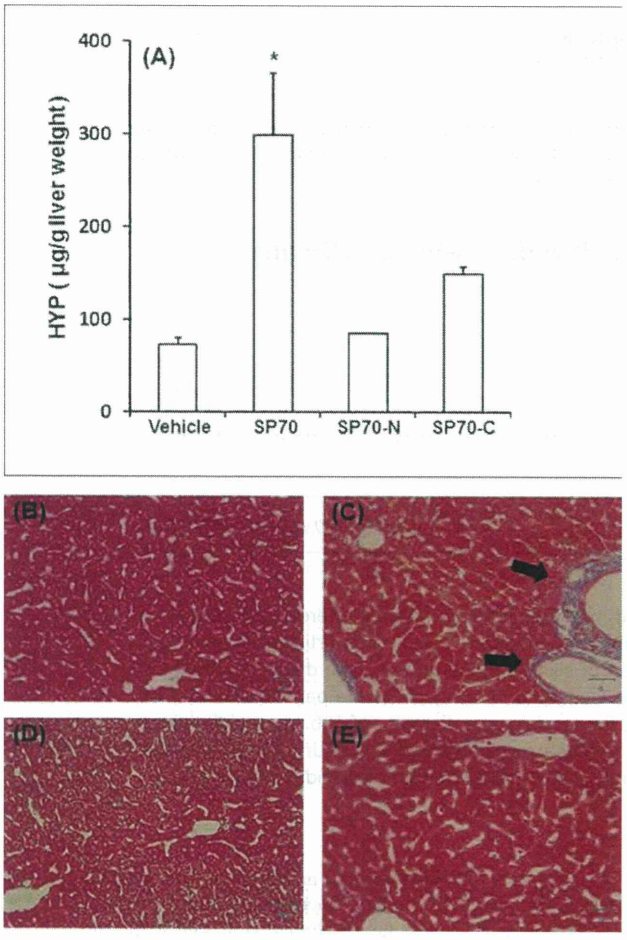

Fig. 4: Effect of SP70-N and SP70-C on chronic liver injury. SP70 was injected into mice every 3 days for 4 weeks at 30 mg/kg. SP70-C and SP70-N was injected into mice every 3 days for 4 weeks at 60 mg/kg. Three days after the last injection, the mice were sacrificed. Hydroxyproline levels (A) in the liver were measured. The liver was excised from mice treated with vehicle (B), SP70 (C), SP70-N (D) or SP70-C (E) and fixed with 4% paraformaldehyde. Tissue sections were stained with Azan and observed under a microscope. The arrows indicate areas of hepatic fibrosis. Data are means ± SEM (n = 4). *$p < 0.05$ as compared to the vehicle-treated group.

3.6. Statistical analysis

The data were analyzed for statistical significance using Dunnett's test. P values less than 0.05 were considered statistically significant.

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Hepatotoxicity of silica nanoparticles with a diameter of 100 nm

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Nanomaterials have potential toxicity that is not found in micromaterials, and it is therefore essential to understand their biological activity and potential toxicity. We focused on silica nanoparticles, since it was previously reported that the intravenous administration of silica nanoparticles with a diameter of 70 nm (SP70) causes hepatic injury. In the present study, we focused on the effects of the particle diameter of silica. We found that silica nanoparticles caused acute liver toxicity at a diameter of 100 nm, and that liver sinusoidal endothelial cells are directly involved in silica nanoparticle-induced liver injury. These findings suggest that the diameter of nanoparticles has great influence on silica nanoparticle-induced liver injury.

1. Introduction

Nanoparticles are generally defined as having diameters of 100 nm or less (Stone et al. 2007; Tsuda et al. 2009). Nanomaterials are used frequently in microelectronics, cosmetics, and semiconductor materials, and research for the development of nanomaterial-based drug delivery systems is promising. As such, there has been a tendency to decrease the grain diameter from the micro to the nano scale in a variety of industrial fields. However, nanosized particles have a potential for toxicity that does not exist for microparticles. It is therefore imperative to understand the biological activity and potential toxicity of nanosized particles (Bystrzejewska-Piotrowska et al. 2009; Warheit et al. 2008).

Silica is the oxide of silicon, and has a large, porous outer structure with a variety of useful characteristics (Kobler and Bein 2008). Silica is commonly used as an industrial material due to its durability and general applicability (Mc Nally et al. 2006). It has been reported that non-crystalloid silica particles at the micrometer scale are completely safe for human exposure (Martin 2007). However, silica nanoparticles are increasingly used as materials in the electronics industry because they serve as a unique substrate (Chung et al. 2009). It is thought that the production of silica nanoparticles will be expanded in the future; however, little is known about their toxicity.

Since nanoparticles are a unique substrate, it follows that their effects on the living body are also unique, and therefore potentially problematic. It is thought that the size and surface area of the particles are important factors in their influence on the living body (Merget et al. 2002). Therefore, decreasing the size of the particles increases their potential influence on living things.

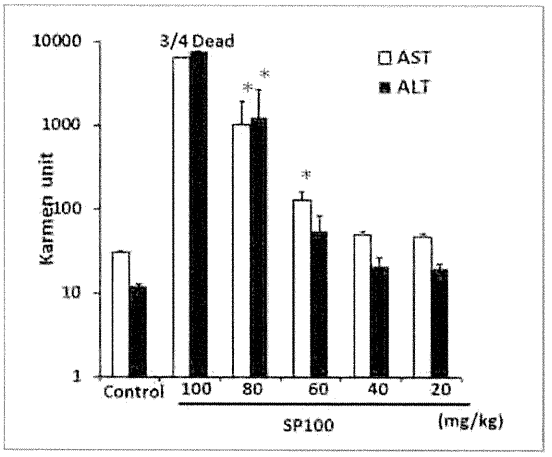
Abbreviations: SP70 70 nm silica particles; SP100 100 nm silica particles; SP300 300 nm silica particles; CDDP cisplatin; PQ paraquat; ALT alanine aminotransferase; AST aspartate aminotransferase; BUN blood urea nitrogen.

We previously reported that silica nanoparticles with a diameter of 70 nm cause liver injury, whereas silica nanoparticles of a diameter of 300 nm do not (Nishimori et al. 2009a, c). This implies that the hepatic toxicity of silica nanoparticles has a specific threshold determined by the grain diameter. In this study, to examine the effects of silica nanoparticles in liver injury, silica nanoparticles of a diameter of 100 nm were used. In addition, we examined the mechanism by which silica nanoparticles caused liver injury, and investigated the synergistic effects on hepatic toxicity of SP100 with pharmaceutical agents.

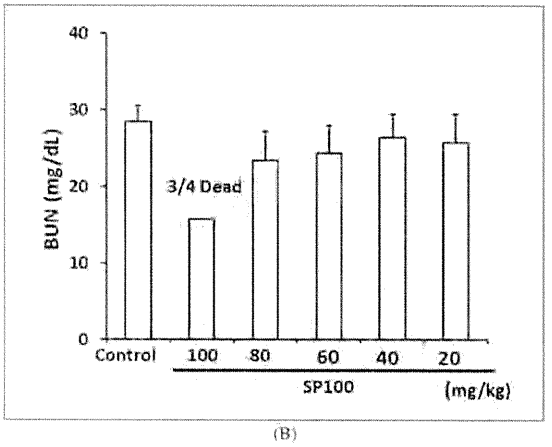
2. Investigations, results and discussion

2.1. Acute toxicity of 100-nm-diameter silica nanoparticles

We initially examined liver injury caused by silica nanoparticles with diameters of 100 nm (SP100), and investigated the acute liver toxicity of silica nanoparticles with diameters of 100 nm at a maximal dose of 100 mg/kg (Fig. 1). Acute liver toxicity of SP100 rose dose-dependently (Fig. 1A). Intravenous injection of SP100 at 100 mg/kg was often lethal in mice. There was acute liver toxicity of SP100 at 60 mg/kg. Moreover, elevation of blood urea nitrogen, a biochemical marker of kidney injury, was not observed (Fig. 1B). These data demonstrated that silica nanoparticles caused acute liver toxicity at a diameter of 100 nm. Next, SP100 at doses of 60 mg/kg, SP70 at 40 mg/kg, and SP300 at 100 mg/kg were administered to mice. As shown in Fig. 2A, at 24 h after silica nanoparticle treatment, the levels of serum ALT after SP70 treatment greatly increased. There was no liver injury caused by SP300, though there was liver injury caused by SP100. Figure 2 (B-E) shows hematoxylin-eosin staining of the liver in silica nanoparticle-injected mice. Liver injury was confirmed in SP70 and SP100 treated mice, but not in SP300 treated mice. These results show that silica nanoparticles with a diameter of 100 nm and below cause liver toxicity.



(A)



(B)

Fig. 1: Dose-dependency of SP100 on liver and kidney injury SP100 was intravenously administered at the indicated doses. Serum ALT, AST, (A) and BUN (B) at 24 h were measured using a commercially available kit, as described in the Experimental section. Data are means ± SEM (n = 4). *Significantly different compared with the vehicle-treated group ($p < 0.05$).

Nanosized particles are defined as having a grain diameter of 100 nm or less. Our results showed that there was hepatic toxicity caused by SP100 and SP70, but not by SP300. Vamanu et al. (2008) reported that TiO_2 nanoparticles of a diameter of 100 nm or less are cytotoxic. Additionally, Shavandi et al. (2010) reported that silver nanoparticles of a diameter of 100 nm or less are cytotoxic. These results show that the grain diameter is critical for determining the level of toxicity to the body and to cells, and in particular, they imply that toxicity is induced by particles of 100 nm or less.

The acute liver toxicity of SP100 increased in a dose-dependent manner (Figs. 1, 2). Moreover, we assessed the presence of liver fibrosis in SP100 treatments. SP100 significantly increased the hepatic hydroxyproline content (data not shown). We previously found that SP70 causes acute liver toxicity and hepatic fibrosis (Nishimori et al. 2009a, c). In the present study, we found that SP70 had a larger effect on liver damage compared to SP100 (Fig. 2). These results show that the level of hepatic toxicity changes according to the grain diameter of the silica particles. In future studies, it will be necessary to examine the level of hepatic toxicity caused by a wide variety of grain diameters of silica nanoparticles.

2.2. Mechanism of acute liver toxicity by silica nanoparticles

We investigated the liver toxicity caused by silica nanoparticles when combined with agents that inhibit the activities of liver sinusoidal endothelial cells or liver Kupffer cells. Cyclophosphamide (CPA) is an alkylating agent that induces apoptosis in liver sinusoidal endothelial cells (DeLeve 1996; Malhi et al. 2002). $GdCl_3$ inhibits phagocytosis by Kupffer cells and transiently eliminates them, and $GdCl_3$ has been widely used to investigate the roles of Kupffer cells in the liver (Hardonk et al. 1992; van Til et al. 2005). We thus investigated the effects of CPA and $GdCl_3$ on silica nanoparticle-induced liver injury. As shown in Fig. 3A, pre-injection of CPA did not affect the ALT levels in mice, whereas in silica nanoparticle-injected mice, CPA dramatically decreased ALT levels to near control values. Moreover, as shown in Fig. 3B, pre-injection of $GdCl_3$ prior to injection of silica nanoparticles elevated serum ALT levels 2-fold or more in the silica nanoparticle-injected group. Next, we investigated the cytotoxicity of SP70, SP100, and SP300 in primary cultured hepatocytes isolated from mice. SP70 and SP100 at 100 μg/ml were toxic to primary hepatocytes to almost the same degree, indicating that the differences in liver injury among these nanoparticles were not due to differences in the sensitivity of hepatocytes to the nanoparticles (Fig. 3C). These data indicated that liver sinusoidal endothelial cells are directly involved in silica nanoparticle-induced liver injury, and that phagocytosis of silica nanoparticles by Kupffer cells attenuates liver injury.

