Hepatitis C virus (HCV) core protein is essential for virus assembly. HCV core protein was expressed and purified. Aptamers against core protein were raised through the selective evolution of ligands by the exponential enrichment approach. Detection of HCV infection by core aptamers and the antiviral activities of aptamers were characterized. The mechanism of their anti-HCV activity was determined. The data showed that selected aptamers against core specifically recognize the recombinant core protein but also can detect serum samples from hepatitis C patients. Aptamers have no effect on HCV RNA replication in the infectious cell culture system. However, the aptamers inhibit the production of infectious virus particles. Beta interferon (IFN-β) and interferon-stimulated genes (ISGs) are not induced in virally infected hepatocytes by aptamers. Domains I and II of core protein are involved in the inhibition of infectious virus production by the aptamers. V31A within core is the major resistance mutation identified. Further study shows that the aptamers disrupt the localization of core with lipid droplets and NS5A and perturb the association of core protein with viral RNA. The data suggest that aptamers against HCV core protein inhibit infectious virus production by disrupting the localization of core with lipid droplets and NS5A and preventing the association of core protein with viral RNA. The aptamers for core protein may be used to understand the mechanisms of virus assembly. Core-specific aptamers may hold promise for development as early diagnostic reagents and potential therapeutic agents for chronic hepatitis C.
In this study, we obtained aptamers for HCV core using \textit{in vitro} SELEX. The selected aptamers against HCV core specifically recognize the recombinant core protein and the serum samples of hepatitis C patients but also inhibit the assembly of virion. Further study shows that these aptamers exert antiviral activity through disruption of the localization of core with lipid droplets and NS5A and blockage of core protein binding to viral RNA.

\textbf{MATERIALS AND METHODS}

\textbf{Cells and reagents.} Huh7.5 cells and mouse monoclonal anti-NS2 antibody were kindly provided by Charles Rice (Rockefeller University, NY) (15), plasmids pJFH1 and pJFH1/GND plasmids were generously provided by Takaji Wakita (National Institute of Infectious Diseases, Tokyo, Japan) (10). Mouse monoclonal anti-NS5A antibody was from Chen Liu (University of Florida).

\textbf{Expression and purification of core protein.} The protein expression vector of full-length core was constructed by PCR amplification using pJFH1 plasmid as the template. The full-length core sequence tagged with 6 His molecules at the N terminus was PCR amplified with primers, digested with NdeI and EcoRI, and inserted into pCM1 to produce pCM1-core. Core protein was expressed in \textit{Escherichia coli} BL21(DE3) cells (Invitrogen, Carlsbad, CA) and purified. The protein was identified using anti-His antibody (Sigma, St. Louis, MO) via Western blotting.

\textbf{In vitro selection of aptamer against core protein.} The synthesized DNA library pool with an overall complexity of \(\approx 10^{10}\) was used for \textit{in vitro} selection. The sequence of the random DNA is 5’-ACGCTCGGATGCCA CTACAG(N40)CTCATGGACGTGCTGGTGA-3’, where N40 represents 40 nucleotides with equimolar incorporation of A, G, C, and T at each position. The selection and amplification procedure was performed as previously described (16, 17). After 8 rounds of selection, the amplified DNA was cloned and several clones were sequenced.

\textbf{ELONA.} For enzyme-linked oligonucleotide assay (ELONA), streptavidin-precoated microtiter plates were coated with biotin-labeled aptamer. Serial dilutions of His-tagged core protein or patient serum were added into the plates and incubated at 37°C for half an hour. After washing to remove the unbound target protein, mouse monoclonal anti-His or core antibody was added into the plates at 37°C for 1 h. Peroxidase-labeled goat anti-mouse IgG was added and incubated at 37°C for 30 min. Color development was performed by addition of freshly prepared substrate solution for 10 min at room temperature. After stopping the reaction with stopping buffer, the plates were read with an enzyme-linked immunosorbent assay (ELISA) reader and the absorbance of each sample was measured at 450 nm.

\textbf{Serum samples.} The sera from HCV-infected patients, hepatitis B virus (HBV)-infected patients, or healthy donors were collected from 2009 to 2012 in Hunan Provincial Tumor Hospital. The experiments were done with the approval of the Ethics Committee of Hunan Provincial Tumor Hospital.

