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Infectivity of hepatitis C virus correlates with the amount of envelope protein E2: Development of a new aptamer-based assay system suitable for measuring the infectious titer of HCV

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ABSTRACT

Various forms of hepatitis C virus (HCV)-related particles are produced from HCV-infected cells. Measuring infectivity of a HCV sample with the conventional 'foci counting method' is laborious and time-consuming. Moreover, the infectivity of a HCV sample does not correlate with the amount of viral RNA that can be measured by real-time RT-PCR. Here we report a new assay suitable for quantifying infectious HCV particles using aptamers against HCV E2, which is named 'Enzyme Linked Apto-Sorbent Assay (ELASA)'. The readout value of HCV ELASA linearly correlates with the infectious dose of an HCV sample, but not with the amount of HCV RNA. We also demonstrated that the activities of anti-HCV drugs can be monitored by HCV ELASA. Therefore, HCV ELASA is a quick-and-easy method to quantify infectious units of HCV stocks and to monitor efficacies of potential anti-HCV drugs.

Introduction

Approximately 170 million people are estimated to be infected by hepatitis C virus (HCV) worldwide (Hoofnagle, 2002). Chronic infection of HCV often leads to liver cirrhosis and hepatocellular carcinoma (Gottwein and Bukh, 2008; Memon and Memon, 2002). No HCV vaccine is available to date, and the current standard therapy, a combination of ribavirin and pegylated interferon-α (peg-IFN-α), cures in only half of patients (Fried et al., 2002). Two new direct-acting antiviral agents (DAA) targeting HCV NS3/4 protease, boceprevir and telaprevir, have become available very recently, and more anti-HCV drugs are being developed (Vermehren and Sarrazin, 2011).

HCV is a member of the *Flaviviridae* family, and the HCV genome consists of a single-stranded positive sense RNA of approximately 9.6 kb (Simmonds et al., 2005). The viral proteins are translated as a polyprotein of about 3010 amino acids that is processed co- and post-translationally by cellular and viral proteases into structural (core, E1 and E2) and nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) viral proteins (Reed and Rice, 2000). The genomic RNA is encapsidated into core proteins, and the nucleocapsid composed of the genomic RNA and core proteins is budded into ER lumen while envelope proteins E1 and E2 are loaded onto the virion particles (Roingeard et al., 2004). Only a minor portion of virion particles (1 out of 100–1000) produced in a model animal (Bukh, 2004) and in a cell culture system (Lindenbach et al., 2005; Zhong et al., 2005) are infectious. Core proteins exist in both infectious and noninfectious HCV particles (Gastaminza et al., 2010, 2006). In contrast, HCV envelope proteins (E1 and E2) are known to be associated with the infectious particles, not with the noninfectious particles (Merz et al., 2011; Vieyres et al., 2010). The envelope proteins have pivotal roles in HCV entry (Dubuisson et al., 2008; Evans et al., 2007; Mazumdar et al., 2011; Pileri et al., 1998; Ploss et al., 2009; Scarselli et al., 2002). The E2 proteins are needed for attachment and entry of virions through interactions

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with cellular proteins (CD81, SRB1, claudin1 and occludin) (Evans et al., 2007; Pileri et al., 1998; Ploss et al., 2009; Scarselli et al., 2002). Therefore, the quantification of HCV E2 in viral stocks could be a logical approach to measure the infectious dose of a virus source. The current state-of-the-art methods of measuring the amounts of HCV are quantitative RT-PCR (Mellor et al., 1999) and immunological measurement of core proteins (Tanaka et al., 2000). None of these methods is suitable for measuring the infectious dose of HCV, but these methods are for quantifying total virion particles including both infectious and noninfectious ones.

Aptamers are oligonucleotides (DNA or RNA) that interact with varieties of target molecules including proteins with high affinity and specificity (Ellington, 1994; Gold et al., 1995). Aptamers have been employed in diverse applications based on molecular recognition as analytical, diagnostic and therapeutic tools (Brody and Gold, 2000; Famulok et al., 2007; Gold, 1995). Aptamers are selected *in vitro* from an oligonucleotide library containing random sequences by the method named 'systematic evolution of ligands by exponential enrichment (SELEX)' (Ellington and Szostak, 1990; Tuerk and Gold, 1990). Theoretically, a SELEX library with 40 nucleotide random sequence has 4^{40} combinational diversities. The SELEX process leads to enrichment of oligonucleotides specifically interacting with a target molecule. In order to increase binding affinity of aptamers to target proteins, we employed the 'advanced SELEX technology' using a modified deoxyribonucleotide (Gold et al., 2010; Vaught et al., 2010).

Here we report a new assay system suitable for quantifying infectious HCV particles using aptamers against HCV E2. In principle, this system is similar to Enzyme-Linked Immuno-Sorbent Assay (ELISA). The direct ELISA, which recognizes an antigen, utilizes two different antibodies specific for the antigen (Engvall and Perlmann, 1971). On the other hand, this new system utilizes two different aptamers recognizing HCV E2. We named this new system as 'Enzyme Linked Apto-Sorbent Assay (ELASA)' since this system is composed of two aptamers instead of antibodies. Quantities of purified HCV E2 proteins of two major genotypes [genotype 1 (1a and 1b) and genotype 2] were measurable by the HCV ELASA system. Moreover, the HCV ELASA was able to detect HCV particles containing envelope proteins of either genotype 1 or 2 generated from *in vitro* cultivation systems. To our surprise, the readout values of the HCV ELASA correlated well with the infectious doses of HCV particles, but not with the amounts of HCV RNAs in virus samples. We also showed that this system is useful for monitoring effects of anti-viral drugs. Therefore, the HCV ELASA is a quick-and-easy tool to quantify infectious units of HCV stocks and to monitor efficacies of potential anti-HCV drugs using *in vitro* cultivation systems.

