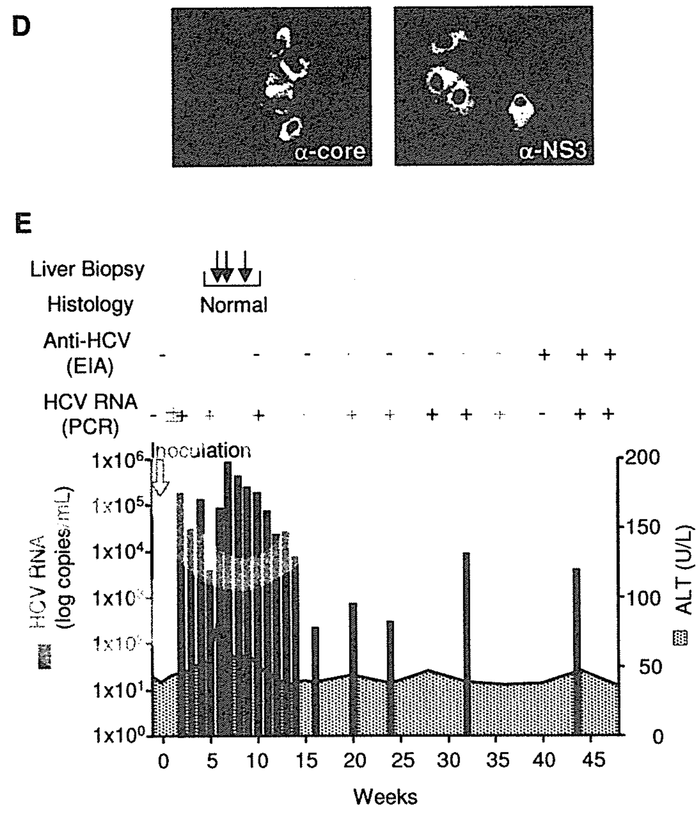

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Figure 1

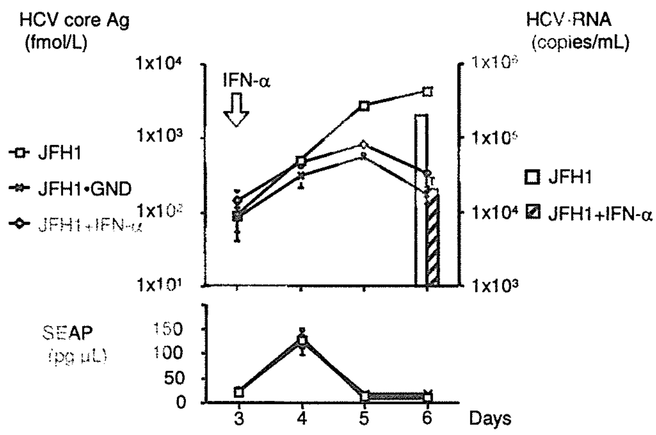

Figure 2

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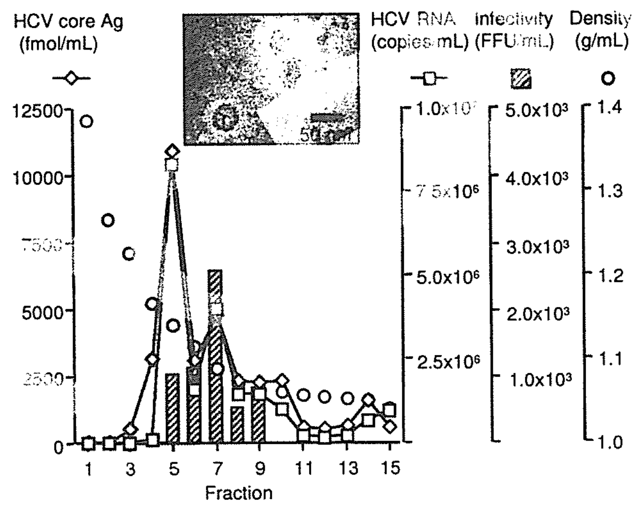