\textbf{MTS assay.} For the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay, the protocol for the FFU assay was performed previously (18). Briefly, cells were washed with PBS and lysed in lysozyme buffer supplemented with protease inhibitor cocktail. Cell lysates were incubated at 4°C for 30 min and centrifuged at 12,000 \times g at 4°C for 15 min. The lysate was diluted before beginning coimmunoprecipitation assay (co-IP) to 2 \(\mu\)g/ml total cell protein with PBS. Two hundred micrograms of lysates was immunoprecipitated with antiserum antibody. The immunocomplex was captured by adding protein G agarose beads slurry. The proteins binding to the beads were boiled in 2× Laemmli sample buffer and then subjected to SDS–12% PAGE. The protocol for immunoblotting is described above.

\textbf{Intracellular virus preparation.} At the indicated time postinfection, cells were washed thrice with PBS and incubated with fresh trypsin-EDTA for 2 min at 37°C. Cells were suspended in PBS and collected by centrifugation at 2,000 rpm for 3 min. The cell pellet was resuspended in Dulbecco’s modified Eagle medium (DMEM)–10% fetal bovine serum (FBS) and lysed by four freeze-thaw cycles in liquid nitrogen and a 37°C water bath, respectively. Cell debris was pelleted by centrifugation for 5 min at 4,000 rpm. The supernatant was collected and used for the focus-forming unit (FFU) assay described below.

\textbf{Co-IP and immunoblotting.} HCV-infected Huh7.5 cells were washed thrice with ice-cold PBS and lysed in lysis buffer supplemented with protease inhibitor cocktail. Cell lysates were incubated at 4°C for 30 min and centrifuged at 12,000 \times g at 4°C for 15 min. The lysate was diluted before beginning coimmunoprecipitation (co-IP) to 2 \(\mu\)g/ml total cell protein with PBS. Two hundred micrograms of lysates was immunoprecipitated with antiserum antibody. The immunocomplex was captured by adding protein G agarose beads slurry. The proteins binding to the beads were boiled in 2× Laemmli sample buffer and then subjected to SDS–12% PAGE. The protocol for immunoblotting is described above.
FIG 1 Selection of aptamers against HCV core protein and binding affinity of the aptamers. (A) His-tagged core was expressed by IPTG (isopropyl-β-D-thiogalactopyranoside) induction in E. coli BL21(DE3). The core protein and control protein LacZ were separated on SDS-PAGE gel and stained using mouse anti-His monoclonal antibody via Western blotting. (B) FITC-labeled DNA pools from library round 1 and round 8 were incubated with agarose beads conjugated with HCV core or control protein LacZ in binding buffer. The density of the fluorescence was measured and normalized to library. (C) Binding affinity of core aptamers. Each biotin-labeled aptamer was added to the microtiter plate, and an ELONA was performed. Purified His-tagged core, NS5A, or control protein LacZ was added to the plates. Mouse monoclonal anti-His antibody and HRP-conjugated goat anti-mouse IgG were used as primary and secondary antibodies, respectively. Color development was performed, and the plates were read with an ELISA reader. The absorbance of each sample was measured at 450 nm and normalized to library. The data represent the averages of five different experiments. **, \( P < 0.01 \) versus library. (D) Binding affinity of core aptamers to core protein from lysates of HCV-infected hepatocytes. Biotin-labeled library, Cnew, C4, C7, C42, C97, C103, or C104 was added to the microtiter plate previously coated with streptavidin. Lysates of HCV-infected Huh7.5 or noninfected Huh7.5 cells were added to the plates. After washing, mouse anti-HCV core, NS2, or NS5A monoclonal antibody was added and incubated at 37°C for 1 h. HRP-conjugated goat anti-mouse IgG was added to the plates. The data were obtained as described for panel C and represent the means of 3 different experiments. **, \( P < 0.01 \) versus library.
and RNA was isolated. RNA was used as the template for viral RNA detection by real-time PCR as described above.

**Statistical analysis.** Differences between means of reading were compared using the Student *t* test. Error bars represent standard deviations.

**RESULTS**

**Cloning and purification of HCV core.** HCV core gene was amplified and cloned into the expression vector pLM1. Core protein was expressed and purified by its N-terminal His tag. The purified core protein and control LacZ protein were detected by immunoblot analysis (Fig. 1A).