Liver sinusoidal endothelial cells form the basic tubular vessels for transvascular exchange between the blood and the surrounding tissue (McCuskey 2008). Kupffer cells are a component of the sinusoidal wall and play a significant role in the removal of particles and cells as well as toxic substances (Wisse et al. 1996). We observed that the ALT values decreased to near control levels by the administration of silica nanoparticles and CPA (Fig. 3A). This indicates that liver sinusoidal endothelial cells greatly influence the hepatic toxicity by silica nanoparticles. There are several receptors on the cell surface of liver sinusoidal endothelial cells, and it is known that they endocytose proteins and large particles (Smedsrod et al. 1997; Steffan et al. 1986). It is likely that silica nanoparticles are englobed by liver sinusoidal endothelial cells, after which they accumulate and are then discharged into hepatocytes. A detailed analysis of the relationship between liver sinusoidal endothelial cells and silica nanoparticles is necessary for future studies.

2.3. Influence of 100-nm-diameter silica nanoparticles with cisplatin or paraquat-induced toxicity

Previously, we reported synergistic toxicity of SP70 with CDDP and PQ (Nishimori et al. 2009b). CDDP is widely used as an anti-tumor agent, and PQ is one of the most widely used and highly toxic herbicides (Ozols and Young 1991; Vandenbogaerde et al. 1984; Witjes 1997). In the present study, we thus investigated the synergistic effects of hepatic toxicity of SP100 with CDDP or PQ. To avoid direct interactions between the chemicals and SP100 before administration and absorption, we injected the chemicals and SP100 intraperitoneally and intravenously, respectively. We observed that the serum levels of ALT were elevated by CDDP (Fig. 4A). We next investigated the interaction between PQ and SP100. Co-administration of PQ and SP100 elevated levels of serum ALT, and SP100 showed synergistic elevation of serum ALT levels from 138.2 to 492.0 KU (Fig. 4B).

We investigated the combined effects of the chemicals on nanoparticle-induced toxicity, and found that CDDP and PQ had synergistic toxic effects with SP100. Verma et al. reported

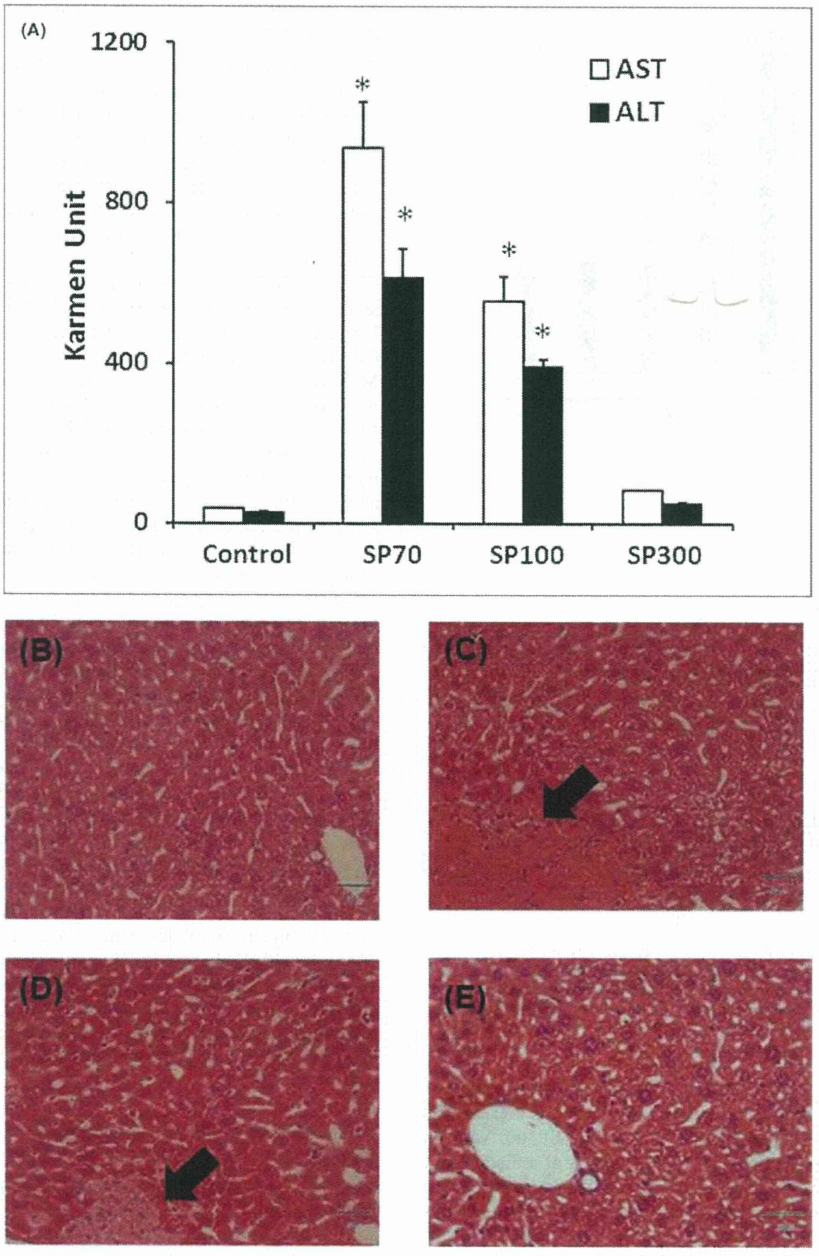


Fig. 2: Comparison of acute liver toxicity of silica nanoparticles Serum ALT and AST (A) at 24 h were measured using a commercially available kit as described in the Experimental section. Histological analysis of silica nanoparticle-treated mice were conduced on tissues fixed with 4% paraformaldehyde 24 h after administration of vehicle (B), SP70 (C), SP100 (D), and SP300 (E). Tissue sections were stained with hematoxylin and eosin and observed under a microscope. Data are representative of at least four mice. Blood was recovered at 24 h after injection. The arrow shows hepatic injury. Data are means ± SEM (n = 4). *Significantly difference compared with the vehicle-treated group ($p < 0.05$).

that the blood circulation levels of CDDP are made to rise by nanomaterial conjugates of CDDP (Verma and Saching 2008). In addition, Moreno et al. (2009) reported that PLGA nanoparticles improve the effects of CDDP. Therefore, nanoparticles could possibly increase both the beneficial effects and toxicity of chemicals and drugs. Further evaluation of such interactions between nanomaterials and pharmaceutical agents for future pharmaceutical applications are necessary.

This report is the first to show that silica nanoparticles with a diameter of 100 nm or less have hepatic toxicity, and that liver injury is mediated by liver sinusoidal endothelial cells. Further studies based on these data should provide useful information regarding the safety of nanomaterials.

3. Experimental

3.1. Materials

Silica particles with a diameter of 70, 100, or 300 nm (SP70, SP100, SP300) were obtained from Micromod Partikeltechnologie GmbH (Rostock,

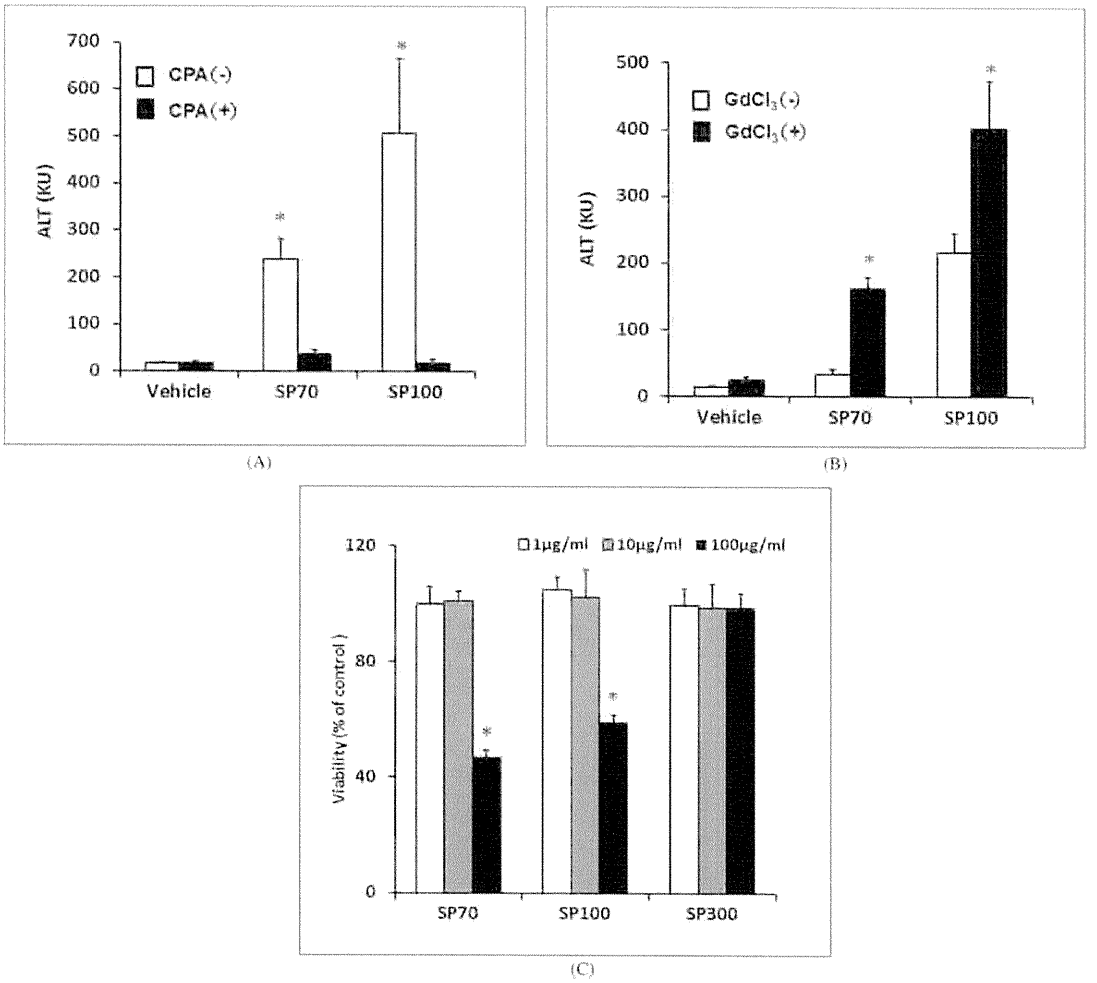


Fig. 3: Analysis of silica nanoparticle-induced liver injury Effect of CPA (A). Vehicle or CPA (300 mg/kg) was intraperitoneally injected into mice 24 h prior to treatment with silica particles (SP70, 40 mg/kg; SP100, 60 mg/kg). At 24 h after administration of particles, blood was recovered, and the resultant serum was used for ALT assay. Data are means ± SEM (n = 4). *Significant difference between vehicle and silica particle-treated groups ($p < 0.05$). Effect of $GdCl_3$ (B). Vehicle or $GdCl_3$ (10 mg/kg) was intravenously injected into mice at 30 h or 6 h prior to treatment with silica particles. 24 h after particle administration, blood was recovered, and the resultant serum was used for ALT assay. Data are means ± SEM (n = 4). *Significant difference between $GdCl_3$ + and $GdCl_3$ - groups ($p < 0.05$). (C) Comparison of cytotoxicity of silica nanoparticles in primary hepatocytes. Hepatocytes were prepared from mouse livers by the collagenase perfusion method, as described in the Experimental section. Cells were seeded onto 96-well plates at 5×10^3 cells/well, and were treated with silica particles at the indicated concentrations. After 48 h of treatment, viability was measured by WST-8 assay as described in the Materials and methods. Data were normalized against vehicle-treated cells (100% control). Data are means ± SD (n = 4).