Figure 3

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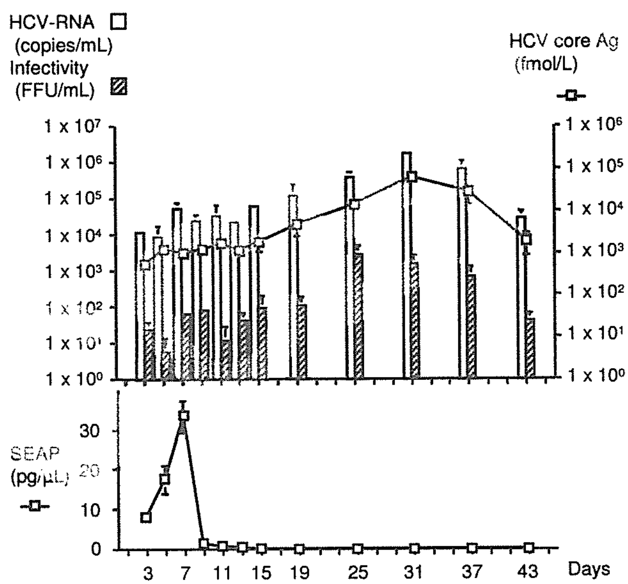

Figure 4

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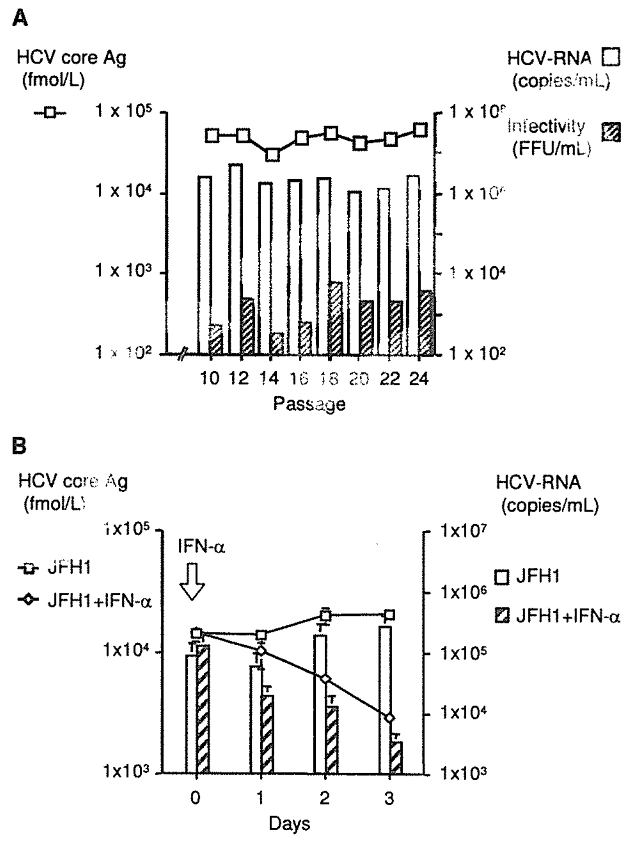

Figure 5

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Journal of Medical Virology 9999:1–10 (2007)

The Roles of CD81 and Glycosaminoglycans in the Adsorption and Uptake of Infectious HCV Particles

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Because appropriate cell-culture systems or small-animal models have been lacking, the early steps in the HCV life cycle have been difficult to study. A cell culture system was developed recently that allows production of infectious HCV. In this study, infectious HCV particles produced in cultured cells were used. To clarify the role of CD81 in HCV attachment and entry, the effect of anti-CD81 antibody was examined. The antibody blocked HCV virion entry but not particle attachment. Only the fraction bound to a heparin affinity column and eluted with 0.3 M NaCl productively infected Huh7 cells, indicating that infectious HCV particles bind to heparin. Both heparin treatment of the virus particles and heparinase treatment of the Huh7 cells reduced virus-cell binding without substantially inhibiting HCV infectivity. Finally, to confirm the role of both heparin sulfate proteoglycan (HSPG) and CD81 in HCV entry, the effects of heparinase I and anti-CD81 antibody were analyzed. No productive RNA replication was detected in the Huh7 cells in the presence of both heparinase I and anti-CD81 antibody. In conclusion, these data suggested that both HSPG and CD81 are important for HCV entry. HSPG may play a role in the initial cell surface binding of infectious HCV particles and CD81 is conceivably correlated with HCV entry after viral attachment. ***J. Med. Virol. 00:1–10, 2007.*** © 2007 Wiley-Liss, Inc.