**Selection of aptamers against HCV core protein.** A nucleotide library was obtained from a pool of ~10^14 single-stranded DNA molecules containing a random fragment of 40 nucleotides flanked by 5′ and 3′ common primers as conserved linkers to amplify the selection process. The DNA library was mixed with beads conjugated with His-tagged core protein. Core-DNA complexes were precipitated with beads, and pellets were washed. DNAs were recovered, amplified with PCR, and used for the next rounds of selection. To remove the nonspecifically bound DNA, we applied the counterselection step using control protein LacZ in each cycle. After 8 rounds of selection, there was an increase in the binding of round 8 DNA pools to core protein compared to that of round 1 pools (Fig. 1B). The selected aptamers were cloned and sequenced. We selected some aptamers and named them Cnew, C4, C7, C42, C97, C103, and C104. Their sequences are listed in Table 1.

**Analysis of binding affinity of aptamers for HCV core protein.** An ELONA was performed to show the binding affinity of aptamers for core protein. Aptamers Cnew, C4, C7, C42, C97, C103, and C104 showed a higher affinity for recombinant core protein than control library, while they did not display apparent binding affinity for recombinant LacZ or NS5A protein (Fig. 1C). Various aptamers bound to core protein, and this interaction was retained in the presence of excess yeast tRNA in the binding buffer, suggesting that their binding to core was specific. We examined whether the aptamer for core could bind core protein in lysates of HCV-infected hepatocytes. The aptamers showed specific binding affinity to core protein from lysates of virally infected cells in comparison with the control library, while they displayed no binding affinity for NS2 or NS5A protein from lysates of virally infected cells (Fig. 1D).

**Detection of core protein in serum samples from HCV-infected patients by core aptamer.** A standard curve for core detected by ELONA or ELISA is shown. Both ELONA and ELISA were performed to determine the detection limitation of HCV core protein using C7 aptamer. The absorbance of each sample was measured at 450 nm. A standard curve was created by plotting the mean absorbance for each standard concentration (y axis) against the core protein concentration (x axis). Mean values and standard deviations for three independent experiments performed in triplicate are shown (Fig. 2B).

**Inhibition of infectious virus production by core-specific aptamers.** Our data showed that core aptamers specifically bind to core protein, so we decided to test whether the aptamers have an effect on HCV infection in an infectious cell culture system. There was no significant difference in the intracellular and extracellular viral RNA levels between aptamer-treated cells and the control library group (Fig. 3A and B). Uptake efficiency for C4 and C7 by HCV-infected Huh7.5 cells is shown in Fig. 3C. Colocalization of core-specific aptamers with core protein in the HCV-infected hepatocytes was demonstrated in Fig. 3D.

---

**TABLE 1 Sequences of core aptamers**

<table>
<thead>
<tr>
<th>Aptamer designation</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cnew</td>
<td>AGTATGCTGTGTCTATCTCAGAGCTCTGCAAGCACATGTGACACAGG</td>
</tr>
<tr>
<td>C4</td>
<td>GACGGCCAGAGGCGCCCTGATCTTCAGCAGGAGGACAGG</td>
</tr>
<tr>
<td>C42</td>
<td>CATATCAGCACAAGACAAAACACATACTACAAGCAGG</td>
</tr>
<tr>
<td>C97</td>
<td>TAAACACACACACAAATGGAAACACAGGAGAGAAGG</td>
</tr>
<tr>
<td>C104</td>
<td>CCAAATCTACCCGAAAAACACCTccccctGTAATAGC</td>
</tr>
<tr>
<td>C7</td>
<td>ACTATACCAAAAAATACAGGAGGACGAGAAAAACACAAAC</td>
</tr>
<tr>
<td>C103</td>
<td>TACCACACATGCAGACACCCACAAAAATACATAGAGAGAGAG</td>
</tr>
</tbody>
</table>