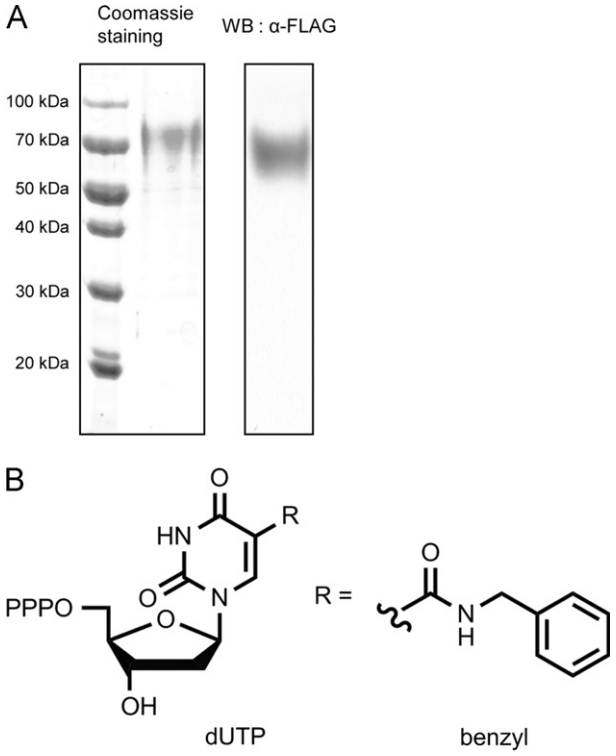

Fig. 1. Generation of DNA aptamers against HCV envelope protein E2. (A) Purification of HCV E2 proteins from yeasts. 6× Histidine- and FLAG-tagged E2 proteins were expressed in yeasts and then purified by affinity chromatography (Materials and methods). The purified E2 proteins were resolved by 12% SDS-PAGE and visualized by Coomassie brilliant blue staining (left) or by Western blotting with an anti-FLAG antibody (right). Numbers on the left side depict the apparent molecular weights of protein size markers. The apparent molecular weight of glycosylated recombinant E2 proteins was approximately 70 kDa. (B) The structure of modified uridine (Bz-dU) used in the advanced SELEX. The 'R' represents benzylaminocarbonyl group (Bz).

Results

Generation of anti-HCV E2 aptamers with high affinities via advanced SELEX using a modified nucleoside.

In order to generate DNA aptamers against HCV E2, we purified a truncated HCV E2 protein containing the luminal domain (383–729 amino acids) of genotype 2a HCV (JFH) that was expressed in yeasts (PBN204 strain). The yeast expression system was used instead of an *Escherichia coli* system since post translational modification [glycosylation (Goffard and Dubuisson, 2003; Beeck et al., 2001) and disulfide bond formation (Dubuisson and Rice, 1996; Fenouillet et al., 2008; Krey et al., 2010)] are essential for proper folding and receptor binding of the polypeptide (Falkowska et al., 2007; Goffard et al., 2005; McCaffrey et al., 2012; Slater-Handshy et al., 2004). Purification of the recombinant E2 protein was performed by affinity chromatography based on 6× Histidine and FLAG tags conjugated to the N-terminal and C-terminal ends of the E2, respectively (Fig. 1A). Advanced SELEX, using 5-benzylaminocarbonyl-dUridine (Bz-dU) instead of Thymidine, was performed to acquire aptamers with high affinity and specificity (Gold et al., 2010; Vaught et al., 2010) (Fig. 1B). After 8 rounds of selection, four aptamers (E2-A, -B, -C, -D) with high affinities to the purified E2 proteins were obtained (Table 1 and Table S1), and the secondary structures of ssDNA aptamers were predicted by using a web-based program Mfold (Supplementary Fig. 1). The affinities and Bmax values of aptamers ranged K_d=0.8–4 nM and 64–80%, respectively.

Table 1
Binding affinities (K_d values) and Bmax values of aptamers against HCV envelope protein E2 (genotype 2a JFH). N/A stands for 'not available'.

	Pool	Round 6	Round 8	E2-A	E2-B	E2-C	E2-D
K_d (nM)	N/A	9.9 ± 1.4	4.6 ± 0.3	1.0 ± 0.5	4.0 ± 0.8	3.5 ± 1.5	0.8 ± 0.2
Bmax (%)	19 ± 2	63 ± 3	56 ± 1	75 ± 6	71 ± 8	64 ± 11	80 ± 5

Development of a new HCV detection method named 'enzyme linked apto-sorbent assay (ELASA)' using aptamers against HCV E2 protein

To quantify HCV E2 proteins or HCV particles containing E2 proteins, we developed a new method named enzyme-linked apto-sorbent assay (ELASA). Schematic diagram of the ELASA is depicted in Fig. 2A. In principle, ELASA is similar to Enzyme-Linked Immuno-sorbent Assay (ELISA) (Engvall and Perlmann, 1971). However, 2 aptamers recognizing 2 different regions of HCV E2 were used in the HCV ELASA instead of 2 antibodies recognizing a target molecule as in ELISA. In the ELASA system, one of the aptamer pair (capture aptamer) is directly conjugated onto the surface of a 96 well plate through NHS linkage, and the other (detection aptamer) is biotinylated to snatch a streptavidin conjugated with horse radish peroxidase (HRP) (Fig. 2A). In this setting, the capture and detection aptamers bind to E2 proteins on the virus surface, and the streptavidin-HRP specifically interacts with the biotin-conjugated detection aptamer. In other words, the HRP is indirectly connected to the well only when HCV E2 proteins or HCV particles with E2 exist in the well after the washing step of ELASA. The amounts of HRP remaining in the well are proportional to the amounts of HCV E2 or HCV particles containing E2. The chemiluminescent signals from HRP are measured at 450 nm. We tested all possible combinations of aptamer pairs in exploring the best pair for ELASA (Fig. 2B). As a source of HCV particles, we used HCV particles produced from an *in vitro* HCV cultivation system based on a highly infectious variant of JFH clone (Keum et al., 2012). Even though all four E2 aptamers have high affinities to recombinant E2 proteins, only aptamers B and D were functional for detecting HCV particles (Fig. 2B). And the pair composed of capture aptamer B and detection aptamer D showed the strongest detection signal among the combinations of aptamer pairs (Fig. 2B). This capture aptamer B and detection aptamer D pair was used for further analyses of the ELASA system.

The sensitivity of the ELASA system was improved by using a multivalent detection aptamer approach.

Avidity of probes can be increased by oligomerizing the probes (Cuesta et al., 2010). Therefore, we tried to improve sensitivity of the HCV ELASA system by oligomerizing the detection aptamer. For this purpose, we used a biotinylated detection aptamer and streptavidin, which forms a tetramer containing 4 biotin-binding sites, conjugated with HRP (Fig. 3A). The multivalent detection aptamers were generated by pre-incubating the streptavidin-HRPs and the biotin-conjugated detection aptamers (Fig. 3B). Several variants (mono-, di-, tri-, and tetramer) of streptavidin–biotinylated aptamer complexes were observed when streptavidin-HRPs and the biotin-conjugated detection aptamers were incubated together. We performed ELASAs of HCV culture media with either monovalent detection aptamers or multivalent detection aptamers (Fig. 3C). The detection limit of the ELASA system with the multivalent aptamers was 3.13–6.25 $\times$ 10^2 FFU/ml,