KEY WORDS: hepatitis C virus; virus entry; infectious HCV particle; HSPG; CD81

INTRODUCTION

Hepatitis C virus (HCV) causes a variety of liver diseases in humans [Choo et al., 1989; Kiyosawa et al., 1990; Saito et al., 1990; Tsukuma et al., 1993], infecting chronically an estimated 170 million persons worldwide [WHO, 2000]. A vaccine for prevention of infection is not available and the most optimal therapy for HCV infection, polyethylene glycol-conjugated alpha interferon (IFN-α) and ribavirin, is successful in only about 50% of treated patients [Manns et al., 2001; Fried et al., 2002]. Although HCV is a very important human pathogen, progress in the understanding of this virus has been slow. The lack of a robust cell culture system to produce infectious virions has hampered the development of anti-viral therapy and research into viral mechanisms.

Several candidate receptors for HCV entry have been proposed, including the cell marker tetraspanin CD81 [Pileri et al., 1998], low density lipoprotein (LDL) receptor [Agnello et al., 1999; Monazahian et al., 1999], scavenger receptor class-B type-I (SR-BI) [Scarselli et al., 2002], the mannose binding lectins DC-SIGN and L-SIGN [Gardner et al., 2003; Lozach et al., 2003; Pohlmann et al., 2003], glycosaminoglycans (GAGs) [Germi et al., 2002a,b; Barth et al., 2003], and asialoglycoprotein receptor [Saunier et al., 2003]. Because of the lack of a cell-culture system or small-animal model in HCV studies, truncated E2 recombinant proteins or virus-like particles or HCV pseudo-particles (HCVpp) have been used as models to study virus-cell or virus-protein interactions. But the results obtained after using the recombinant E2 protein have differed from those after using virus-like particles or HCVpp [Wunschmann et al., 2000; Wellnitz et al., 2002; Bartosch et al., 2003a,b; Hsu et al., 2003; Cormier

Abbreviations used: HCV, hepatitis C virus; GAG, glycosaminoglycan; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; HCVpp, HCV pseudo-particle.

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et al., 2004; Zhang et al., 2004; Lavillette et al., 2005], raising the suspicion that these models might not reflect the in vivo phenomenon. Recently a cell culture model for HCV was developed using the JFH-1 genotype 2a strain of HCV [Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005], allowing investigation of the viral life cycle.

CD81 is a tetraspanin present on nearly all nucleated cells [Levy et al., 1998] and forms multimolecular complexes with a wide variety of cell surface receptors. The interaction between the HCV envelope glycoproteins and CD81 has been documented and confirmed [Flint et al., 1999; Flint and McKeating, 2000; Flint et al., 2000; Yagnik et al., 2000; Roccasecca et al., 2003]. CD81 has been proposed as a putative receptor for HCV. HCV belongs to the *Hepacivirus* genus of the Flaviridae family [Shukla et al., 1995; Major and Feinstone, 1997]. The GAG, heparan sulfate proteoglycan (HSPG), is expressed at the surface of most mammalian cells [Rabenstein, 2002] and constitutes an important cellular-binding molecule for several members of Flaviridae family, such as dengue [Chen et al., 1997; Hilgard and Stockert, 2000; Germi et al., 2002a,b], classical swine fever [Hulst et al., 2001], yellow fever [Germi et al., 2002a,b], tick-borne encephalitis [Mandl et al., 2001] and Japanese encephalitis viruses [Su et al., 2001; Lee et al., 2004; Liu et al., 2004; Zhao et al., 2005]. Internalization of flaviviruses is thought to occur after binding to GAG residues, and other molecules are also involved in virus entry. It has been proposed that the affinity of viral surface molecules for HSPG may be an important determinant of organ tropism and pathogenesis [Putnak et al., 1997; Shukla and Spear, 2001].

