（2）miRNA以外のHCV複製増殖に関わる宿主因子の同定、（3）（2）を標的とした抗HCV剤のリード化合物の同定、を主におこなった。まず培養細胞においてTPFは主にHCV翻訳を阻害することによってHCV複製を阻害することが示唆された。また感染性HCV粒子産生系を用いたスクリーニングにより、TPFとは別系統の抗HCV作用を有するさまざまな低分子化合物が得られた。これらのうちhalopemideは宿主phospholipase Dを阻害することによりHCV放出過程を阻害すること、MA026は感染レセプターの一つであるclaudin-1と結合することによりHCV侵入を阻害すること、SCY-635はHCV感染細胞におけるPKRリン酸化の阻害を介したインターフェロン下流遺伝子群の翻訳上昇によりHCV複製を低下させることなどが明らかとなった。以上の結果は抗HCV剤の創薬標的となる新たな宿主因子およびこれを標的とするリード化合物を提示するものであり、新規抗HCV剤開発に有用な知見を提供するものである。

A.　研究目的

C 型肝炎ウイルス（HCV）感染者は本邦において約 100 万人存在すると推定されている。HCV 治療としては現在まで主にペグインターフェロンとリバビリンの併用療法に加えプロテアーゼ阻害剤が用いられているが、これらの治療法は HCV 遺伝子型によって効果が異なること、インターフェロンベースの治療法では特に副作用が問題になること、プロテアーゼ阻害剤に対しては薬剤耐性ウイルスの出現が否定できないことから、新たな抗 HCV 療法が求められている。特に長期投与に伴う薬剤耐性ウイルス出現を克服するためには、宿主因子を標的とした治療薬開発が重要である。本研究ではマイクロ RNA（miRNA）等の宿主因子を標的とした新規治療法の開発を目指す。miRNA は多様な宿主遺伝子発現を調節する小分子 RNA であり、特に肝臓に高発現する miR-122 は HCV の複製補因子として認知されており、細胞内での HCV ゲノム複製を強く促進する。これまでに研究代表者はさまざまな miRNA 種の遺伝子サイレンシング経路を低分子化合物により阻害できることを初めて示した。そこで本研究ではまず miRNA 機能阻害による HCV 複製制御を目指してこの miRNA 阻害剤 TPF を用いた解析をおこなう。またこれとは別の抗 HCV 作用を有する化合物を探索する。得られた化合物の抗 HCV 分子メカニズムの解析をおこなうことにより、抗 HCV 剤の創薬標的となる新規宿主因子の同定を目指す。

B.　研究方法

（1）miRNA 阻害剤による HCV 複製抑制作

用メカニズムの解析

miR-122 依存的 HCV 複製は、miR-122 を過剰発現した HeLa 細胞に HCV RNA を導入し、コロニー形成アッセイによって評価した。また肝細胞での HCV 複製活性の測定には、HCV レプリコンを有する Huh-7 細胞を用いた。HCV 翻訳は、NS5B 内の酵素活性中心に変異を導入して RNA 複製活性が欠失した変異体レプリコンにより評価した。

(2) 抗 HCV 効果を有する低分子化合物の探索

HCV RNA を導入した Huh7-25 細胞に化合物を 72 時間処理し、培養上清中の HCV 感染力価を測定することにより、それぞれの化合物が感染性 HCV 産生に与える影響を評価した。

(3) halopemide の宿主標的同定および抗 HCV 作用機序の解析

Huh-7.5.1 細胞への siRNA 導入には lipofectamine RNAiMax を用いた。また HCV core タンパク質とさまざまなオルガネラマーカーの細胞内局在は免疫蛍光法により検出した。

(4) MA026 の宿主標的同定および抗 HCV 作用機序の解析

MA026 の宿主標的スクリーニングには、MA026 を固相化したプレートおよびランダムペプチドライブラリーを表出するファージを用いたファージディスプレイ法を用いた。MA026 と claudin-1 との結合は表面プラズモン共鳴により評価した。

(5) SCY-635 のインターフェロン(IFN)経路修飾作用機序の解析

ISG15、MxA、PKR、リン酸化 PKR、eIF2a、リン酸化 eIF2a、HCV core、actin タンパク質はイムノブロット法で検出した。

C. 研究結果

(1) miRNA 阻害剤による HCV 複製抑制作用メカニズムの解析

miRNA 阻害剤 TPF を処理することにより HeLa 細胞における miR-122 依存的 HCV 複製が減少した。また TPF によって Huh-7 細胞内のレプリコン活性も減少した。また HCV 複製抑制メカニズムの一つとして、TPF により用量依存的に HCV 翻訳活性の低下が認められた。