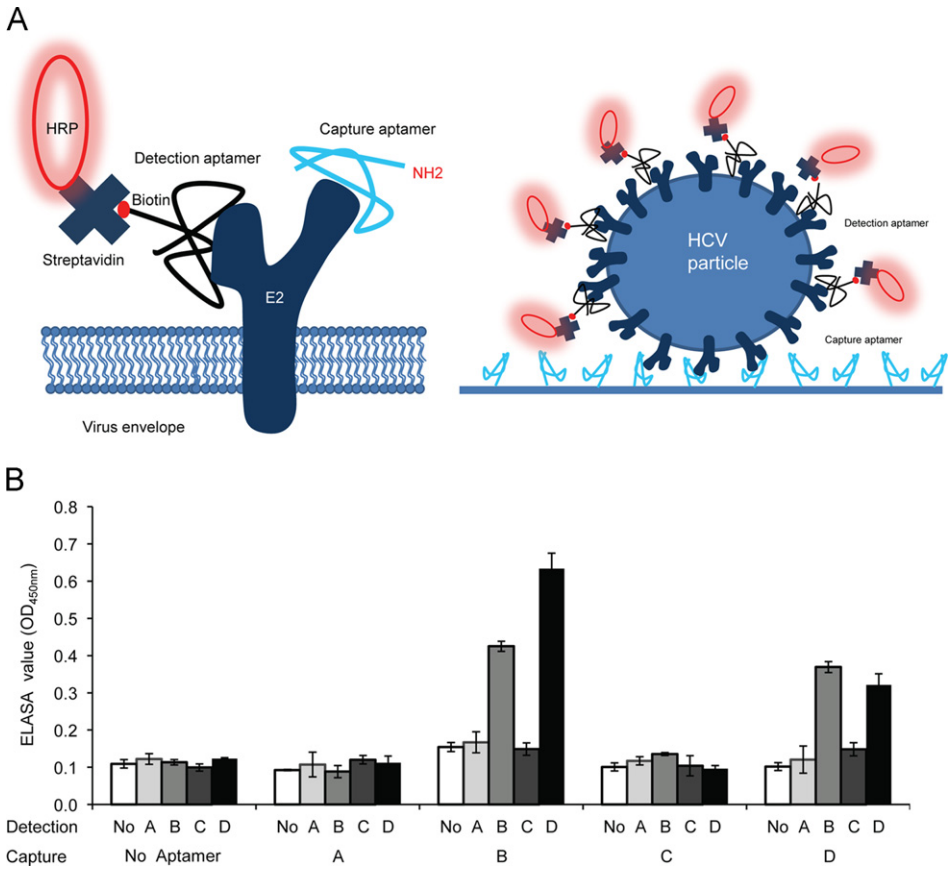

Fig. 2. Development of an ELASA system. (A) Schematic diagram of ELASA. A pair of DNA aptamers specifically interacting with different parts of E2 protein on a HCV virion particle is depicted. The capture aptamer is immobilized on the plate through the amine group, and the detection aptamer is conjugated with biotin that interacts with streptavidin conjugated with horse radish peroxidase (HRP) (left panel). The chemiluminescent signals from HRP were measured at 450 nm (right panel). (B) Efficacies of different pairs of aptamers in ELASAs. HCV culture medium (100 μl, 1.00 $\times$ 10^4 FFU/ml) was loaded on each well in an Immobilizer Amino 96 well plate conjugated with various capture aptamers (aptamers A–D) and then incubated at RT for 1 h. To detect the captured HCV particles, biotinylated detection aptamers (aptamers A–D) were treated in the wells at RT for 1 h, and then streptavidin–HRP conjugates were treated at RT for 30 min (Materials and methods). No aptamer is a negative control. The bars and lines represent the mean values and standard deviations from three independent experiments.

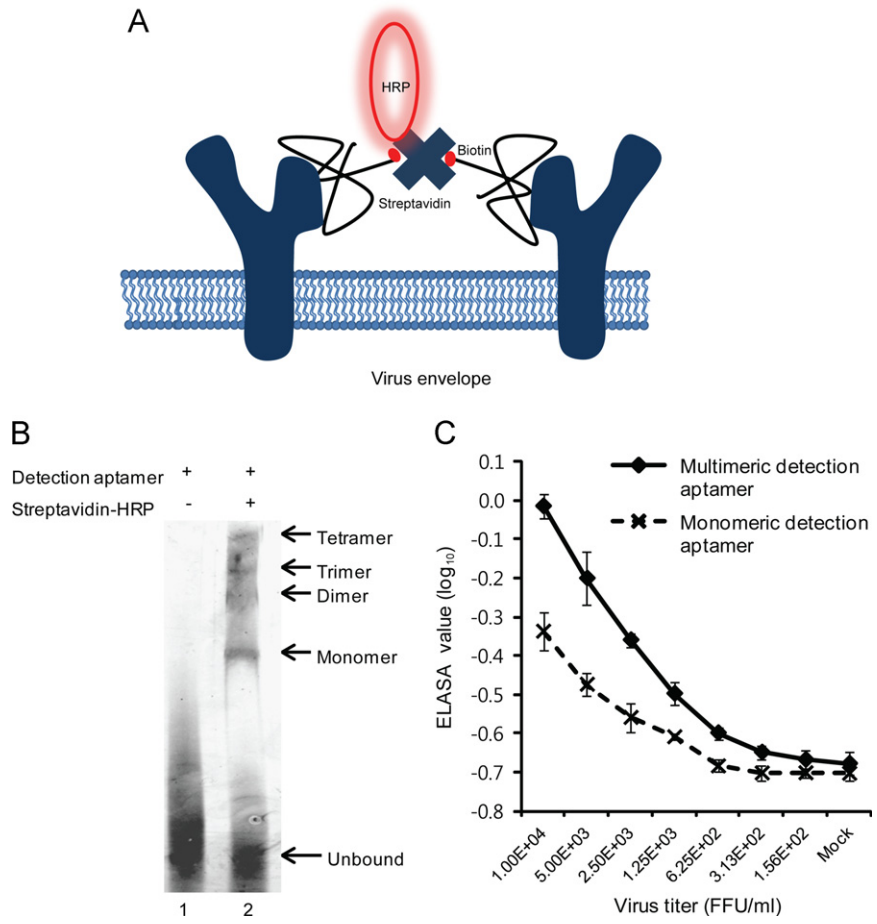

Fig. 3. Improvement of sensitivity of ELASA using multivalent detection aptamers. (A) Schematic diagram of ELASA using multivalent detection aptamers. The multivalent detecting aptamers were formed by pre-incubation of biotinylated detection aptamers and streptavidin that contains four biotin-binding sites. (B) Detection of streptavidin-aptamer complexes. Streptavidin-HRP and biotin-conjugated detecting aptamers were incubated at RT for 2 h and then resolved on a 6% denaturing urea polyacrylamide gel. The positions of aptamers were visualized by Sybro gold nucleic acid staining. The several forms (mono-, di-, tri-, and tetra-meric aptamer–streptavidin complexes) were detected. (C) The sensitivities of ELASA using monovalent or multivalent aptamers. HCV samples were serially diluted from 3.13×10^2 FFU/ml to 2.00×10^4 FFU/ml and ELASAs were performed. Chemiluminescent signals were measured at 450 nm. The ELASA values are depicted in logarithmic scale. The dots and lines represent the mean values and standard deviations from three independent experiments.

while the detection limit of the ELASA system with monovalent aptamers was $1.25–2.50 \times 10^3$ FFU/ml (Fig. 3C). These results indicated that the ELASA system using the multivalent detection aptamers has about 4 fold higher sensitivity than that using the monovalent detection aptamers. Therefore, the further experiments were performed by the multivalent detection aptamers.