Germany). The size distribution of the particles was analyzed using a Zetasizer (Sysmex Co., Kobe, Japan), and the mean diameters were 57.5 ± 20.3, 137 ± 32.1, and 296 ± 36.3 nm, respectively. The particles were spherical and nonporous and were stored at 25 or 50 mg/mL in an aqueous suspension. The suspensions were thoroughly dispersed with sonication before use and then diluted in ultrapure water. Paraquat (PQ) and cisplatin (CDDP) were dissolved in saline and stored at −20 °C before use. All reagents used were of research grade.

3.2. *Animals*

Eight-week-old BALB/c male mice were purchased from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan) and were maintained in a controlled environment (23 ± 1.5 °C; 12-h light/dark cycle) with access to standard rodent chow and water *ad libitum*. The mice were left to adapt to the new environment for 1 week before commencing with the experiment. Mice that received a single treatment of silica nanoparticles were anesthetized and sacrificed 24 h after intravenous injection. The experimental protocols conformed to the ethical guidelines of the Graduate School of Pharmaceutical Sciences, Osaka University.

3.3. *Biochemical analysis*

Serum alanine aminotransferase (ALT) and blood urea nitrogen (BUN) were measured with commercially available kits according to the manufacturer's protocols (Wako Pure Chemical Industries, Osaka, Japan).

3.4. *Histological analysis*

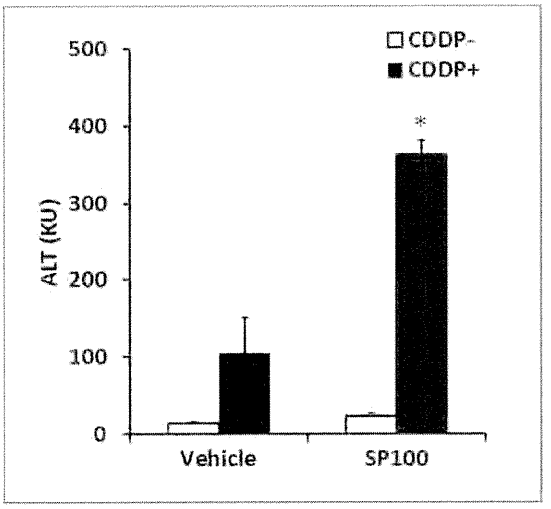
The liver was removed and fixed with 4% paraformaldehyde. After sectioning, thin tissue sections were stained with hematoxylin and eosin for histological observation.

3.5. *Cyclophosphamide assay*

Disruption of liver sinusoidal endothelial cells was carried out by intraperitoneal injection of 300 mg/kg body weight cyclophosphamide (CPA) at 24 h prior to administration of nanoparticles. Blood was recovered at 24 h after injection of nanoparticles for the ALT assay.

3.6. *Gadolinium chloride assay*

For Kupffer cell blockage of phagocytosis and partial depletion in the liver, mice were injected intravenously with gadolinium chloride ($GdCl_3$) at



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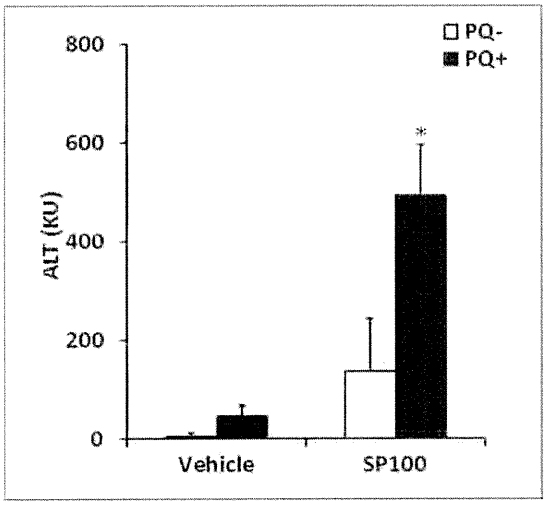

(B)

Fig. 4: Effect of SP100 on cisplatin- and paraquat-induced toxicity (A) Mice were injected with cisplatin (CDDP) at 0 or 50 μmol/kg and SP100 at 10 mg/kg, intraperitoneally and intravenously, respectively. At 24 h post-injection, the serum was recovered. ALT levels were assayed as described in the Experimental section. Data are means ± SEM (n = 4). *Significant difference between vehicle and CDDP-treated group ($p < 0.05$). (B) Mice were injected with paraquat (PQ) at 0 or 50 mg/kg and SP100 at 60 mg/kg, intraperitoneally and intravenously, respectively. At 24 h post-injection, the serum was recovered. ALT levels were assayed as described in the experimental section. Data are means ± SEM (n = 4). *Significant difference between vehicle and PQ-treated group ($p < 0.05$).

10 mg/kg body weight at 30 h and 6 h prior to intravenous administration of nanoparticles. Blood was then recovered 24 h after injection of nanoparticles for the ALT assay.

3.7. Cytotoxicity in primary hepatocytes

Hepatocytes were isolated from BALB/c mice by Seglen's method using perfusion of collagenase (Seglen 1976). Viability of the isolated hepatocytes was assayed by trypan blue staining, and cells (over 90% viability) were seeded onto 96-well plates at 5×10^3 cells/well, and the cells were treated with silica particles for 48 h. Cell viability was then assayed with the Cell Counting Reagent SF, according to the manufacturer's protocol (Nacalai Tesque, Kyoto Japan).

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Adenovirus vector-mediated assay system for hepatitis C virus replication

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ABSTRACT

The efficient delivery of the hepatitis C virus (HCV) RNA subgenomic replicon into cells is useful for basic and pharmaceutical studies. The adenovirus (Ad) vector is a convenient and efficient tool for the transduction of foreign genes into cells *in vitro* and *in vivo*. However, an Ad vector expressing the HCV replicon has never been developed. In the present study, we developed Ad vector containing an RNA polymerase (pol) I-dependent expression cassette and a tetracycline-controllable RNA pol I-dependent expression system. We prepared a hybrid promoter from the tetracycline-responsive element and the RNA pol I promoter. Ad vector particles coding the hybrid promoter-driven HCV replicon could be amplified, and interferon, an inhibitor of HCV replication, reduced HCV replication in cells transduced with the Ad vector coding HCV replicon. This is the first report of the development of an Ad vector-mediated HCV replicon system.

INTRODUCTION

Hepatitis C virus (HCV) is a member of *Flaviviridae* that contains a 9.6-kb positive-sense RNA genome. A total of 170-million people worldwide are infected with HCV, leading to chronic hepatic inflammation, hepatic fibrosis, hepatic cirrhosis and hepatocellular carcinoma (1). Chronic infection with HCV is a major cause of hepatocellular carcinoma (1). Interferon (IFN) therapy is the gold standard method for HCV patients, but it is effective in only 50% of patients and its use has been limited because of severe side effects (2–4). Additional pharmaceutical therapies are needed to overcome HCV. However, the tropism of HCV is limited to chimpanzees and humans, and the mechanism of HCV infection and replication is not fully understood. The HCV genome encodes a polyprotein precursor of about 3000 amino acids that is cleaved into at least 10 proteins: core, envelope 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B (5). An HCV subgenome replicon (called HCV replicon in the present study) consisting of a reporter gene and HCV NS genes has allowed various studies of HCV replication and the development of anti-HCV agents (6–8). The delivery of the HCV genome or HCV replicon is a powerful tool for basic and pharmaceutical research, and the transduction of *in vitro* translated HCV RNA genome is often performed by electroporation. However, a convenient and efficient method to transfer the 9.6-kb HCV RNA genome or the 8–9-kb HCV replicon has never been fully developed.

Transcribed RNAs are classified into rRNAs, mRNAs and short RNAs (tRNAs) in mammalian cells. RNA polymerases differ among the transcribed RNA species: RNA polymerase (pol) I for rRNAs, RNA pol II for mRNA and RNA pol III for short RNAs. RNA pol I transcribes RNA without a 5′-cap structure or a 3′-poly-A tail, and a plasmid vector encoding RNA pol I promoter and terminator has been applied to the development of RNA virus-expression system. For instance, influenza viruses, arenavirus and uukuniemi viruses are generated using RNA pol I-driven expression plasmid vectors coding each segment of negative-sense RNA (9–12). Recombinant adenovirus (Ad) vectors have been widely used to deliver foreign genes to a variety of cell types and tissues *in vitro* and *in vivo* in basic research and clinical therapy. Ad vector can be easily prepared, grown to a high titer, and used to efficiently transfer genes into dividing and non-dividing cells. Furthermore, several types of Ad vectors have been developed to expand their tropism and to increase the size of encoded genes (13,14). Ad vector encoding RNA pol I-driven expression of influenza virus

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

RNA has been developed for the generation of vaccine seed strains and for basic influenza virus studies (15). These findings indicate that the RNA pol I Ad vector system can be a promising tool for basic and pharmaceutical studies on HCV. However, the development of an RNA pol I-driven vector system expressing the HCV RNA genome has never been reported.

In the present study, we developed an RNA pol I-driven vector system to monitor HCV replication using an HCV replicon in which structural genes were replaced by the luciferase gene. We prepared an Ad vector containing a tetracycline (tet)-regulated RNA pol I-expression cassette consisting of an RNA pol I-driven responsive vector and a *trans*-activator vector, and we successfully developed an Ad vector-mediated HCV replication system.

MATERIALS AND METHODS

Cell culture

Huh7.5.1 1bFeo [genotype 1b HCV replicon cell line, (8)] were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and G418 (Nacalai Tesque, Kyoto, Japan) at 500 μg/ml. Huh7 and 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS. The cells were maintained in a 5% CO_2 atmosphere at 37°C.