In this study, the roles of CD81 and HSPG in the adsorption and uptake of infectious HCV particles were investigated.

MATERIALS AND METHODS

Cell Culture

Huh7 [Nakabayashi et al., 1982] cells were cultured and maintained in conditions described previously [Kato et al., 2003].

Plasmid Construction

pJFH-1 constructs were generated as previously reported [Wakita et al., 2005].

Production of Infectious HCV Particles

The plasmid pJFH-1 was linearized at the 3′-end of the HCV cDNA by Xba I digestion and then treated with a mung bean nuclease (New England Biolabs, Berkeley, MA) as described elsewhere [Kato et al., 2003]. Linearized plasmid DNAs were purified and used as template for in vitro transcription with the MEGAscript^{TM} T7 kit (Ambion, Austin, TX). In vitro synthesized RNAs were transfected into Huh7 cells by electroporation as described [Lohmann et al., 1999; Kato et al., 2003]. Cell culture supernatants were collected at 72 hr after transfection, cleared the superantants using low-speed centrifugation, and passed them through a 0.45-μm filter. Part of the filtrate was concentrated 1/20 using an Amicon Ultra-15 (cut-off: 1×10^5 Da; Millipore, Bedford, MA) in accordance with manufacturer's instructions. Infectious titers of about 7.5×10^4 focus forming units (ffu) per ml in concentrated viral stocks were estimated on the basis of immunofluorescence detection of infected foci, following infection of Huh7 cells. RNA quantification was performed by real-time detection reverse transcription polymerase chain reaction (RTD-PCR) analysis as described previously [Takeuchi et al., 1999] using the ABI Prism 7500 sequence detector system (Applied Biosystems Japan, Tokyo, Japan). The titer was determined to be 1.38×10^8 RNA copies/ml. Concentrated culture medium samples were stored at −80°C until use.

Quantitation of HCV RNA by Real-Time Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated from cell lysates using the Isogen (Nippon Gene Co. Ltd., Tokyo, Japan). Copy numbers of HCV RNA were determined by real-time detection reverse transcription polymerase chain reaction (RTD-PCR) analysis described previously [Takeuchi et al., 1999] using the ABI Prism 7500 sequence detector system (Applied Biosystems Japan, Tokyo, Japan).

HCV Particles Infection

Huh7 cells were seeded 24 hr before infection at a density of 8×10^4 cells/well in a 6-well plates (Corning Inc., Corning, NY), each well was inoculated with concentrated culture medium (100 μl) containing 1×10^7 copies of infectious HCV particles, incubated 3 h with periodic rocking at 37°C, washed three times with PBS, and incubated with 2 ml of complete culture medium at 37°C for 0, 12 , 24 , and 48 hr. The cells (washed three times with PBS) were harvested and the amount of cellular HCV RNA was determined by RTD-PCR.

Anti-CD81 Antibody Treatment

To determine the role of CD81, binding experiments were conducted on Huh7 cells (8×10^4 cells/well) seeded for 24 hr in 6-well plates (Corning) before infection. Infectious HCV (1×10^7copies) inoculate to Huh7 cells in a final volume of 100 μl /well in 6-well plates at 4°C to prevent viral internalization for 3hr to allow binding. After binding, unbound virus particles were removed by washing three times with PBS. Cells were treated with anti-CD81 antibody (JS-81; BD Biosciences, Franklin Lakes, NJ) at 4°C for 1 hr either before or after incubation with virus. After treatment, anti-CD81 antibody

was removed by washing three times with PBS. Cells were switched to 2 ml of complete culture medium and warmed to 37°C to allow internalization and replication. Cells were incubated for 0, 12, 24, and 48 hr. At each time point culture medium was removed and the cells (washed three times with PBS) were harvested for RTD-PCR. Cells incubated with virus using same procedure with anti-CD9 antibody (BD Biosciences, Franklin Lakes, NJ) were used as controls.