The ELASA system detects purified HCV E2 proteins of various genotypes

We investigated whether the HCV ELASA system can detect and quantify the recombinant E2 protein of genotype 2a (JFH), which was used in the SELEX of HCV E2 aptamers. Proteins were serially diluted from 500 ng/ml to 4 ng/ml with $SB18T_{0.05}$. The E2 proteins of genotype 2a were quantitatively detected with the detection limit of approximately 16 ng/ml (Fig. 4A). Bovine serum albumin (BSA), which was used as a negative control, showed a basal level of signal even when tested at the highest concentration. Generally, aptamers recognize the tertiary structures of target molecules in contrast to most antibodies recognizing linear epitopes (Patel et al., 1997). Therefore, we investigated whether the HCV ELASA system can detect E2 proteins of various genotypes having diverse primary sequences since the tertiary structures of E2 proteins of various genotypes should share common features for receptor-binding (Pileri et al., 1998; Scarselli et al., 2002), entry (Bartosch et al., 2003; Hadlock et al., 2000), and assembly (Bianchi et al., 2011). The E2 of HCV genotype 1b (con1), which is the most prevalent genotype in the world (Zeuzem, 2004), and that of genotype 1a (H77), which is widely distributed in northern Europe and in the United States (Armstrong et al., 2006; Esteban et al., 2008), were detected equally well by the HCV ELASA system (Fig. 4C and B, respectively). These data suggested that the ELASA is suitable for detecting and quantifying HCV E2 proteins of various genotypes.

The HCV ELASA system detects HCV particles of genotypes 1a and 2a

To explore whether the HCV ELASA system is suitable for detecting virion particles of various genotypes, we investigated whether the ELASA can detect HCV containing E2 proteins of genotypes 1a and 2a. Viral stocks of genotypes 1a and 2a were subjected to the HCV ELASA. As a source of HCV particles with structural proteins of genotype 1a, we produced infectious HCV that is a chimera encoding structural proteins of H77S and nonstructural proteins of JFH (Yi et al., 2007', 2006). Virion particles of genotypes 1a and 2a were detected with detection limits of about $3.8–7.8 \times 10^2$ FFU/ml (Fig. 5B and A, respectively). These results showed that the ELASA specifically detect the virus associated E2 proteins. In conclusion, the above data suggested

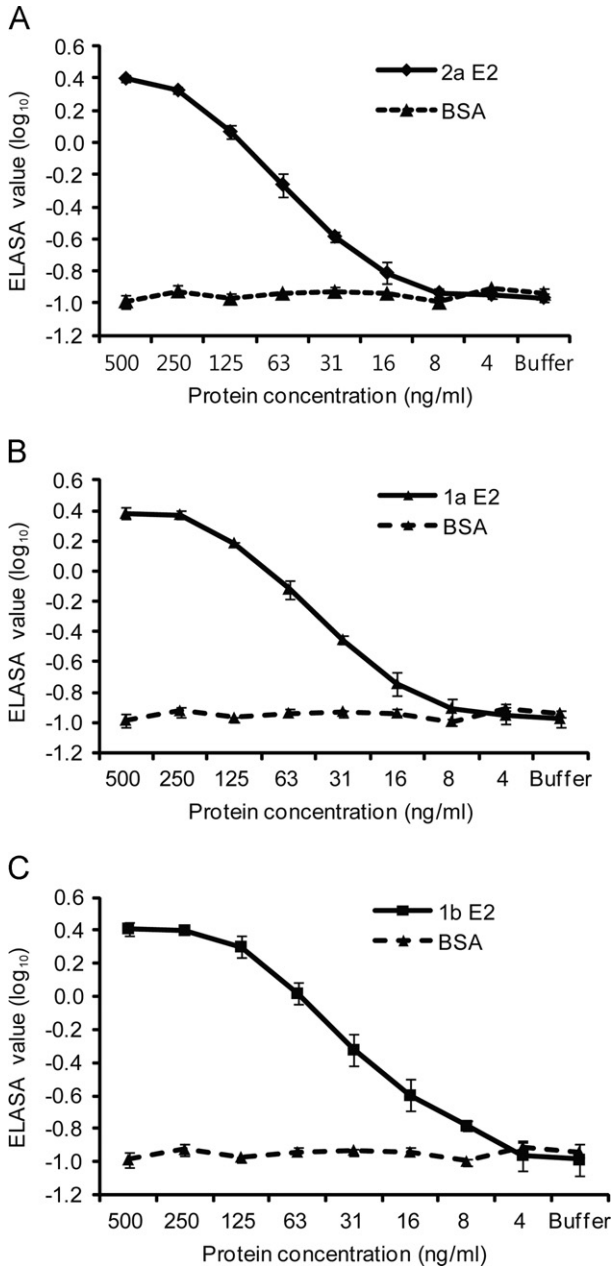

Fig. 4. Detection of purified E2 proteins of various genotypes (1a, 1b and 2a) by ELASA. The E2 proteins of various genotypes were purified by using a yeast expression system. The purified E2 proteins of genotype 2a (A), 1a (B), and 1b (C) were serially diluted from 4 ng/ml to 500 ng/ml with $SB18T_{0.05}$ and detected by ELASA. Bovine serum albumin (BSA) was used as a negative control. The Chemiluminescent signals were measured at 450 nm. The ELASA values are depicted in logarithmic scale. The dots and lines represent the mean values and standard deviations from three independent experiments.