Preparation of RNA pol I-driven plasmid vectors

An RNA pol I expression-cassette was subcloned as follows: pHH21 (kindly provided by Dr Kawaoka, Tokyo, Japan) containing RNA pol I expression cassette was digested with *Afl*III, blunted by the Klenow fragment of DNA polymerase, ligated with *Eco*RI linker and digested with *Eco*RI/*Nhe*I, resulting in a fragment of the RNA pol I expression-cassette. The RNA pol I cassette was inserted into the *Eco*RI-*Xba*I site of pHM5 (16), generating pPol I. A fusion gene of enhanced green fluorescent protein and firefly luciferase (EGFPLuc, Clontech, Mountain View, CA, USA) was inserted into pPol I, resulting in pP_IWT-EL.

The subgenomic HCV sequence and the replication-incompetent subgenomic HCV sequence deleting GDD motif (MLVNGDDLVV) in NS5B were amplified by polymerase chain reaction (PCR) using pRepFeo as a template (8). The PCR fragments were inserted into pPol I, generating pPol I-1bFeo and pPol I-1bFeoΔGDD. The Feo fragment in pPol I-1bFeo or pPol I-1bFeoΔGDD was replaced with firefly luciferase, generating pPol I-HCV or pPol I-ΔGDD coding firefly luciferase reporter, HCV NS3, NS4A, NS5A and NS5B or mutated NS5B, respectively. A plasmid expressing β-galactosidase, pCMVβ, was purchased from Marker Gene Inc. (Eugene, OR, USA).

Preparation of tet-controllable RNA pol I-driven plasmid vectors

To develop the tet-controllable RNA pol I promoter expression system, the minimal cytomegalovirus promoter was replaced by fragments of RNA pol I promoters (from −235 to −1, from −311 to −1 or from −412 to −1) in pHM5-TREL2 (17), generating pP_I235, pP_I311 or pP_I412. These RNA pol I plasmid vectors were used for optimization of the tetracycline responsive element (TRE)/RNA pol I chimeric promoter. pHM5-tTA, pHM5-rtTA and pHM5-TREL2 were used in tet-regulated experiments (17).

Preparation of Ad vector expressing HCV replicon

The HCV replicon fragments cloned from pPol I-HCV or pPol I-ΔGDD were inserted into pP_I235, and then the firefly luciferase was replaced by the renilla luciferase to form pP_I235-HCV or pP_I235-ΔGDD. Ad vectors were constructed by an improved *in vitro* ligation method (18). Briefly, pP_I235-EL, pP_I235-HCV and pP_I235-ΔGDD were digested with I-*Ceu*I and PI-*Sce*I, and then ligated with I-*Ceu*I/PI-*Sce*I-digested pAdHM4 and pAdHM36, respectively. The resulting plasmids were digested with *Pac*I and transfected into 293 cells with SuperFect (Qiagen, Valencia, CA, USA). AdP_I235-EL, AdP_I235-HCV and AdP_I235-ΔGDD were purified by $CsCl_2$ gradient centrifugation and dialyzed with a solution containing 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$ and 10% glycerol. The multiplicity of infection (MOI) of Ad vectors was measured using an Adeno-X rapid titer kit (Clontech). Ad-tTA vectors were prepared as previously described (17).

Expression of plasmid-based HCV replicon

Huh7 cells were transfected with 0.8 μg of pPol I-HCV. After 24 h of incubation, the cells were lysed in LCβ (Toyo Ink, Tokyo, Japan). The cell lysates were frozen-thawed and centrifuged at 32 000 *g* for 5 min. The luciferase activity in the resulting supernatant was measured using a commercially available kit (PicaGene; Toyo Ink).

Inhibition assays of HCV replication in plasmid- or Ad-based RNA pol I HCV system

Huh7 cells were transfected with 0.8 μg of pPol I-HCV and 0.2 μg of pCMVβ or infected with AdP_I235-HCV (10 MOI) and Ad-tTA (50 MOI). After 2.5 or 1.5 h of transfection, the cells were treated with recombinant human interferon-α8 (IFN-α8) at the indicated concentration. After an additional 72 h of incubation, the cells were lysed in LCβ. Luciferase activity and β-galactosidase activity in the lysates was measured with PicaGene and a Luminescent β-gal Kit (Takara Bio Inc., Shiga, Japan), respectively. The cell viability was measured with a WST-8 kit according to the manufacturer's instruction (Nacalai Tesque).

Evaluation of tetracycline-controllable promoters in plasmid vector

Huh7 cells were co-transfected with 0.1 μg of reporter plasmid (pP_I235-EL, pP_I311-EL, pP_I412-EL or pP_IWT-EL), 0.8 μg of tet-responsive *trans*-activator plasmid (pHM5-rtTA in the tet-on system or pHM5-tTA in the tet-off system) and 0.1 μg of pCMVβ. After 2.5 h, the cells were treated with doxycycline (Dox) at the indicated

concentration for 48 h. Then, luciferase and β-galactosidase activities in the lysates were measured.

Expression of Ad vector containing tetracycline-controllable promoter system

Huh7 cells were transfected with a reporter Ad vector (AdP_I235-EL or AdP_I235-HCV at MOI of 5 or 10) and a *trans*-activator vector (Ad-tTA at MOI of 10 and 50). After an additional 48 h of incubation, luciferase activity in the cell lysates was measured.

Western blotting

Huh7 cells were co-infected with AdP_I235-HCV at 10 MOI and Ad-tTA at 50 MOI. The cells were lysed in RIPA buffer [50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA] containing a cocktail of protease inhibitors (Sigma, St Louis, MO, USA). The cell lysates (30 μg of protein) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by western blotting onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking in 5% skim milk, the filter was incubated with mouse anti-NS5A (Meridian Life Science, Sacao, ME, USA) or anti-β-actin Ab (Sigma). Then, the peroxidase-labeled secondary antibodies were added. The immunoreactive bands were visualized by chemiluminescence reagents (GE Healthcare, Buckinghamshire, UK).

Evaluation of NS5B-dependent replication

Huh7 cells were transfected with AdP_I235-HCV or AdP_I235-ΔGDD at 3 MOI and Ad-tTA at 15 MOI. After 24 h, the cells were treated with 10 μg/ml of Dox for 48 h. Then, luciferase activities in the lysates were measured.

Detection of a fragment of the HCV negative strand RNA

Huh7 cells were co-infected with AdP_I235-HCV or AdP_I235-ΔGDD at 3 MOI and Ad-tTA at 15 MOI. After 24 h, the cells were treated with 10 μg/ml of Dox for 48 h. The total RNAs were purified with High Pure RNA Isolation kit (Roche, Mannheim, Germany). The RNAs were reverse-transcribed to cDNA using a commercial available kit [TaKaRa RNA PCR Kit (AMV) Ver. 3.0] and a primer for the HCV negative strand RNA (5′-GCCAGCCCCCGATTGGGG-3′) or a primer for GAPDH (5′-TCTACATGGCAACTGTGA-3′), respectively. The transcription products of NS3 and GAPDH were amplified by PCR using paired primers (5′-ATGG CGCCTATTACGGCC-3′ and 5′-TGGTCTACATTAG TGTAC-3′) and (5′-GGTGGTCTCCTCTGACTTCAA CA-3′ and 5′-GTGGTCGTTGAGGGCAATG-3′), respectively. The putative sizes of the PCR products were 242-bp for NS3 and 89-bp for GAPDH. The PCR products were separated on 2% agarose gel.

RESULTS

RNA pol I-driven plasmid vector

First, we constructed an RNA pol I-driven plasmid coding an HCV replicon in which structural coding genes were replaced by the luciferase gene (Figure 1A). To investigate the expression of the HCV replicon from the RNA pol I plasmid vector, we transfected the plasmid vector into Huh7 cells. As shown in Figure 1B, the luciferase activity was observed in the RNA pol I vector-transfected cells. IFN is the most popular agent used to inhibit HCV replication. To examine whether the RNA pol I plasmid vector functions as an assay system for anti-HCV activity, we investigated the effect of IFN on the expression of the HCV replicon in the RNA pol I plasmid-transfected Huh7 cells. IFN dose-dependently reduced the replication of the HCV genome (Figure 1C), reaching 29.2% of the control at 5 pg/ml. IFN treatment did not cause any cytotoxicity (Figure 1D). These data suggest that the RNA pol I plasmid coding the HCV replicon works as an assay system for HCV replication.

RNA pol I-driven Ad5 vector

The Ad vector is the most efficient gene transfer vector for a variety of mammalian cells *in vitro* and *in vivo* (13,14,19,20). There are more than 51 serotypes of Ad. The Ad type 5 (Ad5) vector has been frequently used in basic research and clinical studies (21). Ad5 vectors are 100- and 1000-fold more efficient at mediating gene transduction than cationic lipids, an effective non-viral vector (22). A reverse genetics system for the generation of influenza virus using RNA pol I-driven Ad5 vector produced 1000-fold the virus titer of the RNA pol I plasmid system (15). These findings indicate that the Ad5 vector may have advantages for the preparation of an HCV replicon system. We prepared RNA pol I-driven Ad5 vectors and confirmed the expression of a reporter gene from the Ad5 vectors coding luciferase (Supplementary Figure S1). However, we did not succeed in preparing Ad5 vector particles coding the HCV replicon. Indeed, there have been no previous reports of the preparation of Ad5 vector expressing the HCV RNA genome.

We think that two problems must be solved in order to develop Ad5 vectors coding the HCV RNA genome. These problems are the influence of the HCV replicon on the preparation of Ad5 particles and the packaging limit of Ad5 vectors.

Preparation of the TRE/RNA pol I chimeric promoter

The tet-regulated system comprises a regulator vector that expresses tet-controlled *trans*-activators and a response vector consisting of TRE within the promoter that controls expression of the gene of interest. The tet-controlled *trans*-activators are classified into tTA and rtTA that binds to the TRE promoter and activates expression from the TRE promoter in the absence and presence of Dox, respectively (23,24). We speculated that a tet-regulated vector system would minimize the influence of the HCV replicon on the preparation of Ad vector