(2) 抗 HCV 効果を有する低分子化合物の探索

HCV RNA を導入した Huh7-25 細胞における感染性 HCV 産生を測定することにより、抗 HCV 作用を有する化合物をスクリーニングした。その結果、感染性 HCV 産生を 1/3 以下に低下させるものとして 17 化合物が得られた。この際、いずれも細胞毒性はほとんど示さなかった。

(3) halopemide の宿主標的同定および抗 HCV 作用機序の解析

halopemide は HCV RNA 複製および粒子形成には影響を与えることなく HCV 放出を有意に低下させた。また halopemide の宿主標的の一つである phospholipase D (PLD)に対する siRNA を導入したところこの細胞からの感染性 HCV 産生が約 1/3 に減少した。PLD 阻害効果をもつ halopemide 以外の化合物を処理することによっても感染性 HCV 産生が減少した。また PLD 阻害によってアルブミン、アポリポタンパク質などの宿主分泌タンパク質の放出にはほとんど影響せず、またこの時、HCV core タンパク質がゴルジ

に蓄積していることが観察された。

(4) MA026 の宿主標的同定および抗 HCV 作用機序の解析

pseudomonas より抽出された新規天然有機化合物 MA026 が HCV 侵入を阻害することを、感染性 HCV 粒子産生系で明らかにした。MA026 の標的分子をファージディスプレイ法により探索したところ、MA026 結合配列として単離されたペプチド配列の一つが、claudin-1 の細胞外ループと相同性を有していた。さらに表面プラズモン共鳴により MA026 とリコンビナント claudin-1 タンパク質の結合が確認された。

(5) SCY-635 のインターフェロン経路修飾作用機序の解析

SCY-635 を処理した HCV 感染細胞では ISG15、MxA などの IFN 下流遺伝子の mRNA 発現は変化することなく、これらのタンパク質量が増加することが明らかとなった。また SCY-635 処理によってリン酸化 PKR およびリン酸化 eIF2a の顕著な低下が認められた。この SCY-635 のリン酸化 PKR 低下作用はシクロフィリンの阻害を介していることが示唆された。

D. 考察

以上のように miRNA 阻害剤 TPF および他の宿主因子を標的とする抗 HCV 化合物 halopemide, MA026, SCY-635 の作用機序を解析した。その結果、halopemide は PLD 阻害によりゴルジからの HCV 輸送を阻害し、HCV 放出を低下させることが示唆された。MA026 は claudin-1 と結合する初めての低分子化合物であることが明らかとなり、これは HCV 侵入阻害効果を有することが示された。また SCY-635 はこれまで HCV RNA を直接阻害することが報告されていたが、それに加えて今回の結果より IFN 下流遺伝子の発現上昇を介する HCV 複製抑制作用も有することが示唆された。

E. 結論

以上のように本研究では、miRNA およびそれ以外の宿主因子を標的とすることにより抗 HCV 作用を示す低分子化合物を同定した。これらの化合物を至適化することによりさらに良好な抗 HCV プロファイルを持つ化合物が同定できると期待される。

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30) 青柳東代、相崎英樹、松本喜弘、松田麻未、SuSu Hmwe、渡邉則幸、渡士幸一、鈴木亮介、市野瀬志津子、松浦知和、鈴木哲朗、和気健二郎、宮村達男、脇田隆字。PhospholipaseA2 および Autophagy による C 型肝炎ウイルス(HCV)分泌過程の制御-グリチルリチンによる抗 HCV 作用-。**第 36 回日本分子生物学会年会**、神戸、2013