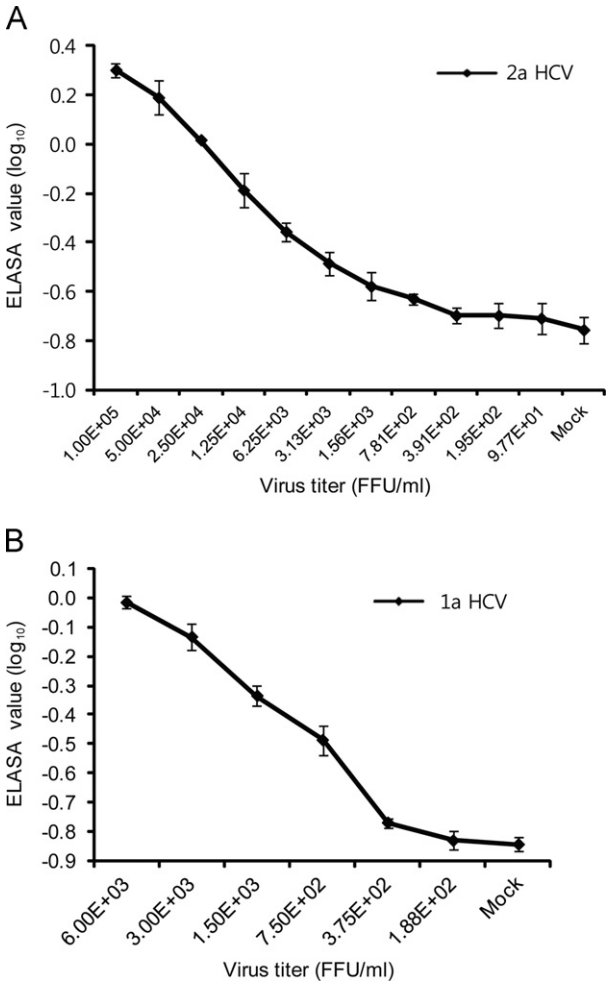

Fig. 5. Detection of HCV particles of genotype 2a and 1a by ELASA. HCV particles containing E2 of genotype 2a (A) or 1a (B) were concentrated by centrifugation and then re-suspended in SB18 (1:100 of the initial volume). HCV samples were serially diluted with $SB18T_{0.05}$ from 9.77×10^1 FFU/ml to 1.00×10^5 FFU/ml for genotype 2a virus or from 1.88×10^2 FFU/ml to 6.00×10^3 FFU/ml for genotype 1a virus, and the amounts of HCV particles were measured by ELASAs. The Chemiluminescent signals were measured at 450 nm. The ELASA values are depicted in logarithmic scale. The dots and lines represent the mean values and standard deviations from three independent experiments.

that the ELASA is suitable for detecting and quantifying HCV particles of various genotypes.

Monitoring HCV proliferation by ELASA

Quantitative analyses of virus proliferation are essential for monitoring the effects of medication of anti-viral drugs and for identifying anti-viral drug candidates during drug development. The quantification of HCV RNA by real-time RT-PCR and the titration of infectious virion particles are currently used for monitoring proliferation of HCV. However, these methods are laborious and time-consuming. Moreover, the amount of viral RNA and the infectious virus titer do not correlate well (Bukh, 2004; Lindenbach et al., 2005; Zhong et al., 2005). Recently, we reported that the specific infectivity (defined herein as focus-forming unit per viral RNA molecule) of HCV changes during HCV infection cycle (Keum et al., 2012). Therefore, a convenient assay system suitable for measuring the infectious dose of a HCV sample has been required in academic and medical fields.

We investigated whether the HCV ELASA is suitable for monitoring anti-HCV effects of anti-HCV drugs. We observed HCV proliferations using an *in vitro* HCV cultivation system (Keum et al., 2012) with or without treatment of anti-HCV agents interferon-alpha (IFN-α) and BMS-790052. IFN-α is currently used for treatment of chronic HCV infection, and BMS-790052 is a NS5A inhibitor in phase III clinical trials (Gao et al., 2010). The anti-HCV agents were treated for 5 days from day 0 when HCV was inoculated. The viral titers were measured by using the HCV ELASA system with the samples taken every day from the Huh 7.5.1 cells culture media inoculated with HCV [0.3 multiplicity of infection (MOI)]. The HCV titers increased gradually up to 5 days post infection in the mock-treated virus culture. On the other hand, viral titers remained at the basal level in the HCV culture

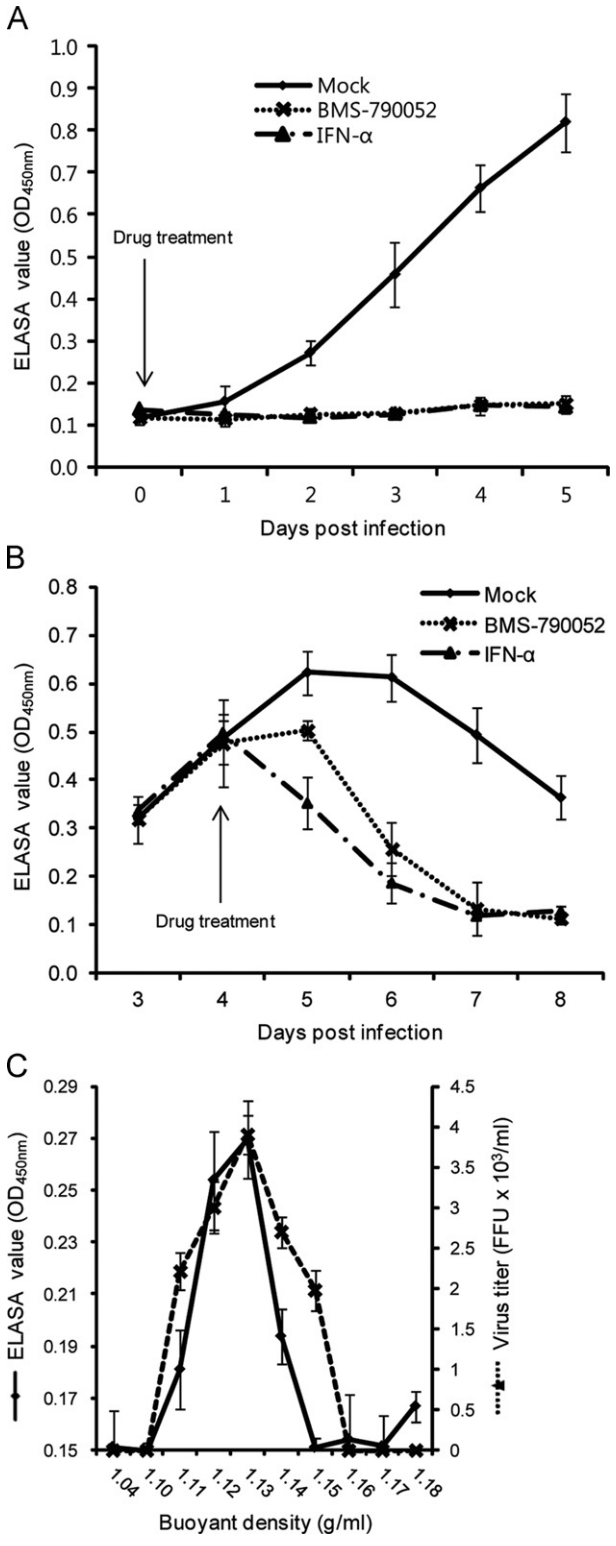

Fig. 6. Monitoring the effects of anti-viral drugs and characterization of virion particles using HCV ELASA. (A) HCV was cultivated in the presence or absence of antiviral drugs [BMS-790052 (1 nM) and interferon-α (500 IU/ml)] from the virus inoculation time. The amounts of HCV particles were monitored by the HCV ELASA until 5 days after infection. (B) The amounts of HCV particles were monitored by the HCV ELASA from 3 days post infection to 8 days post infection. Anti-HCV drugs were treated at 4 days after inoculation of HCV. (C) Comparison between ELASA values and infectious HCV titers measured by foci counting method in the fractions of a sucrose density gradient centrifugation. The ELASA values and the virus titers are depicted in $OD_{450\ nm}$ and FFU $\times 10^3$/ml, respectively. The dots and lines represent the mean values and standard deviations from three independent experiments.

treated with either IFN-α or BMS-790052 (Fig. 6A). Changes of viral titers, which were measured by foci formation, showed similar patterns to the ELASA values (Table S2 A).