**FIG 2** Detection of HCV core in serum samples from HCV-infected patients by core aptamer. (A) A standard curve for core detected by ELONA or ELISA is shown. Both ELONA and ELISA were performed to determine the detection limitation of HCV core protein using C7 aptamer. The absorbance of each sample was measured at 450 nm. A standard curve was created by plotting the mean absorbance for each standard concentration (y axis) against the core protein concentration (x axis). Mean values and standard deviations for three independent experiments performed in triplicate are shown. (B) Detection of core in the serum samples from hepatitis C patients by core aptamer and HCV viral titer is proportional to core protein concentration in the samples. Streptavidin-precoated microtiter plates were coated with biotin-labeled core aptamer C7. The serum samples from HCV-infected patients, HBV-infected patients, or healthy donors were added into the plates. Color development was performed, and the absorbance of each sample was measured at 450 nm. HCV RNA copy numbers in the sera measured by quantitative real-time PCR were plotted against core concentration in serum samples.
FIG 3 Inhibition of infectious virus production by aptamers against core. (A and B) Effects of core aptamers on the intracellular (A) or extracellular (B) viral RNA level in hepatocytes. JFH1 virus suspension at a multiplicity of infection (MOI) of 0.1 was used to infect Huh7.5 cells for 3 days. The cells were treated by aptamer or library for 72 h. Intracellular or extracellular HCV RNA was measured by real-time PCR. The data represent the means of 3 different experiments. (C) Uptake efficiency of core-specific aptamers by HCV-infected hepatocytes. HCV-infected Huh7.5 cells were inoculated with different doses of Cy5-labeled aptamers. At the indicated time points, the virus-containing supernatant and the cells were collected separately. The cells were suspended in 1 ml fresh DMEM and then subjected to four freeze-and-thaw cycles to collect the intracellular Cy5-labeled aptamers. The intracellular and extracellular supernatants were transported to the black 96-well plate at 100-μl volume per well. Then, the extracellular and intracellular Cy5 signal absorbances at 647 nm were measured by microplate reader. The percentages of the intracellular and the extracellular efficiencies were relative to the positive ones, which were the fresh culture media to which were added the Cy5-labeled aptamers at the same concentration. The data represent three independent experiments performed in triplicate. (D) Colocalization of core-specific aptamers with HCV core protein in the HCV-infected hepatocytes. HCV-infected Huh7.5 cells were treated with Cy5-labeled aptamers for 24 h. The cells were fixed with ice-cold acetone for 10 min at −20°C. The cells were washed with PBS, blocked with 1:50 goat serum for 30 min at room temperature, and then incubated for 1 h with mouse monoclonal anticore antibody. The cells were stained with Texas Red-labeled goat anti-mouse antibody for 45 min at room temperature. The nuclei were counterstained with DAPI. Fluorescent images were obtained under a fluorescence microscope. (E and F)
As a surrogate for virus production, we quantified the secretion of core protein into the culture medium of virus-infected cells in either aptamer-treated cells or the control group using ELISA. There were no differences in the levels of extracellular core protein between aptamer-treated cells and the control group (Fig. 3E). To determine whether the secreted core protein was consistent with the expression of intracellular core protein, we measured the intracellular core protein using ELISA. No difference in the intracellular core protein level between the aptamer-treated and the control groups was observed (Fig. 3F). The release efficiency (the percentage of extracellular core over total core protein) for the aptamer-treated cells was comparable to that of the library-treated group.

HCV core protein plays an essential role in infectious virus production (20). To determine whether core aptamers affect infectious virus production, we collected the supernatants of virus-infected Huh7.5 cells in the presence of core aptamers or library and used them to infect naive Huh7.5 cells. The titers of infectious viruses in the supernatants of naive Huh7.5 cells were determined. The levels of extracellular infectious viruses were reduced in the cells with aptamer treatment in comparison with library-treated cells (Fig. 3G). To examine whether a decrease in infectious HCV in the supernatants was attributable to defective virion assembly, we determined cell-associated infectivity. Intracellular infectious virions in aptamer-treated cells were lower than those in library-treated cells (Fig. 3G). The concentration of aptamers used in the study showed no apparent toxic effect to the cells (Fig. 3H). All the data indicate that core-specific aptamers inhibit the infectious virus production by suppressing the assembly of infectious virus particles. Aptamers did not affect hepatitis B viral DNA infection (Fig. 3I), implying that aptamers against core specifically inhibit HCV infection.