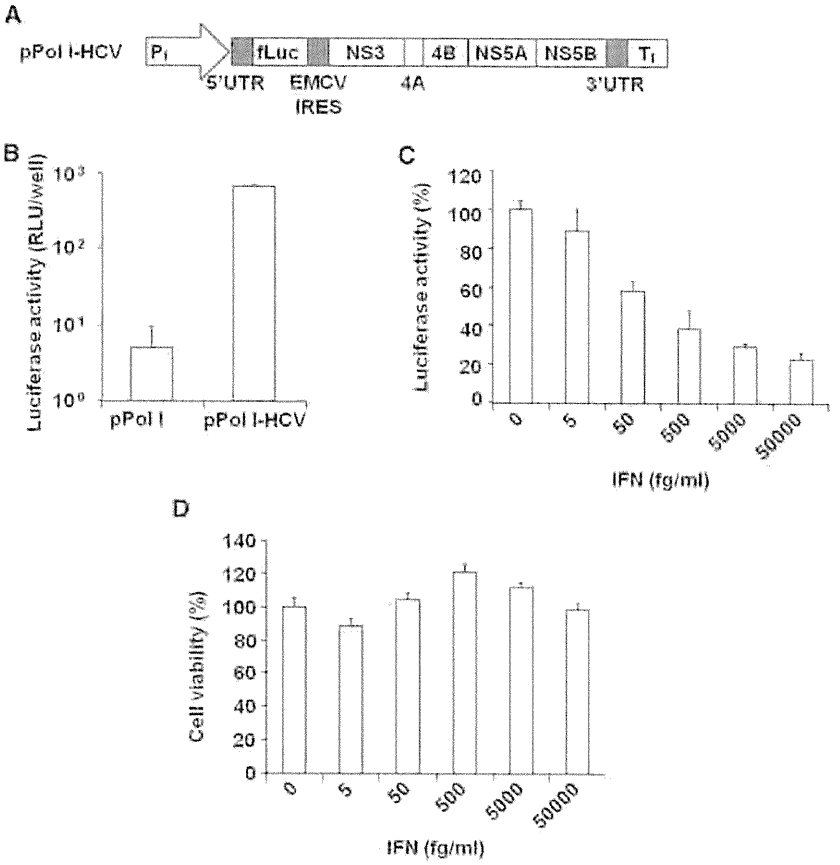

Figure 1. Preparation of plasmid expressing HCV replicon driven by RNA pol I promoter. (**A**) Schematic construct of HCV replicon-expression cassette. The HCV replicon gene was driven by the RNA pol I promoter (P_I) and terminator (T_I). (**B**) Transgene expression in Huh7 cells. Cells were transfected with pPol I-HCV. After 24 h of transfection, the luciferase activities were measured. Data are mean ± SD (n = 3). (**C** and **D**) Effect of IFN on HCV replication in RNA pol I vector-transfected cells. Huh7 cells were transfected with pPol I-HCV. After 2.5 h of transfection, the cells were treated with IFN at the indicated concentration. After an additional 72 h of incubation, the luciferase activity (C) and the cell viability (D) were measured. The luciferase activity (%) was calculated as a percentage of that in the vehicle-treated cells. Data are mean ± SD (n = 3).

particles. First, we optimized the chimeric promoter of TRE and the RNA pol I promoter. As shown in Figure 2A, the RNA pol I promoter is a 412-bp fragment containing an upstream control element (UCE) and the binding site of a transcription factor (Core). We constructed three chimeric promoter-driven plasmid vectors and checked the expression profiles using luciferase as a reporter gene. The chimeric vector was co-transfected into Huh7 cells with response vectors coding tTA or rtTA (23,24). As shown in Figure 2B and C, co-transfection with tTA exhibited a higher expression level than that of rtTA. The P_I235 promoter had the lowest luciferase expression in the absence of response vectors (Supplementary Figure S2). We used tTA and the P_I235 promoter in further studies. To investigate whether the chimeric RNA pol I promoter works in the Ad vector, we prepared Ad5 vector coding the chimeric RNA pol I-driven luciferase gene. AdP_I235-EL (MOI of 5) was co-transduced with Ad-tTA at MOI of 10 and 50. As shown in Figure 2D, the luciferase expression was increased in an Ad-tTA concentration-dependent manner.

Expression of the HCV replicon from Ad vector

The packaging limit of a foreign gene in the conventional Ad5 vector has been estimated to be 8.1–8.2-kb (25). The HCV replicon is ~8.9-kb and contains a 1.7-kb firefly luciferase gene and sequence derived from the HCV genome. Thus, another reason for no previous reports regarding the preparation of Ad5 vector coding the HCV replicon appears to be the packaging limit of the Ad5 vector. Mizuguchi and Hayakawa found that Ad5/35 vector containing chimeric fibers of Ad5 and Ad35 increased the size limit of foreign genes to 8.8-kb (26). We were successful in preparing Ad5/35 vector particles (9.53×10^8 IFU/ml) coding the TRE/RNA pol I chimeric promoter-driven HCV replicon containing the 1.0-kb renilla luciferase gene and sequence derived from the HCV genome (Figure 3A). To investigate the expression of the HCV replicon, Huh7 cells were transfected with the Ad vector coding the HCV replicon and Ad-tTA at MOI of 10 and 50, respectively. As shown in Figure 3B, western blot analysis showed that NS5A was expressed in Huh7 cells transfected with the vectors in the absence of Dox.

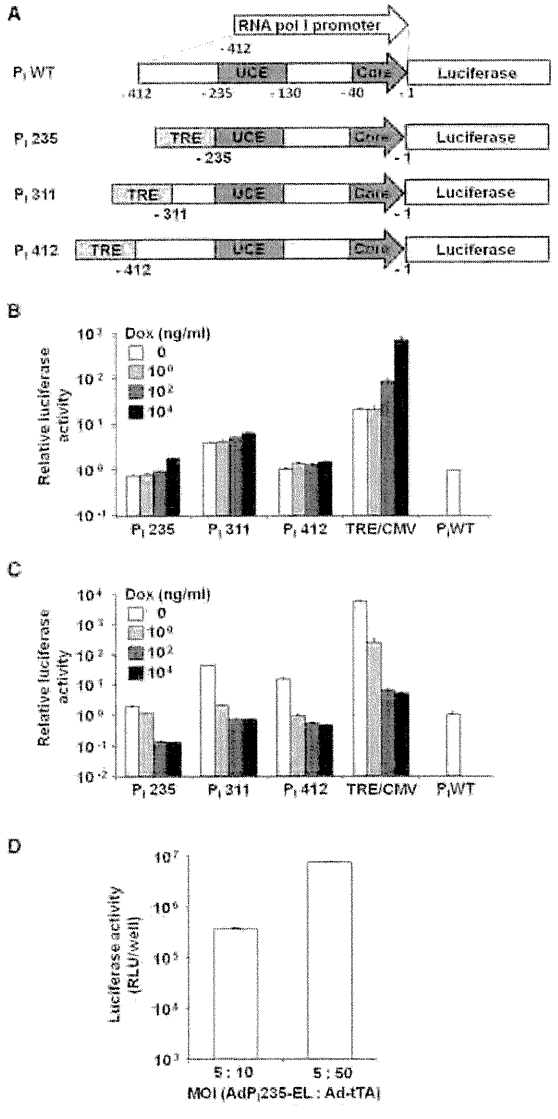

Figure 2. Development of tet-controllable RNA pol I promoter. (**A**) Construct of the chimeric RNA pol I promoter. The 412-bp human RNA pol I promoter contains Core (from −40 to −1), the binding site of the transcription factor, and UCE (from −235 to −130). TRE is connected to the full or partial RNA pol I promoter at the indicated sites, resulting in P_I235, P_I311 and P_I412. (**B** and **C**) Promoter activities of the chimeric promoter in Huh7 cells. Huh7 cells were co-transfected with the chimeric RNA pol I plasmid coding EGFPLuc, pCMVβ and *trans*-activator plasmid [rtTA (B) or tTA (C)]. After 2.5 h of transfection, the cells were treated with Dox at the indicated dose. After an additional 48 h of incubation, the luciferase and β-galactosidase activities were measured. The luciferase activity was normalized by the β-galactosidase activity and expressed relative to that of pP$_I$WT-EL-transfected cells. Data are mean ± SD (n = 3). (**D**) Transgene activity of Ad vector coding the chimeric promoter construct. Huh7 cells were co-infected with AdP$_I$235-EL and Ad-tTA. The MOI ratio of AdP$_I$235-EL to Ad-tTA was 5:10 or 5:50. After an additional 48 h of incubation, the luciferase activity was measured. Data are the mean ± SD (n = 3).

Luciferase was also expressed (Figure 3C). Dox dose-dependently attenuated expression of luciferase (Supplementary Figure S3). To discriminate between translation of the RNA pol I-transribed HCV RNA derived from the vector DNA and translation of HCV RNA derived from autonomous HCV replication in the transcribed cells, we prepared replication-incompetent HCV replicon deleting GDD motif in NS5B. Luciferase expression was attenuated in the cells transfected with the GDD-deleted Ad vector (AdP$_I$235-ΔGDD) (Figure 3D). A fragment of the HCV negative strand RNA, an essential replication intermediate, amplified by RT-PCR has been detected in the cells transfected with AdP$_I$235-HCV but not AdP$_I$235-ΔGDD (Figure 3E). Autonomous replication of the HCV RNA may occur in this system. To evaluate whether the Ad vector systems could be used to evaluate inhibitors of HCV replication, we investigated the effect of IFN on luciferase expression from HCV replicon in the Ad vector. As shown in Figure 3F, treatment of cells with 5 pg/ml of IFN reduced luciferase expression (33.3% of vehicle-treated cells). Cell viability was not affected by IFN treatment (Figure 3G). These findings indicate that the tet-controllable RNA pol I Ad vector may be useful for evaluation of anti-HCV activity.

DISCUSSION

HCV is an RNA virus containing the positive strand of a 9.6-kb RNA genome. A technique to transfer all or part of the HCV RNA genome to cells could be widely applicable for basic studies on HCV and pharmaceutical therapy against HCV. However, efficient and convenient methods to transduce the HCV RNA genome have never been fully developed. Electroporation of *in vitro* translated HCV RNA genome into cells is the most popular method. In the present study, we used a tet-controllable expression system to successfully develop an Ad vector system expressing the HCV RNA genome.

To our knowledge, development of Ad vector expressing HCV subgenome or genome has never been succeeded. The NS3 protease is essential for processing most of the NS proteins from the HCV polyprotein (27–30). The cleavage site of the NS3 protease is estimated to be between the P1 and P1′ position of an acidic amino acid at the P6 position, a Cys or Thr residue at the P1 position, and a Ser or Ala residue at the P1′ position (31). E1A, pIIIa, pol and V proteins of Ad have the cleavage site of the NS3 protease. The lack of previous success in generating Ad vectors coding the HCV genome and subgenome might be partly due to the degradation of Ad components by the NS3 protease during the preparation of Ad particles. In the tet-regulated system, when Ad vectors coding foreign genes driven by the TRE hybrid promoter are co-transfected with tTA or rtTA vector, the foreign gene can be expressed. Expression of the foreign gene could be suppressed during amplification of Ad vector particles in 293 cells, resulting in the preparation of Ad vector particles. The critical factor in the HCV replicon must be determined in a future study.