We also monitored the curing processes of HCV-infected cells using the ELASA and the HCV cultivation system. Huh 7.5.1 cells inoculated with HCV (0.3 MOI) were cultivated for 2 days (about 70% of cells were infected at this time point), splitted on 12 well plates, and then cultivated for 2 more days. Infected cells were treated with IFN-α or BMS-790052, and then the HCV titers in the media were monitored by the ELASA. Precipitant decreases of viral titers were observed from the culture media at 2 days after the drug treatments (dotted lines in Fig. 6B). On the other hand, viral titer was increased in the culture media of mock-treated samples during this time (solid line in Fig. 6B). The viral titer in the mock-treated cell culture media was decreased after 7 days post infection due to the death of infected cells (Keum et al., 2012). Changes of viral titers, which were measured by foci formation, showed similar patterns to the ELASA values (Table S2B). These data indicated that the ELASA system is suitable for monitoring efficacies of anti-HCV drug candidates and for therapeutic follow-ups of anti-HCV drug treatments.

Density gradient analyses of viruses cultivated *in vitro* showed that virion particles with densities of 1.10 to 1.14 g/ml have the highest specific infectivity (Gastaminza et al., 2006; Grove et al., 2008; Lindenbach et al., 2005). On the other hand, the majority of viral RNAs was fractionated at buoyant densities between 1.15 g/ml and 1.17 g/ml (Keum et al., 2012). This indicates that most of the virus-related particles in the high density fractions are not infectious. We measured viral titers with the HCV ELASA (solid line in Fig. 6C) and with foci formation (dotted line in Fig. 6C). The viral titers measured by the ELASA and foci formation were peaked at buoyant density 1.11–1.14 g/ml as previous reported (Gastaminza et al., 2006; Grove et al., 2008; Lindenbach et al., 2005). These results indicated that the HCV ELASA can be used for analysis of HCV infectivity since the ELASA values well-matched with the viral infectivity values.

HCV infectivity correlates well with HCV ELASA value

HCV E2 has an essential role(s) in HCV infection of more than 95% of infectious HCV particles since neutralizing antibodies targeting E2 reduced infectivity of viral stocks more than 95% (Brimacombe et al., 2011). Therefore, it is plausible that the quantity of HCV E2 reflects the infectivity of a HCV stock unlike the quantities of core and HCV RNA that are known to exist in both infectious and noninfectious particles (Merz et al., 2011; Vieyres et al., 2010). We investigated the relationships among infectious viral dose, quantity of HCV RNA and ELASA value in the culture media at the indicated times after infection of HCV into Huh 7.5.1 cells (5 MOI). The infectious virus titers and the amount of HCV RNA were measured by foci formation and real-time RT-PCR, respectively. The ELASA values correlated well with the infectious virus titers regardless of the collecting time ($R^2=0.99$) (Fig. 7A). On the other hand, no correlation ($R^2=0.40$) was observed between the ELASA values and the quantities of viral RNA (Fig. 7B). In addition, no correlation ($R^2=0.39$) was observed between the virus titers and the quantities of viral RNA (Fig. 7C). In conclusion, the infectious virus titers strongly correlated with the ELASA values, and the HCV ELASA is a quick, easy and reliable method to measure the infectious dose of a HCV sample.

Discussion

Measuring infectivity of HCV samples using a conventional method (counting foci of HCV infected cells immunostained with