HCV core aptamers do not induce IFN-β and interferon-stimulated genes (ISGs) in virus-infected hepatocytes. Our data showed that aptamer against core inhibits the assembly of infectious viral particles. The presence of DNA molecules inside or outside the cells may cause a nonspecific induction of IFN, which is likely to lead to an antiviral effect. To exclude the possibility that inhibition of infectious virus production by core aptamers is due to the aptamer-induced innate response, we examined the expression of IFN-β in aptamer- or library-treated cells. IFN-β was not induced in aptamer-treated hepatocytes (Fig. 4A). Core-specific aptamers did not induce G1P3 and 1-8U in HCV-infected hepatocytes (Fig. 4B and C). All the data suggest that the inhibition of infectious virus production by core aptamers is not due to aptamer-induced innate response.

Domain I and domain II of core protein are involved in the antiviral effects mediated by core-specific aptamers. To identify the residues of core protein involved in aptamer binding and inhibition of virus assembly, we generated different truncated versions of core. The expression of different truncated versions of core protein was confirmed using Western blotting (Fig. 5A). ELONA was used to determine the truncated version to which the aptamers bind. Deletion of domain III of core did not affect the affinity for core aptamers. Domain I and domain II of core are involved in the binding of aptamers to the protein (Fig. 5B). The data suggest that the binding region of aptamers may localize inside domains I and II of core protein. To further confirm that core aptamers bind to domains I and II of core protein and inhibit the production of infectious viral particles, we conducted competition experiments. The levels of both extracellular and intracellular infectious viruses in cells transfected with plasmid containing domains I and II of core with aptamer treatment were markedly higher than those in control cells (Fig. 5C). All the data suggested that domain I and domain II of core protein are involved in the inhibition of virus assembly by core-specific aptamers.
Core aptamers disrupt the localization of core with lipid droplets and block the interaction between core and NS5A protein. The region of core required for association with lipid droplets, a prerequisite of virion production, is located in domain II. Localization of core protein with lipid droplets is critical for infectious HCV production (21). Disrupting the interaction between core and lipid droplet may prevent virion production. To address this hypothesis, we examined the subcellular localization of core protein with lipid droplets in HCV-infected Huh7.5 cells with aptamer treatment by immunofluorescence analysis. Core colocalized with lipid droplets in cells with library treatment, whereas their localization was decreased in aptamer-treated cells (Fig. 6A).

Interaction of core with NS5A protein is critical for infectious virus production (20). Disruption of the interaction of core with NS5A protein may decrease infectious virus production. To prove this, we analyzed the interaction of core with NS5A protein by co-IP experiments. The amounts of core or NS5A protein were comparable in aptamer-treated or library-treated cells (Fig. 6B). Core protein in immunoprecipitates with an equal amount of NS5A protein in aptamer-treated HCV-infected Huh7.5 cells was lower than that in the library group (Fig. 6B). The data showed that core-specific aptamers block the association of core protein with viral RNA, thereby inhibiting the assembly of infectious viral particles.

Isolation and characterization of JFH1-resistant variants. Viral target-based inhibitor allows for the selection of resistant viruses. To identify amino acid mutations that confer resistance to core aptamer, JFH1-infected Huh7.5 cells were treated with 100 nM C4 for 3 weeks. Control cells were maintained with 100 nM library. Mutations within core associated with reduced suscepti-
bility to C4 were selected and identified by sequence analysis of core cDNA isolated by RT-PCR from control and aptamer-treated cells. The substitution at core residue 31 (V31A substitution) was identified.

To evaluate the contribution of the selected specific amino acid substitution to resistance, the V31A substitution was introduced into JFH1. The sensitivity of the variant to C4 was assessed in the infectious cell culture system. The V31A substitution caused a decrease in C4 potency (Fig. 8). The data suggested that the selected V31A substitution within core is the major resistance substitution identified.