G.　知的所得権の所得状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

II. 研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻・号	ページ	出版年
Watashi K, Sluder A, Daito T, Matsunaga S, Ryo A, Nagamori S, Iwamoto M, Nakajima S, Tsukuda S, Borroto-Esoda K, Sugiyama M, Tanaka Y, Kanai Y, Kusuhara H, Mizokami M, Wakita T	Cyclosporin A and its analogs inhibit hepatitis B virus entry into cultured hepatocytes through targeting a membrane transporter NTCP.	Hepatology	in press		
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Shimura S, Ishima M, Nakajima S, Fujii T, Himeno N, Ikeda K, Izaguirre-Carbonell J, Murata H, Takeuchi T, Kamisuki S, Suzuki T, Kuramochi K, Watashi K, Kobayashi S, Sugawara F	Total Synthesis and Anti-Hepatitis C Virus Activity of MA026	J Am Chem Soc	135(50)	18949-18956	2013
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Matsumoto Y, Matsuura T, Aoyagi H, Matsuda M, Hmwe SS, Date T, Watanabe N, Watashi K, Suzuki R, Ichinose S, Wake K, Suzuki T, Miyamura T, Wakita T, Aizaki H	Antiviral activity of glycyrrhizin against hepatitis C virus in vitro	PLoS One	8(7)	e68992	2013
渡士幸一	薬学的視点からのウイルス学研究—肝炎ウイルス複製阻害化合物の同定とその作用機序	薬学雑誌	133(11)	1169-1175	2013

III. 研究成果の刊行物・別刷

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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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).

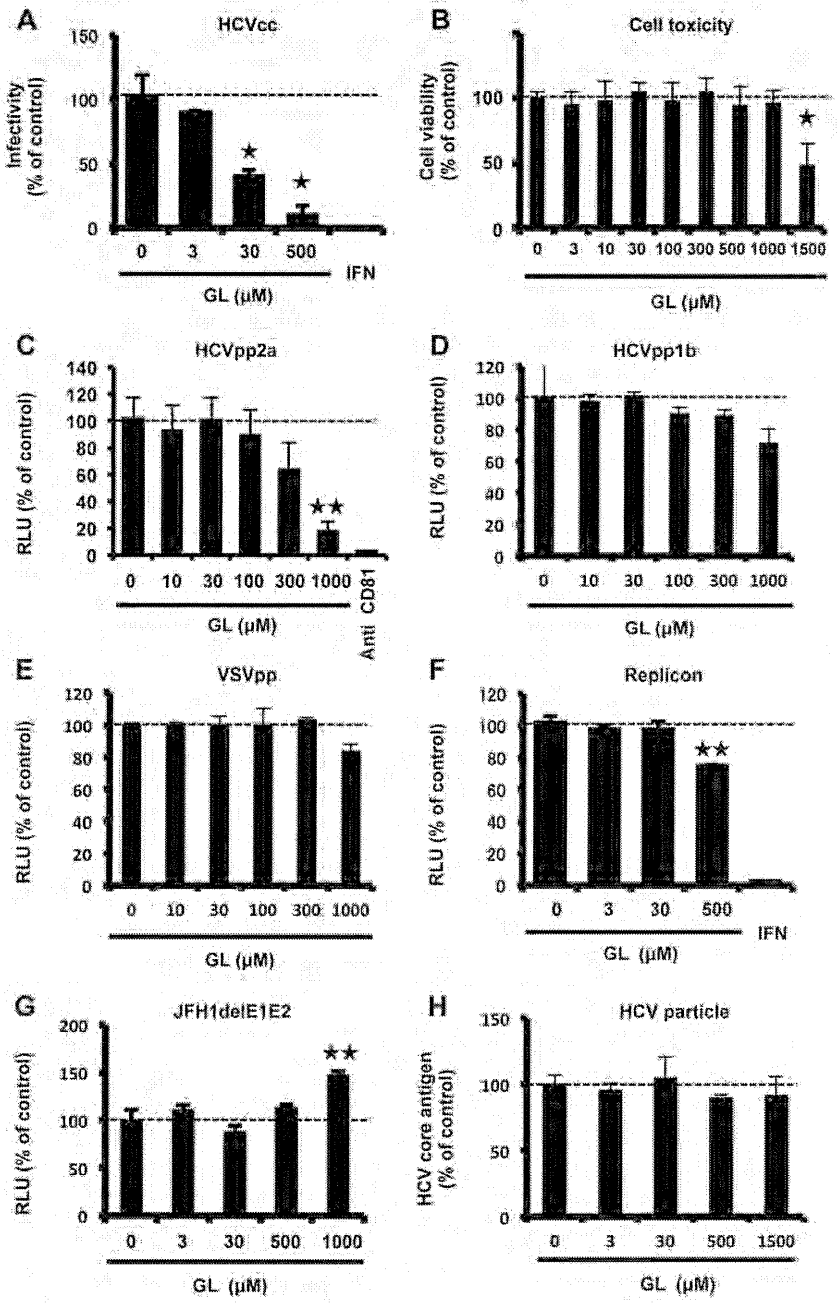

Figure 1. Anti-HCV effects of GL. (A) HCVcc-infected cells were treated with various concentrations of GL for 72 hours. Naïve Huh7 cells were inoculated with supernatant and cultured for 72 hours. Infectivity was determined by immunostaining. (B) Cell viability was assessed using Cell Titer-Glo Luminescent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