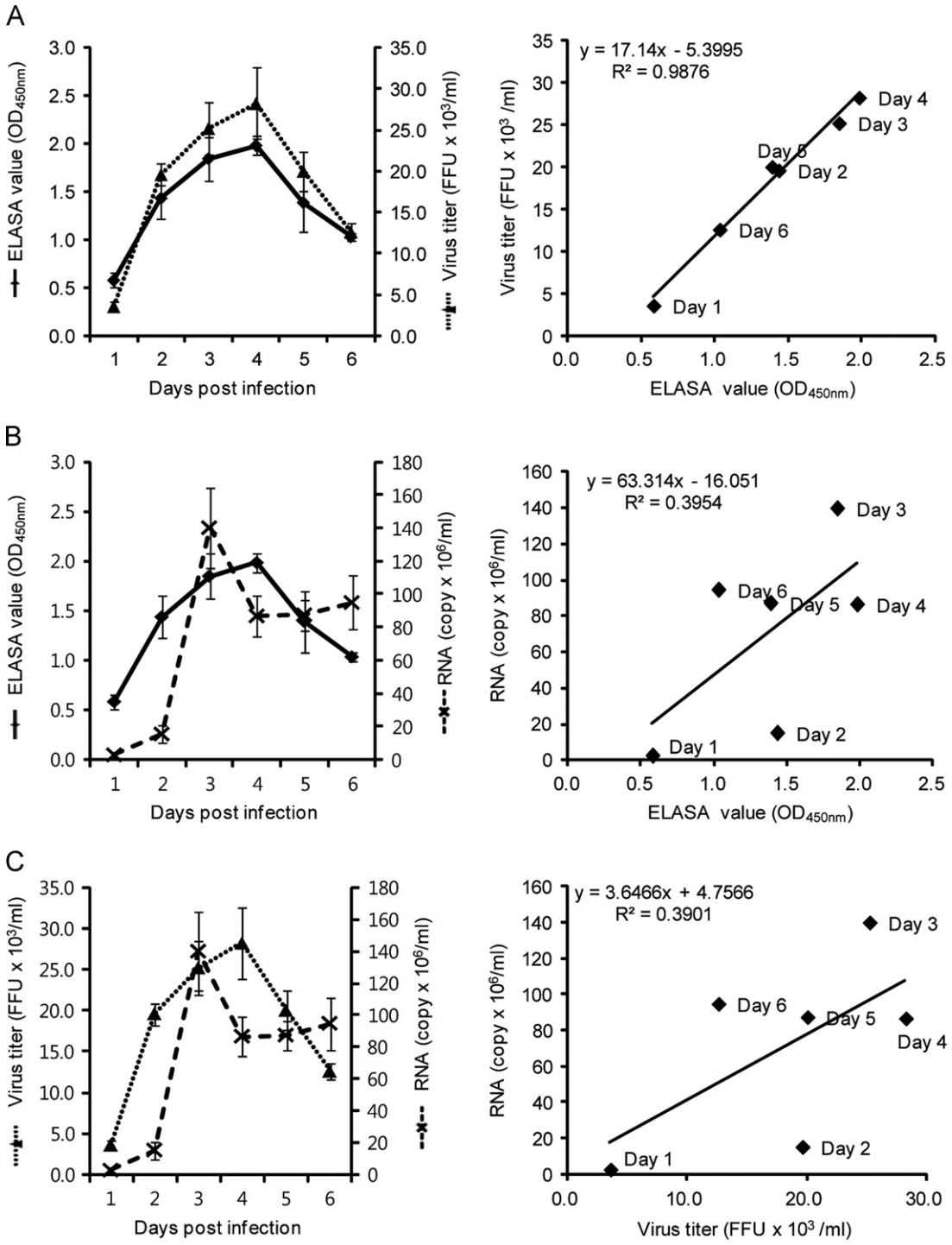

Fig. 7. Correlations among ELASA values, infectious HCV titer and the amount of HCV RNA. ((A)–(C)) Huh 7.5.1 cells were infected with HCV of genotype 2a (5 MOI), and the culture media were collected every 24 h for 6 days. ELASAs of the HCV samples were performed as described in Materials and methods. The amounts of HCV RNAs and infectious HCV titers were measured by quantitative RT-PCR and foci counting method, respectively. (Left panels) Infectious HCV titers and ELASA values (A), the amounts of HCV RNAs and ELASA values (B), and the amounts of HCV RNAs and infectious HCV titers (C) in media collected in various days after infection are depicted in separate graphs. (Right panels) Correlations between infectious HCV titers and ELASA values (A), the amounts of HCV RNAs and ELASA values (B), and the amounts of HCV RNAs and infectious HCV titers (C) are depicted. The correlation coefficient values (R^2 values) and the best-fitting lines by linear regression analyses are also depicted. The dots and lines represent the mean values and standard deviations from three independent experiments.

an antibody against a viral protein after cultivation of virus inoculated cells) takes long time (3–5 days), and the process is laborious. Here we report a quick-and-easy method for measuring the amount of infectious HCV. We developed an aptamer-based assay system named as ELASA. The advanced SELEX technology using BzdU instead of Thyamine, which was developed by Gold et al. (2010), was applied to obtain aptamers with high affinities. Four HCV E2-specific aptamers, which do not compete each other for binding to E2, were obtained from the advanced SELEX. No competition among the E2 aptamers indicates that the binding sites of aptamers on HCV E2 do not overlap each other. Among the different combinations of aptamer pairs, aptamers A and C did not show positive signals in detecting HCV particles in ELASA (Fig. 2B) even though these aptamers bind strongly to the purified HCV E2

proteins (Table 1). On the other hand, the aptamer pair B and D showed strong positive signals in the ELASA (Fig. 2B). An explanation for the discrepancy between the functionality of aptamers A and C in the ELASA system and the binding of the aptamers to purified E2 protein is as follows: Aptamers B and D, but not A and C, may bind to E2 proteins on HCV virion particles due to the following reasons. (1) Aptamers B and D, but not A and C, may bind to the native form of HCV E2 proteins on HCV particles. (2) The binding sites for aptamers A and C on the E2 protein might be shielded by other component(s) in virion particles such as the E1 protein that interacts with E2 (Beeck et al., 2000). (3) The binding sites of aptamers A and C might reside very close to the transmembrane domain of HCV E2, which sterically hinders binding of aptamers A and C to E2 proteins on virions. Related to this issue, it is worth to note that aptamers B and D, but not A and C, inhibit infection of HCV (data not shown). No effect of aptamers A and C on HCV infection could be attributed to an inability of these aptamers to bind to E2 proteins on HCV virions.

The HCV ELASA system using aptamers B and D could detect E2 proteins of different genotypes of HCV including genotypes 1a, 1b and 2a (Fig. 4). This indicates that the HCV ELASA system is useful for monitoring the infection of major genotypes of HCV (genotypes 1 and 2). Moreover, the HCV ELASA may be able to detect other genotypes of HCV such as genotypes 3–6 even though we have not tested whether the ELASA can detect these genotypes. The broad spectrum of the HCV ELASA in detecting various genotypes suggests that the aptamers B and D recognize the conserved regions in HCV E2 that are essential for infection of the virus. Indeed we found that both aptamers B and D blocked the infection of HCVcc into a Huh-7.5 cell (data not shown). On the other hand, the aptamers A and C, which could not recognize HCV virions, did not affect the HCVcc infection (data not shown). Determination of the binding sites of aptamers B and D on HCV E2 remains to be investigated.

One interesting aspect of the HCV ELASA is that the ELASA value correlates well with the infectious HCV titer of culture media but not with the HCV RNA titer (Fig. 7). This indicates that the ELASA system is a quick-and-easy method to measure the infectious virus titer of HCV culture media. The ELASA values showed linearity from 3.91×10^2 FFU/ml to 1.00×10^5 FFU/ml (Fig. 5A). This indicates that the quantification of infectious virus titer is possible within this range of infectious virus titer. The linear relationship between the infectious HCV titer and the concentration of the E2 protein suggests that the E2 protein has an important role(s) in infection of virion particles. Several roles of HCV E2 during infection of virus have been reported. E2 protein participates in the recognition of viral receptors such as CD81 and SRB1 (Pileri et al., 1998; Scarselli et al., 2002). Moreover, the E2-viral receptor interactions modulate host cell functions to facilitate virus infection (Liu et al., 2012). However, it is noteworthy that there exists a subpopulation of HCV virions that is not detected by the ELASA (fraction 1.15 g/ml in Fig. 6C). The infectious virion particles in this fraction 1.15 g/ml, which were not detected by the HCV ELASA, could be the same viral entity that can infect the neighboring cells in the presence of neutralizing E2 antibodies (Brimacombe et al., 2011). Further investigations are required to confirm this hypothesis.

Previously, an assay system for detection of HCV using an aptamer against HCV E2 was reported (Chen et al., 2009). Their system contained an antibody against HCV E2 (Li et al., 2007) as a capture of HCV and an aptamer as a detector. In other words, their system utilizes both an antibody and an aptamer. The ELASA system, on the other hand, utilizes two aptamers recognizing distinct parts of HCV E2 as a capture and a detector, respectively. Moreover, the previous report did not demonstrate the relationship between the infectivity of the HCV and the assay value.