Separation of Heparin Bound and Unbound HCV Fractions

Culture medium containing secreted virus particles was collected from transfected Huh7 cells. Cleared and filtered culture medium was purified using a HiTrap™ Heparin HP affinity column (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The culture medium which passed through the heparin column contained the heparin unbound fraction, and the heparin bound fractions were eluted with buffer containing 0.3 M NaCl and 1 M NaCl. Each fraction was concentrated using an Amicon Ultra-15 centrifugal filter unit.

Heparin and Heparinase-Treatment

To determine whether heparin inhibits HCV infection of Huh7 cells, infectious HCV (1×10^7copies) reacted with different concentrations heparin (Mochida Pharmaceutical Co. Ltd., Tokyo, Japan) in a final volume of 100 µl /well at 4°C for 1 hr was added to Huh7 cells in 6-well plates at 4°C for 3 hr to allow binding. After binding, unbound virus particles were removed by washing three times with PBS. Cells were switched to 2 ml of complete culture medium and warmed to 37°C to allow internalization and replication. Cells were incubated for 0, 12, 24, and 48 hr. At each time point culture medium was removed and the cells (washed three times with PBS) were harvested for RTD-PCR. Cells infected with HCV without heparin pre-treatment were used as controls.

To determine whether heparinase treatment inhibits HCV infection, Huh7 cells were pre-treated with different concentrations heparinase I, II, and III (Sigma Aldrich, St. Louis, MO) at 37°C for 1 hr, washed three times with PBS, and incubated with virus particles (1×10^7copies/well) for 3 hr at 4°C. Unbound virus particles were removed by washing three times with PBS, and cells were switched to complete culture medium and warmed to 37°C. Cells were incubated for 0, 12, 24, and 48 hr and harvested for RTD-PCR. Cells not treated with heparinase were used as controls.

Statistical Analysis

Data from repeated experiments were averaged and expressed as means ± standard deviation of the mean. Statistical analysis was performed using the Student's *t*-test. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Anti-CD81 Antibody did not Reduce HCV Binding to Huh7 Cells, But CD81 Was Closely Correlated With HCV Particle Entry

To determine the infectivity of infectious HCV particles, Huh7 cells (8×10^4 cells/well in 6-well plates) were incubated with equal RNA copy numbers of HCV particles (1×10^7 copies/well) for 3 hr at 37°C, washed three times with PBS, and incubated at 37°C for 0, 12, 24, and 48 hr. Cell-associated HCV RNA titers were measured using RTD-PCR [Takeuchi et al., 1999]. Changes in RNA titer over time is shown in Figure 1 [Wakita et al., 2005]. RNA titers decreased up to 12 hr. But 12 hr later, RNA titers increased and reached a peak at 48 hr.

To determine the role of CD81, cells were treated with anti-CD81 antibody at 4°C for 1 hr either before (Fig. 2A) or after incubation with HCV (Fig. 2C) for 3 hr at 4°C to prevent viral internalization, washed three times with PBS, and incubated at 37°C for 0, 12, 24, and 48 hr. Pre-treatment before infection resulted in increased binding but decreased RNA titer at 48 hr relative to the control (Fig. 2B). However, post-treatment had no effect on HCV binding but dose-dependently suppressed RNA titer at 48 h (Fig. 2D). Thus, CD81 does not play an important role in HCV particle attachment, but may be correlated with HCV entry after viral attachment.