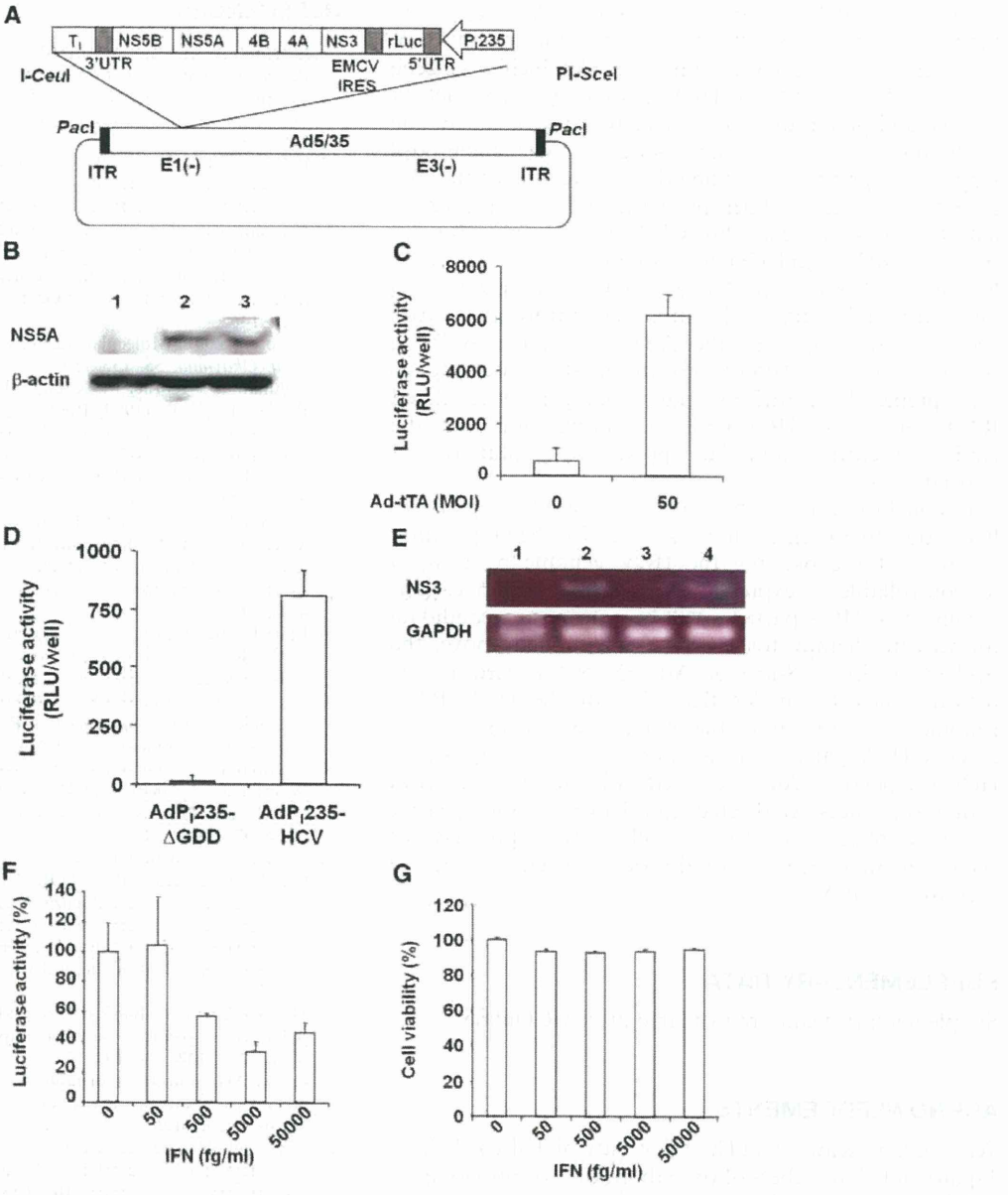

Figure 3. Preparation of Ad vector to monitor HCV replication. (**A**) Construct of Ad vector. The Ad vector contained the chimeric RNA pol I promoter (P_I235) and the HCV replicon to monitor HCV replication as the luciferase expression. (**B**) Expression of HCV NS5A protein in Huh7 cells transfected with AdP_I235-HCV. The cells were transfected with AdP_I235-HCV (10 MOI) and Ad-tTA (50 MOI). After 72 h of incubation, the cells were harvested, and the lysates (30 μg) were subjected to SDS–PAGE, followed by immunoblotting with antibody against NS5A. Huh7 cells and Huh7.5.1 1bFeo cells were used as the negative and positive controls, respectively. Lane 1, Huh7 cells; lane 2, Huh7 cells infected with AdP_I235-HCV; lane 3, Huh7.5.1 1bFeo cells. (**C**) Expression of luciferase in the Ad vector-transfected cells. Huh7 cells were co-infected with AdP_I235-HCV (10 MOI) and 0 or 50 MOI of Ad-tTA. After an additional 48 h of incubation, the luciferase activity was measured. Data represent the mean ± SD ($n = 3$). (**D**) Involvement of NS5B in expression of luciferase in the Ad vector-transfected cells. Huh7 cells were infected with AdP_I235-HCV or AdP_I235-ΔGDD (3 MOI) and Ad-tTA (15 MOI). After 24 h, the cells were treated with 10 μg/ml of Dox for 48 h. Then, the luciferase activity was measured. Data represent the mean ± SD ($n = 3$). (**E**) Expression of minus-stranded HCV RNA in the Ad vector-transfected cells. Huh7 cells were co-infected with AdP_I235-HCV or AdP_I235-ΔGDD at 3 MOI and Ad-tTA at 15 MOI. After 24 h, the cells were treated with 10 μg/ml of Dox for 48 h. Then RT-PCR analysis was performed for detection of minus-stranded HCV NS3 and GAPDH. The PCR products were separated on 2% agarose gel. Huh7 cells and Huh7.5.1 1bFeo cells were used as the negative and positive controls, respectively. Lane 1, Huh7 cells; lane 2, Huh7.5.1 1bFeo cells; lane 3, Huh7 cells infected with AdP_I235-ΔGDD; lane 4, Huh7 cells infected with AdP_I235-HCV. (**F** and **G**) Effect of IFN on the replication of HCV replicon. Huh7 cells were infected with AdP_I235-HCV (10 MOI) and Ad-rtTA (50 MOI). After 1.5 h of infection, the cells were treated with IFN at the indicated concentration for 72 h. Then, the luciferase activity (F) and the cell viability (G) were measured. Data represent the percentage of vehicle-treated cells. Data are the mean ± SD ($n = 3$).

Transgenes delivered by a conventional Ad5 vector are limited to a size of 8.1–8.2-kb (32), and the size of HCV replicon is ~8.2-kb (containing a 1.0-kb luciferase gene and a 7.2-kb fragment of HCV genome) (8). The lack of a successful preparation of Ad5 vector may be partly due to limitation of packaging transgene. Mizuguchi and Hayakawa prepared a chimeric Ad vector containing type 5 and type 35 fiber proteins, which is a package 8.8-kb of foreign gene (26). CD46 is a receptor for Ad type 35 (Ad35), and CD46 is ubiquitously expressed in human cells (33,34). The Ad5/35 chimera vector can transduce various human cells more effectively than Ad5 vectors, indicating that the Ad5/35 vector may be a better system than Ad5 (26,35). In this study, we successfully prepared an Ad5/35 vector coding a tet-regulated RNA pol I-driven HCV replicon, and we found that the Ad5/35 vectors could be applied to evaluation of anti-HCV activity.

In conclusion, to the best of our knowledge, this is the first report to establish a novel strategy for the preparation of Ad vector expressing the HCV genome by using a tet-controllable expression system. Replication-incompetent HCV particles will be a promising candidate for vaccine therapy for HCV. As mentioned above, the packaging size (8.8-kb) of Ad5/35 vector used in the present study is smaller than that of the HCV RNA genome (9.6-kb), and, therefore, the preparation of inactive HCV particles using Ad5/35 vector is impossible. Helper-dependent Ad vector (HDAd), in which all viral coding sequences are deleted, can deliver a large capacity of ~37-kb to cells (36). Tet-controllable RNA pol I HDAd vector might contribute to the development of vaccine therapy for HCV.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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Use of human hepatocyte-like cells derived from induced pluripotent stem cells as a model for hepatocytes in hepatitis C virus infection

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ABSTRACT

Host tropism of hepatitis C virus (HCV) is limited to human and chimpanzee. HCV infection has never been fully understood because there are few conventional models for HCV infection. Human induced pluripotent stem cell-derived hepatocyte-like (iPS-Hep) cells have been expected to use for drug discovery to predict therapeutic activities and side effects of compounds during the drug discovery process. However, the suitability of iPS-Hep cells as an experimental model for HCV research is not known. Here, we investigated the entry and genomic replication of HCV in iPS-Hep cells by using HCV pseudotype virus (HCVpv) and HCV subgenomic replicons, respectively. We showed that iPS-Hep cells, but not iPS cells, were susceptible to infection with HCVpv. The iPS-Hep cells expressed HCV receptors, including CD81, scavenger receptor class B type I (SR-BI), claudin-1, and occludin; in contrast, the iPS cells showed no expression of SR-BI or claudin-1. HCV RNA genome replication occurred in the iPS-Hep cells. Anti-CD81 antibody, an inhibitor of HCV entry, and interferon, an inhibitor of HCV genomic replication, dose-dependently attenuated HCVpv entry and HCV subgenomic replication in iPS-Hep cells, respectively. These findings suggest that iPS-Hep cells are an appropriate model for HCV infection.

1. Introduction

Hepatitis C virus (HCV), a hepatotropic member of the *Flaviviridae* family, is the leading cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma. Approximately 130–200 million people are estimated to be infected with HCV worldwide. Each year, 3–4 million people are newly infected with HCV [1]. Thus, overcoming HCV is a critical issue for the World Health Organization.

HCV contains a positive strand ~9.6 kb RNA encoding a single polyprotein (~3000 aa), which is cleaved by host and viral proteases to form structural proteins (core, E1, E2, and p7) and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [1]. These virus proteins might be potent targets for anti-HCV drugs. However, combination therapy with interferon and ribavirin, which often causes severe side-effects leading to treatment termination, has been the only therapeutic choice [2]. Very recently, new direct antiviral agents have been approved or are under clinical trials; these agents include NS3 protease inhibitors, NS5A inhibitors, and NS5B polymerase inhibitors [2–4]. However, the emergence of drug resistance is a serious problem associated with the use of direct antiviral agents [5].

Host targets are alternative targets for the development of anti-HCV drugs. A liver-specific microRNA (miRNA), miR-122, facilitates the replication of the HCV RNA genome in cultured liver cells [6]. Administration of a chemically modified oligonucleotide complementary to miR-122 results in long-lasting suppression of HCV with no appearance of resistant HCV in chimpanzees [7]. Epidermal

Abbreviations: HCV, hepatitis C virus; iPS-Hep cells, human induced pluripotent stem cells-derived hepatocyte-like cells; HCVpv, HCV pseudotype virus; SR-BI, scavenger receptor class B type I; miRNA, microRNA; EGF-R, epidermal growth factor receptor; EphA2, ephrin factor A2; iPS cells, human induced pluripotent stem cells; FCS, fetal calf serum; Ad, adenovirus; HNF-4α, hepatocyte nuclear factor-4α; RT, reverse transcription; PCR, polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; VSV, vesicular stomatitis virus; VSVpv, VSV pseudotype virus; tet, tetracycline; pol, polymerase; MOI, multiplicity of infection; Dox, doxycycline; IFN, interferon-α8; ES cells, embryonic stem cells.

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growth factor receptor (EGF-R) and ephrin factor A2 (EphA2) are host cofactors for HCV entry [8]. Inhibitors of EGF-R and EphA2 attenuated HCV entry, and prevented the appearance of viral escape variants [8]. These findings strongly indicate that identification of host factors associated with infection of human liver by HCV is a potent strategy for anti-HCV drug development. Because the host tropism of HCV is limited to human and chimpanzee [9], there is no convenient model for the evaluation of HCV infections. This has led to a delay in the development of anti-HCV agents targeting host factors.