In summary, we developed a HCV ELASA that is suitable for measuring infectious units in HCV stocks by using a pair of aptamers against HCV E2. This ELASA is a quick-and-easy assay system detecting both genotypes 1 and 2. Currently we are trying to find the right conditions for ELASA to quantify HCVs in HCV patients' sera.

Materials and methods

Modified systematic evolution of ligands by exponential enrichment (SELEX)

The advanced SELEX technology was used as described by Gold et al. (2010). For positive selection of the 1st round, 50 pmol of E2 protein was used in SELEX. For additional rounds of SELEX (rounds 2–8), the amount of protein was reduced to 10 pmol. Altogether, 8 rounds of SELEX were performed to enrich target-specific oligonucleotides.

Cloning and sequencing of selected aptamers

After 8 rounds of SELEX, aptamers were amplified by QPCR using 5′- and 3′-primers, and then cloned into TA cloning Kit (Solgent, Korea). Fifty colonies were picked for each sample and the cloned parts were sequenced by Solgent. Sequences were aligned using the 'aptamer motif searcher', an in-house program of Aptamer Sciences (Inc.), and a pattern analysis was performed.

Enzyme linked apto-sorbent assay (ELASA)

A pair of aptamers was used in developing an ELASA system. NH_2-aptamers (100 nm) were conjugated on the bottom of an Immobilizer™ Amino 96-well plate (Thermo scientific) by incubating in coupling buffer [100 mM sodium phosphate (pH 7.0), 120 mM NaCl, 5 mM KCl, and 5 mM $MgCl_2$] for 2 h at room temperature (RT). After washing the wells three times with phosphate-buffered saline (PBS) with 0.05% (w/v) Tween-20 (PBST), 100 μl of recombinant protein solutions or virus samples (HCVcc) in $SB18T_{0.05}$ [40 mM HEPES (pH 7.5), 120 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$ and 0.05% Tween-20] were added to each well and incubated for 1 h at RT. The wells were washed three times, and the mixture (100 μl) of the biotin-conjugated aptamer (10 nM) and streptavidin-conjugated HRP (0.5 μl, R&D System), which was pre-incubated for 2 h at RT, was added and then further incubated for 1 h at RT. The wells were washed three times. Finally, the samples were incubated with 100 μl of TMB solution (Invitrogen), and the chemiluminescent signals from HRP were measured at 450 nm.

Protein expression and purification

For constructions of plasmids expressing the luminal domains of E2 of genotypes HCV 1a, 1b and 2a, the nucleotides 1150–2175 of H77 (genotype 1a), nucleotides 1150–2175 of con1 (genotype 1b), and nucleotides 1150–2187 of JFH1 (genotype 2a) were amplified by PCR with 1a primer pair (5′-GAAACCCACGT-CACCGGGGG-3′ and 5′-CAGAAGGAACAGGAGAACG-3′), 1b primer pair (5′-GGAACCTATGTGACAGGGGG-3′ and 5′-AAGAAGGAAGAG-CAACAGGA-3′), and 2a primer pair (5′-GGCACCACCACCGTTG-GAGG-3′ and 5′-GAGCAGGAATAAGAGTACCA-3′), respectively. The PCR products were treated with Nhe1/Hind3 and cloned into the corresponding sites of plasmid p425-GPD containing 6 Histidine coding region and a FLAG tag. E2 proteins were expressed in yeasts (strain PBN204) after transforming the cells with plasmid p425-GPD-E2. Yeasts were harvested at 1 OD_{600} and resuspended

in lysis buffer [20 mM sodium phosphate (pH 7.5), 300 mM NaCl, 1 mM PMSF and 1% (w/v) Triton × -100]. E2-His-FLAG proteins were allowed to bind to talon metal affinity resin (Clontech Laboratories) for 2 h at 4 °C, and the resin-bound proteins were eluted by 200 mM Imidazole in lysis buffer. The eluted proteins were allowed to bind to ANTI-FLAG M2 affinity gel (Sigma Aldrich) in lysis buffer for 2 h at 4 °C and eluted by 100 ug/ml 3 × FLAG peptide (Sigma Aldrich) in lysis buffer. The proteins were dialyzed in PBS.

Production of infectious HCV

RNAs for production of HCVcc were synthesized by *in vitro* transcription using T7 RNA polymerase (Stratagene). The plasmids pJFH-m4 (Kim et al., 2007) and pH-(NS2)-J (Yi et al., 2006, 2007) were linearized with *Xba*I and then used as templates for transcription. The plasmid Jc1 E2 FLAG containing an infectious HCV sequence with a FLAG epitope in the E2 gene was linearized with Mlu1 (Merz et al., 2011) and then used as a template for transcription. Huh7.5.1 cells were transfected with RNAs as previously described (Zhong et al., 2005). At 3–5 days after transfection, the culture media containing viruses were collected, filtered through a 0.45 μm filter, and inoculated onto naïve Huh 7.5.1 cells for 5 h at 37 °C. The cells were cultivated for 48–72 h prior to the analyses of HCV infection. The infectious titers (focus forming units [FFU]/ml) of HCVcc were determined as described previously (Zhong et al., 2005).

RNA purification and real-time RT-PCR

Viral RNAs were isolated from clarified cell supernatants by trizol/chloroform extraction method using a TRI reagent (Invitrogen). Total RNAs were reverse transcribed using Improm II reverse transcriptase (Promega), and the cDNAs were subjected to real-time PCR analyses for quantification using Sybr premix Ex *Taq* (Takara). Primer sequences for reverse transcription-PCR and real-time PCR were 5′-GTCTAGCCATGGCGTTAGTA-3′ and 5′-CTCCCGGGGCACTCGCAAGC-3′, respectively.

Sucrose gradient density centrifugation

The HCV-infected cell culture medium was centrifuged at 4000 rpm for 7 min at 4 °C to remove cellular debris, and the supernatant was loaded on a 20% sucrose cushion and centrifuged in a 45Ti rotor (Beckman Coulter) at 36,000 rpm for 4 h at 4 °C. The pellets were resuspended in TNC buffer [10 mM Tris–HCl (pH 7.4), 150 mM NaCl, and 1 mM $CaCl_2$] and applied onto a 20–60% sucrose gradient (10.5 ml volume) in a SW41 tube (Beckman Coulter) and centrifuged at 40,000 rpm for 16 h at 4 °C. A total of 10 fractions (1 ml each) were collected from the top to bottom of the sucrose gradient. The sucrose in each fraction was diluted with PBS to a 1:12 ratio, and then the samples were centrifuged at 40,000 rpm for 16 h at 4 °C in a Beckman SW41 rotor. The resulting HCV pellets were dissolved in PBS and aliquoted for subsequent studies.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.01.014.

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