Infectious HCV Particles Bind to the Heparan Sulfate Homologue Heparin

Heparin is a GAG and the closest homologue of liver HSPG. To determine whether infectious HCV particles bind to GAGs, culture medium containing secreted virus particles was collected from JFH-1 RNA transfected

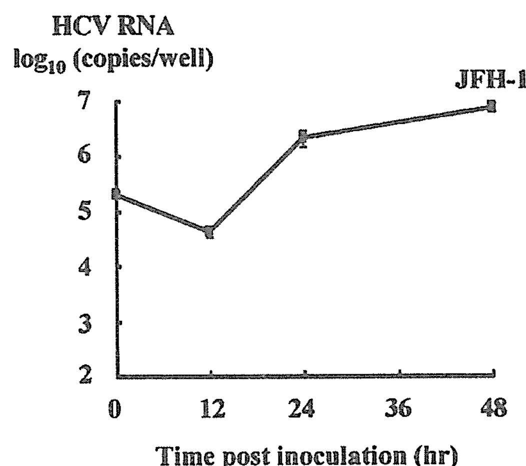

Fig. 1. HCV particles produced in cultured cells infected Huh7 cells. Huh7 cells (8×10^4 cells/well) were seeded 24 hr before incubation with HCV (1×10^7 copies/well) for 3 hr with periodic rocking at 37°C. After inoculation, unbound virus particles were removed by washing three times with PBS and cells were supplemented with complete culture medium. Cells were harvested at 0, 12, 24, and 48 hr and amounts of HCV RNA were determined by RTD-PCR. The bars represent the mean and standard deviation of the means of six wells for triplicate experiments.