Takahashi and Yamanaka developed human induced pluripotent stem (iPS) cells from human somatic cells [10]. The stem cells can be redifferentiated *in vitro*, leading to new models for drug discovery, including iPS-based models for drug discovery, toxicity assessment, and disease modeling [11,12].

Recently, several groups reported that iPS cells can be successfully differentiated into hepatocyte-like (iPS-Hep) cells that show many functions associated with mature hepatocytes [13–19]. However, whether iPS-Hep cells are suitable as a model for HCV infection has not been fully determined. Here, we investigated HCV entry and genomic replication in iPS-Hep cells by using HCV pseudotype virus (HCVpv) and HCV subgenomic replicons, respectively.

2. Materials and methods

2.1. Cell culture

Huh7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). An iPS cell-line (Dotcom) generated from the human embryonic lung fibroblast cell-line MCR5 was obtained from the Japanese Collection of Research Bioresources Cell Bank [20,21]. The iPS cells were maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts (Millipore, Billerica, MA) in iPSellon culture medium (Cardio, Hyogo, Japan) supplemented with 10 ng/ml fibroblast growth factor-2.

2.2. In vitro differentiation

Before the initiation of cellular differentiation, the medium of the iPS cells was replaced with a defined serum-free medium, hESF9, and the cells were cultured as previously reported [22]. The iPS cells were differentiated into iPS-Hep cells by using adenovirus (Ad) vectors expressing SOX17, the homeotic gene HEX or hepatocyte nuclear factor 4α (HNF-4α) in addition to the appropriate growth factors, cytokines, and supplements, as described previously [19].

2.3. Reverse transcription (RT)-polymerase chain reaction (PCR) analysis of HCV receptors

Total RNA samples were reverse-transcribed using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA), and the resultant cDNAs were PCR amplified by using Ex Taq DNA polymerase (TaKaRa Bio Inc., Shiga, Japan) and specific paired-primers for CD81 (5′-cgccaaggatgtgaagcagttc-3′ and 5′-tcccggagaagaggtcatcgat-3′), scavenger receptor class B type I (SR-BI; 5′-attccgatcagtgcaacatga-3′ and 5′-cagttttgcttcctgcagcacag-3′), claudin-1 (5′-tcagcactgccctgccccagt-3′ and 5′-tggtgttgggtaagaggttgt-3′), occludin (5′-tcagggaatatccacctatcacttcag-3′ and 5′-catcagcagcagccatgtactcttcac-3′), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (5′-tcttcaccaccatggagaag-3′ and 5′-accacctggtgctcagtgta-3′). The expected sizes of the PCR products were 245 bp for CD81, 788 bp for SR-BI, 521 bp for claudin-1, 189 bp for occludin, and 544 bp for GAPDH. The PCR products were separated on 2% agarose gels and visualized by staining with ethidium bromide.

2.4. HCVpv infection

Pseudotype vesicular stomatitis virus (VSV) bearing HCV envelope glycoproteins (HCVpv) and VSV envelope glycoproteins (VSVpv) were prepared as described previously [23]. iPS, iPS-Hep and Huh7 cells were treated with HCVpv or mixtures of HCVpv or VSVpv and anti-CD81 monoclonal antibody (JS-81; BD Biosciences, Franklin Lakes, NJ) or control mouse IgG for 2 h. After an additional 24 h of culture, the luciferase activities were measured by using a commercially available kit (PicaGene, Toyo Ink, Tokyo, Japan).

2.5. Preparation of Ad vector expressing the HCV replicon

Ad vectors expressing a tetracycline (tet)-controllable and RNA polymerase (pol) I promoter-driven HCV subgenomic replicon containing renilla luciferase (AdP_I235-HCV), a replication-incompetent HCV subgenomic replicon containing renilla luciferase (AdP_I235-ΔGDD), tet-responsive trans-activator (Ad-tTA) or a tet-controllable RNA pol-I driven firefly luciferase (AdP_I235-fluc) were prepared by using an *in vitro* ligation method as described previously [24–26]. The biological activity (infectious unit) of the Ad vectors was measured by using an Adeno-X rapid titer kit (Clontech, Mountain View, CA).

2.6. HCV replication assay

iPS, iPS-Hep and Huh7 cells were infected with AdP_I235-HCV or AdP_I235-ΔGDD at multiplicity of infection (MOI; infectious unit per cell) of 3, and Ad-tTA at MOI of 15. After 24 h, the cells were treated with 10 μg/ml of doxycycline (Dox) for 48 h. Renilla luciferase activities in the lysates were then measured with the use of the Renilla Luciferase Assay System (Promega, Madison, WI). To normalize for the infectivity of Ad vector, iPS, iPS-Hep and Huh7 cells were co-infected with AdP_I235-fluc (3 MOI) and Ad-tTA (15 MOI). After a 72-h incubation, the firefly luciferase activities in the lysates were measured, and the renilla luciferase activities were normalized by dividing by the corresponding firefly luciferase activities.

2.7. Quantitative analysis of plus- and minus-strand HCV RNA

iPS, iPS-Hep and Huh7 cells were co-infected with AdP_I235-HCV or AdP_I235-ΔGDD (3 MOI), and Ad-tTA (15 MOI). After 24 h, the cells were treated with 10 μg/ml of Dox for 48 h. Total RNA was reverse-transcribed into cDNA by using the Thermoscript reverse transcriptase kit (Invitrogen) as described previously [27,28]. Real-time PCR was performed with SYBR Premix Ex Taq (TaKaRa Bio Inc.) by using Applied Biosystems StepOne Plus (Applied Biosystems, Foster City, CA). The transcription products of the HCV plus-strand RNA, minus-strand RNA, and GAPDH gene, were amplified by using specific primers for HCV plus-strand RNA (RC1 primer, 5′-gtctagccatggcgttagta-3′; and RC21 primer, 5′-ctcccggggcactcgcaagc-3′), HCV minus-strand RNA (tag primer, 5′-ggccgtcatggtggcgaataa-3′; and RC21 primer), and GAPDH (5′-ggtggtctcctctgacttcaaca-3′ and 5′-gtggtcgttgagggcaatg-3′), respectively. The copy numbers of the transcription products of the HCV plus- and minus-strand RNA were normalized with those of the GAPDH gene and infectivity of Ad vector as described in the Section 2.6.

2.8. Inhibition of HCV replication by interferon-α8

iPS-Hep and Huh7 cells were infected with AdP_I235-HCV (3 MOI) and Ad-tTA (15 MOI). After 24 h of infection, the cells were treated with 10 μg/ml of Dox and recombinant human interferon-α8 (IFN) at the indicated concentration. After an additional 48-h incubation, renilla luciferase activity in the lysates was measured with the use of the Renilla Luciferase Assay System. Cell

viability was measured with the use of a WST-8 kit (Nacalai Tesque, Kyoto, Japan).

3. Results

3.1. Infection of iPS-Hep cells with HCVpv

HCV entry requires sequential interaction between the envelope proteins and multiple cellular factors, including CD81, SR-BI, claudin-1, and occludin [29]. To investigate expression of these receptors in iPS-Hep cells, we performed RT-PCR analysis. iPS cells expressed CD81 and occludin, but not SR-BI and claudin-1. In contrast, iPS-Hep and Huh7 cells expressed all four receptors (Fig. 1A). HCVpv have been widely used in studies of the mechanism of HCV entry and in screens for inhibitors of HCV infection [30]. We therefore investigated HCVpv infection in iPS-Hep cells. iPS cells showed no susceptibility to HCVpv infection. In contrast, HCVpv dose-dependently infected iPS-Hep cells as well as Huh7 cells, a popular model cell line for HCV research (Fig. 1B). Treatment of the cells with IgG did not affect susceptibility of iPS-Hep or Huh7 cells to HCVpv infection, even at IgG concentrations of 1 μg/ml. In contrast, anti-CD81 antibody dose-dependently inhibited HCVpv infection of iPS-Hep and Huh7 cells, and the antibody treatment did not affect infection of VSVpv with iPS-Hep (Fig. 1C). These findings suggest that iPS-Hep cells are a useful model for HCV infection.

3.2. Replication of subgenomic HCV RNA in iPS-Hep cells

We previously developed Ad vectors containing tet-controllable and RNA pol I-driven HCV RNA subgenomic replicons (AdP_I235-HCV [replication competent], and AdP_I235-ΔGDD [replication incompetent]). The replicons encoded luciferase, and monitoring of luciferase activity in infected cells was a simple and convenient method to evaluate HCV replication [24]. Here, we found cells transduced with the replication-competent HCV replicon expressed luciferase in iPS-Hep cells, but not in iPS cells (Fig. 2A). In contrast, cells transduced with the replication-incompetent HCV replicon did not express luciferase (Fig. 2A). Taken together, these results suggest that replication of the HCV RNA genome occurred in the iPS-Hep cells. To confirm replication of the HCV genome, we investigated production of minus-strand HCV RNA from the positive-strand HCV RNA genome by performing real time-PCR analysis. The results of this analysis showed that minus-strand HCV RNA was produced in iPS-Hep cells and Huh7 cells, but not in iPS cells (Fig. 2B). To investigate whether the iPS-Hep cells could be used to screen for drugs that suppress HCV replication, we treated the cells with a suppressor of HCV replication, IFN. Treatment with IFN resulted in dose-dependent attenuated replication of the HCV genome with no cytotoxicity (Fig. 3A and B). These findings suggest that the iPS-Hep cells are a suitable system to use for monitoring the replication of the HCV RNA genome.