DISCUSSION

Although the common serological tests to detect HCV infection rely on the detection of antibodies against the virus, these tests cannot distinguish between individuals who have resolved their infection and those who remain actively infected with the virus. The HCV core antigen test detects circulating core protein and...
The core protein (aa 2 to 114) of genotype 1a. RNA aptamer is whereas those obtained in the other study were isolated by using the core (aa 1 to 191) from genotype 2a JFH1, selected by using the core protein, since our aptamers were sequenced by the source of core protein, whereas our aptamers were used in their study. Moreover, the differences might be due to the selection of core protein, the detection of HCV infection, and the suppression of core protein with viral RNA. Our aptamers have been reported to inhibit the detection of HCV infection against core protein in our study, while the RNA aptamers for core protein, although detection of HCV core present in serum is highly sensitive and specific (3). An aptamer-based detection assay is recognized as a promising molecular diagnostic method (23).

Many chronic hepatitis C patients do not respond to the current therapy. Future regimens will incorporate multiple new agents directly targeting the virus to increase the efficacy of treatment. The protease inhibitors have entered into the clinic for HCV-infected individuals recently (24). There will be plenty of patients resistant to the current therapy (25, 26). It is desirable to seek a combination therapy for HCV infection that targets different steps of the virus life cycle. It is logical to design antiviral therapies targeting the core and cellular proteins critical for the virus assembly.

Here, we reported the selection of aptamers against HCV core protein, the detection of HCV infection, and the suppression of infectious virus production by these aptamers. Our aptamers have sequences that are different from other recently described RNA aptamers selected for core (23). The difference might be due to the selection technique used. We used SELEX to select DNA aptamers against core protein in our study, while the RNA aptamers for core were used in their study. Moreover, the differences may be attributed to the source of core protein, since our aptamers were selected by using the core (aa 1 to 191) from genotype 2a JFH1, whereas those obtained in the other study were isolated by using the core protein (aa 2 to 114) of genotype 1a. RNA aptamer is more unstable than DNA aptamer and is particularly susceptible to nuclease degradation.

This study showed that our aptamers bound core protein of genotype 2a. Importantly, the core-specific aptamers can detect the virus in serum samples from patients infected by HCV with different genotypes, suggesting that different genotypes of HCV share aptamers binding sites. This method is highly consistent with the detectable result of HCV RNA in the serum samples of HCV patients. The data indicate that our aptamers may be utilized to diagnose early HCV infection by detecting core antigen present in the serum during the early stage of infection and before virus clearance.

Our data showed that core-specific aptamers disrupt the localization of core with lipid droplets and prevent the association of core protein with viral RNA. Although the aptamers against core were found to bind core protein, the details of the residues involved in the interaction between aptamers and core protein remain to be explored. Determination of these residues may provide knowledge about the essential functional regions of core protein. In addition, the aptamers can be used with core protein to understand the mechanisms of virus assembly and the interaction between core and cellular proteins. Anti-HIV Gag protein aptamers can be used to examine the Gag-HIV RNA interactions (27). The interaction between the aptamers for HIV reverse transcriptase and reverse transcriptase provides a model for the study of the mechanisms of how these aptamers act as broad-spectrum inhibitors of HIV reverse transcriptase (28). All these examples illustrate the potential use of aptamers in virology.

One recent study reported that inhibition of viral infection by aptamers might be due to the aptamer-induced innate immune response (29). However, most studies suggest that inhibition of virus infection by aptamers targeting different viral proteins can be attributed to suppression of viral protein by the aptamers themselves (30–33). Consistent with these reports, our study showed that the aptamers for core protein do not induce IFN-β and ISGs, indicating that inhibition of infectious virus production by core aptamers is not due to the innate immune response.

In summary, our study provides the first evidence of direct antiviral activity of aptamers for HCV core protein in the infectious cell culture system. These results demonstrate the power of the SELEX approach for the selection of inhibitors for viral infection and the exploration of the mechanisms of viral replication. The data suggest that aptamers against core protein inhibit the production of infectious viral particles by disrupting the localization of core with lipid droplets and preventing the association of core protein with viral RNA. Core-specific aptamers may hold promise for development as early diagnostic reagents for HCV infection and potential therapeutic agents for hepatitis C patients.

ACKNOWLEDGMENTS

We acknowledge Charles Rice for the gift of Huh7.5 cells and Takaji Wakita for pJFH1 and pJFH1/GND plasmids. We thank Chen Liu for helpful discussions.

This work was supported by a National Science and Technology Major Project of the Ministry of Science and Technology of China (2009ZX10004-312) and by the National Natural Science Foundation of China (81271885).

REFERENCES