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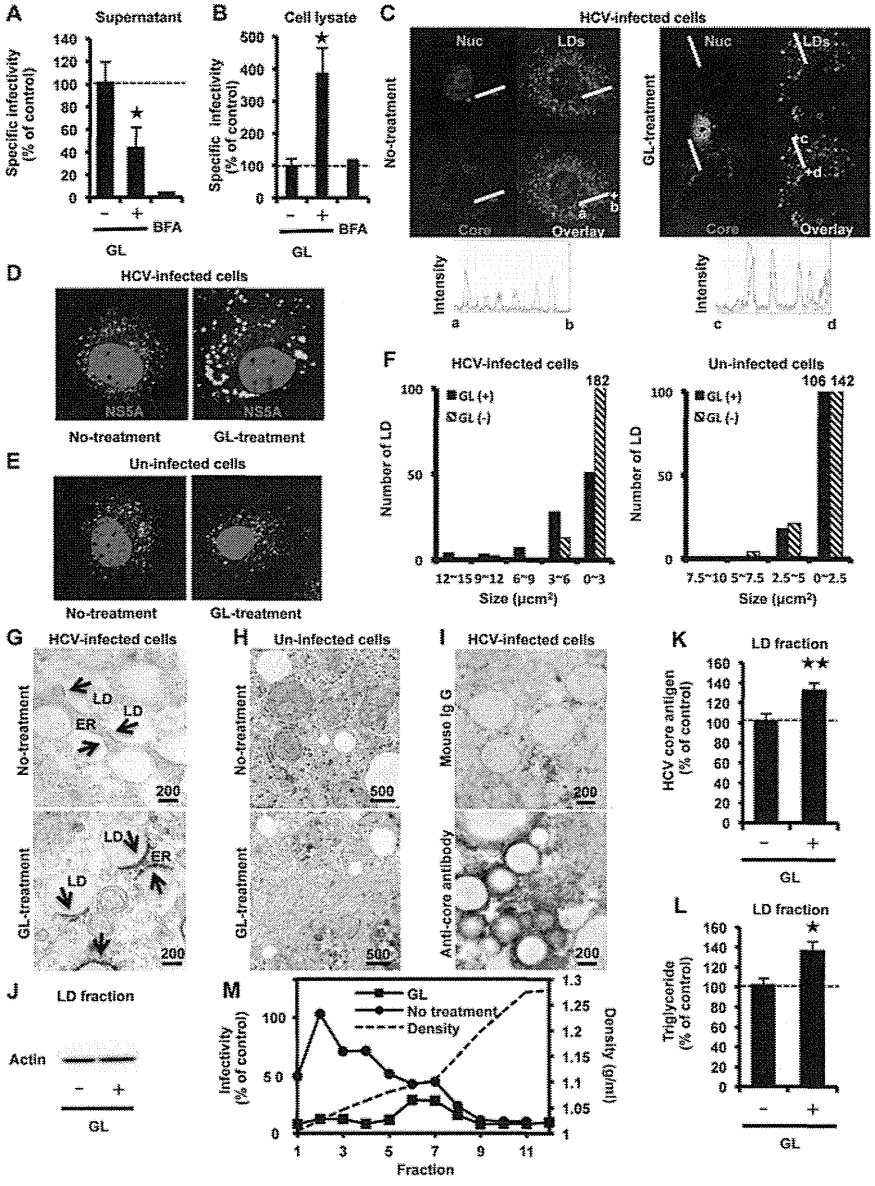

Figure 2. Effects of GL in release of infectious HCV particles. HCVcc-infected cells were treated with GL at a concentration of 500 μM for 72 hours. Untreated cells were used as controls. Extra- (A) and intracellular specific infectivity (B) were determined. Subcellular co-localization of HCV core (C)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