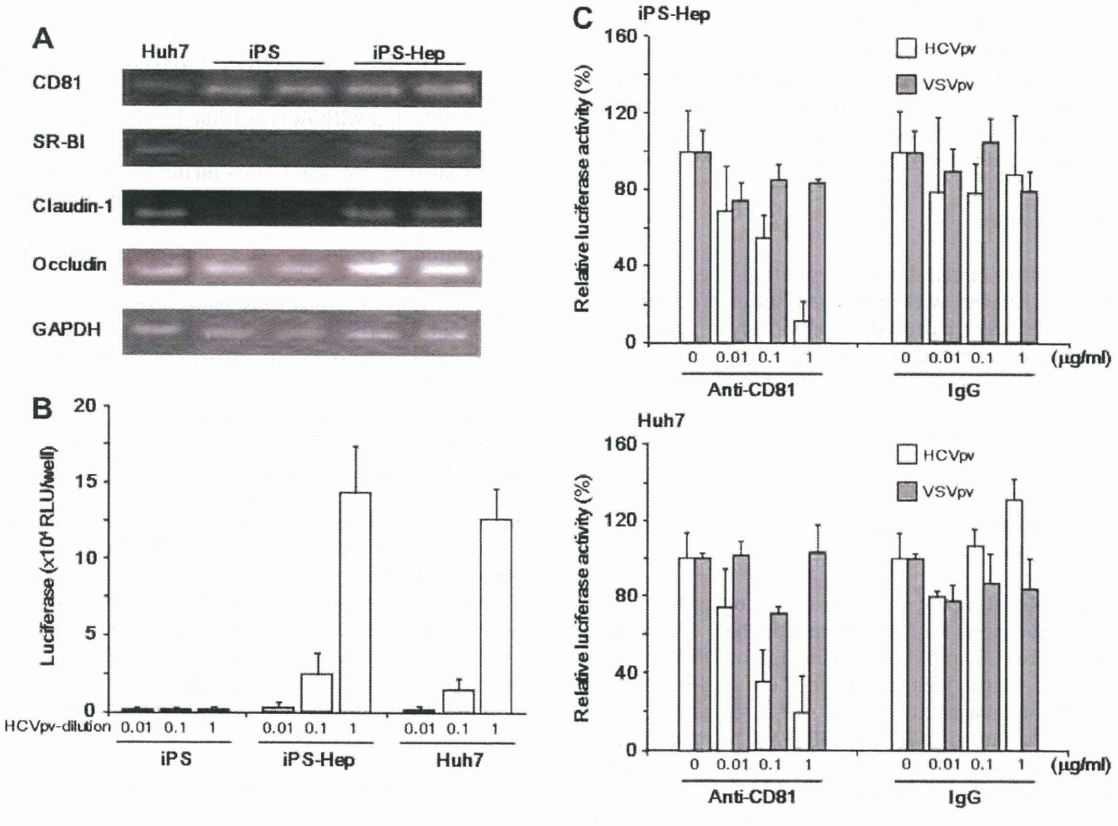

Fig. 1. HCV infection assay in iPS-Hep cells. (A) Expression of HCV receptors in iPS-Hep cells. Total RNA samples from Huh7, iPS, and iPS-Hep cells were subjected to RT-PCR analysis as described in the Section 2. The PCR products were separated on 2% agarose gels, followed by staining with ethidium bromide. (B) Infection of iPS-Hep cells with HCVpv. iPS, iPS-Hep and Huh7 cells were infected with HCVpv at the indicated dilution. After 2 h of infection, the cells were cultured with fresh medium for 24 h. Then, luciferase activities were measured. Data are presented as means ± SD ($n = 3$). (C) Effect of anti-CD81 antibody on infection of iPS-Hep cells with HCVpv. iPS-Hep (upper panel) and Huh7 (lower panel) cells were treated with mixtures of HCVpv (open column) or VSVpv (gray column) and anti-CD81 antibody or control mouse IgG at the indicated concentrations. After a 2-h incubation, the cells were cultured with fresh medium for 24 h. Then, the luciferase activities were measured. Data represent the percentage of vehicle-treated cells. Data are presented as means ± SD ($n = 3$).

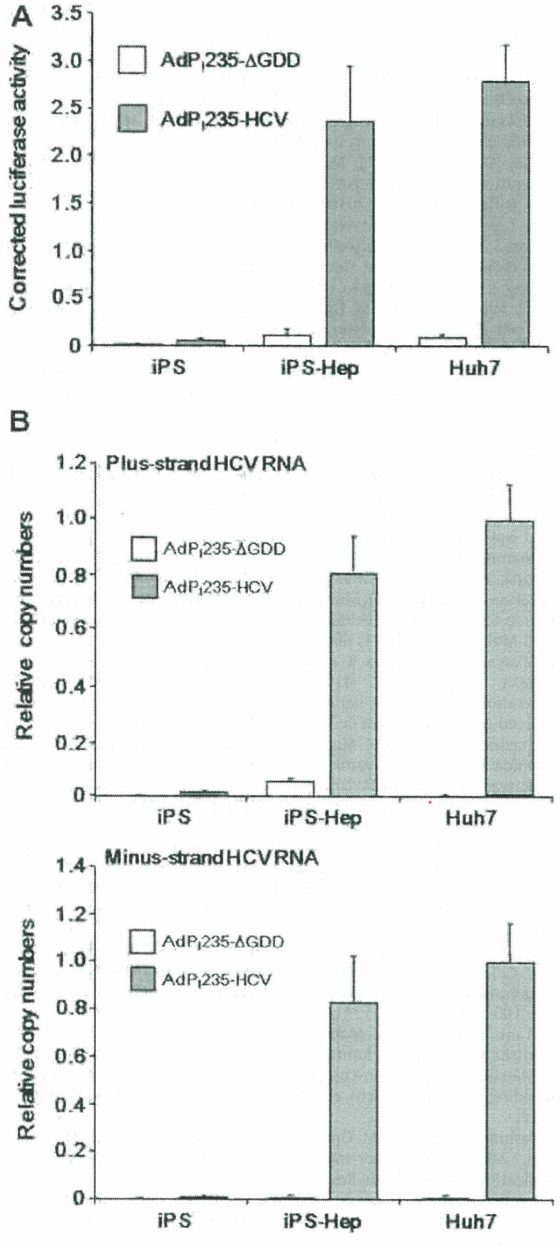

Fig. 2. HCV replication assay in iPS-Hep cells. (A) Comparison of replication of HCV subgenomic replicons, AdP$_i$235-HCV (gray column) and AdP$_i$235-ΔGDD (open column), in iPS, iPS-Hep and Huh7 cells. The cells were infected with replicons, treated with Dox, and renilla luciferase activity was measured, as described in the Section 2. To normalize for infectivity of Ad vector, cells were co-infected with AdP$_i$235-fluc and Ad-tTA. After 72 h, firefly luciferase activity was measured. Corrected luciferase activity was calculated as the ratio of renilla luciferase activity to firefly luciferase activity. (B) Real-time PCR analysis of HCV plus- and minus-strand RNA in iPS-Hep cells. iPS-Hep cells were infected with replicons, and total RNA was subjected to real-time PCR analysis, as described in the Section 2. The copy numbers were shown as ratio of those of Huh7. Data are presented as means ± SD ($n = 3$).

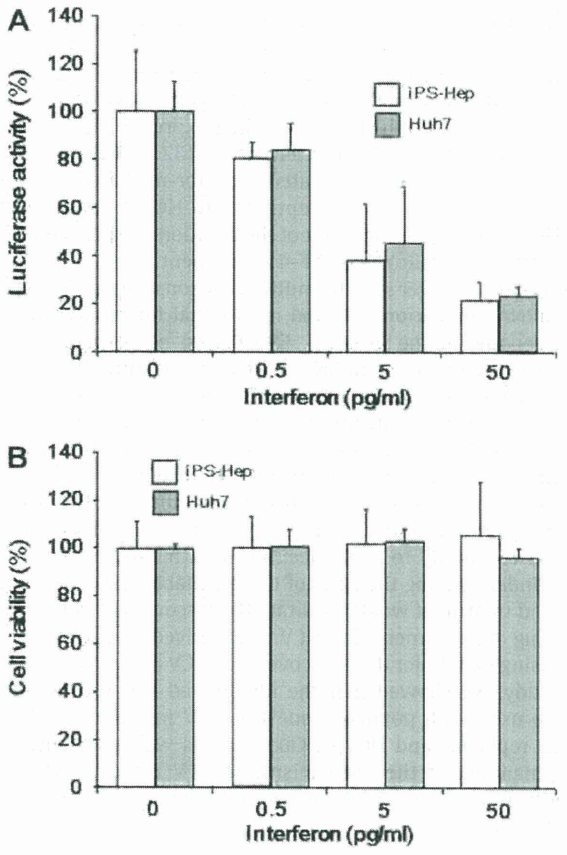

Fig. 3. Effect of interferon on HCV replication in iPS-Hep cells. iPS-Hep (open column) and Huh7 (gray column) cells were infected with AdP$_i$235-HCV and Ad-tTA. After 24 h, the cells were treated with Dox and the indicated concentration of interferon for 48 h. Luciferase activities (A) and cell viabilities (B) were measured as described in the Section 2. Data represent the percentage of the value for vehicle-treated cells, and are presented as means ± SD ($n = 3$).

4. Discussion

Tropism of HCV is limited to human and chimpanzee. Our understanding of HCV infection has been delayed by the lack of appropriate model systems. In the present study, we demonstrated that iPS-Hep cells are suitable *in vitro* models of hepatocytes for use in the study of HCV infection.

Other *in vitro* model systems of hepatocytes may not accurately reflect the biology of hepatocytes *in vivo*. For instance, expression profiles of mRNAs in embryonic stem (ES) cell-derived hepatocyte-like cells are different from those of primary human hepatocytes [31]. The development of efficient methods to differentiate stem cells into hepatocytes has been a critical issue in the application of stem cell technology to drug discovery. Recently, Mizuguchi and colleagues established efficient differentiation protocols for iPS cells by using adenoviral transfer of SOX17 [17], HEX [18], and HNF-4α [19] in addition to growth factors. Approximately 80% of the differentiated cells showed expression of hepatic-specific proteins, including cytochrome P-450s (CYP2D6, CYP3A4, and CYP7A1) [19]. The iPS-Hep cells were also used as a simple system to evaluate the hepatotoxicity of drugs that are metabolized into toxic substances by cytochromes [19]. Here, we showed that the essential host factors for HCV infection (occludin, claudin-1, SR-BI, and CD81) are expressed in the iPS-Hep cells. HCV RNA genome replication occurred in the cells, and HCVpv infected the cells. An inhibitor of HCV entry (anti-CD81 antibody), and an anti-HCV agent (IFN), attenuated the entry of HCVpv and the replication of the HCV genome in the cells, respectively. These findings suggest that the iPS-Hep cells are useful for understanding HCV infection and for screening anti-HCV drugs.

We found that iPS cells express CD81 and occludin, and are not susceptible to HCV entry, whereas iPS-Hep cells express all four HCV receptors and are susceptible to HCV entry. These findings are consistent with previous studies showing that CD81, occludin,

SR-BI, and claudin-1 are key receptors for HCV [29]. HNF-4α, which promotes the differentiation of iPS cells to iPS-Hep cells, is essential for the expression of a multitude of genes encoding cell junction and adhesion proteins during embryonic development of the mouse liver [32]. For instance, claudin-1 expression is not detected in the liver of HNF-4α-deficient mice [32]. HNF-4α enhances peroxisome proliferator-activated receptor-mediated SR-BI transcription [33]. Thus, the susceptibility to HCV entry observed in iPS-Hep cells may be the result of the additional expression of claudin-1 and SR-BI following HNF-4α treatment.

miR-122 is a liver specific miRNA that constitutes 70% of the total miRNA population [34] and is essential for replication of the HCV genome in the liver [6]. ES cells do not express miR-122, whereas expression of miRNA is observed during differentiation into hepatocyte-like cells [35]. Replication of HCV subgenomic replicons was observed in iPS-Hep cells, but not iPS cells (Fig. 2A). Expression of miR-122 might be a key factor controlling the replication of the HCV RNA genome in iPS-Hep cells.

The reasons that 15–20% of people infected with HCV can clear the virus without pharmaceutical intervention, and patients vary in their sensitivity to pharmaceutical treatments, are still unclear [36]. Understanding the basis of these variable responses to infection and treatment would facilitate the discovery of potent targets for drug development for HCV. iPS-derived hepatocytes are a promising system for drug discovery for HCV infection. In the present study, we showed that the iPS-derived hepatocyte-like cells can be used with popular models of HCV infection: HCV subgenomic replicons and HCVpv. Our findings will contribute to our understanding of the mechanisms of HCV infection and to the identification of novel targets for HCV therapy by means of iPS technology.

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