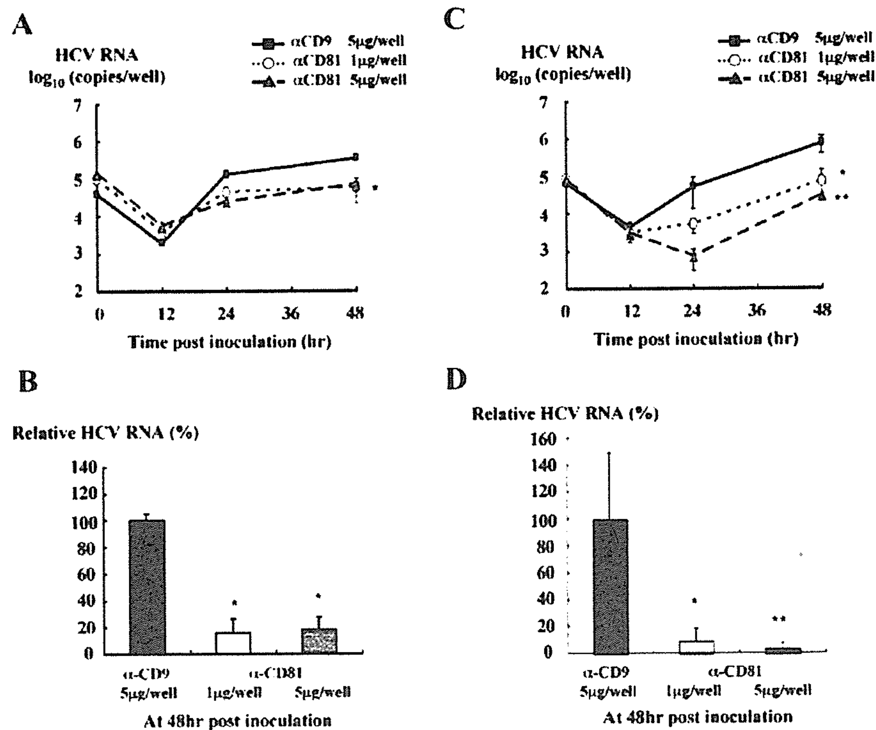

Fig. 2. Inhibition of virus particles infection by anti-CD81 antibody. Huh7 cells (8×10^4 cells/well) were seeded 24 hr before incubation with HCV (1×10^7 copies/well) at 4°C to prevent viral internalization. Inoculation was terminated after 3 h by three times washing cells with PBS and supplementing them with complete culture medium without antibodies. (**A**) Before incubation with HCV, Huh7 cells were pre-treated at 4°C for 1 hr with 5 μg/well (black squares) of anti-CD9 antibody as a control, or 1 μg/well (white circles), or 5 μg/well (grey triangles) of anti-CD81 antibody. (**B**) The efficiencies of infection by pre-treatment for Huh7 cells with antibodies were determined 48 hr post-inoculation and are expressed relative to the amount observed in the presence of anti-CD9 antibody (black bar). White and grey bars represent infections in the presence of anti-CD81 antibodies at a concentration of 1 and 5 μg/well, respectively. The treated HCV with anti-CD9 antibody associated RNA titer was arbitrarily set at 100%. (**C**) After incubation with HCV, cells were treated at 4°C for 1 hr with 5 μg/well (black squares) of anti-CD9 antibody as a control, or 1 μg/well (white circles), or 5 μg/well (grey triangles) of anti-CD81 antibody. (**D**) The efficiencies of infection by treatment for HCV particles bound Huh7 cells with antibodies were determined 48 hr post-inoculation. Relative HCV RNA titers are shown. The RNA titers at 0, 12, 24, and 48 hr were determined by RTD-PCR. Columns and bars represent the mean and standard deviation of the means of six wells for triplicate experiments. Asterisks indicate that anti-CD81 antibody significantly suppressed HCV particle infectivity. $^{*}P < 0.05$; $^{**}P < 0.01$.

Huh7 cells and separated on a heparin column into three fractions (a flow-through unbound fraction and bound fractions eluted by 0.3 and 1 M NaCl). To determine the infectivity of each fraction, Huh7 cells were incubated with equal RNA copy numbers of HCV particles and harvested at 0, 12, 24, and 48 hr after addition of the fraction. Cell-associated HCV RNA titers were measured using RTD-PCR [Takeuchi et al., 1999]. Only the heparin-bound and eluted with 0.3 M NaCl fraction was capable of productive infection (Fig. 3), indicating that infectious HCV particles were bound to heparin.

Heparin and Heparinase Treatments Reduced the HCV Particle Binding to Huh7 Cells

In the previous reports, GAGs such as HSPG on the target cell surface were needed for the initial viral attachment of several viruses and viral surface proteins [Jackson et al., 1996; Chen et al., 1997; Rostand and Esko, 1997; Laquerre et al., 1998; Summerford and Samulski, 1998; Feldman et al., 1999; Joyce et al., 1999; Klimstra et al., 1999; Hilgard and Stockert, 2000]. Thus heparin was tested as an inhibitor of HCV particle

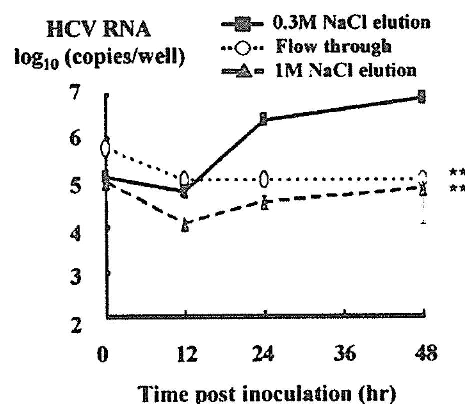

Fig. 3. Heparin bound and eluted with 0.3 M NaCl fraction caused productive infection. The infectivity of the heparin unbound fraction (white circles) was compared with that of the heparin bound and then eluted with 0.3 M NaCl fraction (black squares) and 1 M NaCl fraction (grey triangles). Huh7 cells (8×10^4 cells/well) were incubated with each fraction containing HCV particles (1×10^7 copies/100 μl) for 3 hr with periodic rocking at 37°C. The amount of cell-associated RNA was measured at 0, 12, 24, and 48 hr by RTD-PCR. The bars represent the mean and standard deviation of the means of six wells for triplicate experiments. Asterisks indicate that both of the unbound fraction and the eluted with 1 M NaCl fraction titers were significantly lower than the eluted with 0.3 M fraction titer. $^{**}P < 0.01$.

binding. Binding experiments were performed at 4°C, to prevent viral internalization. Preincubation of HCV particles with increasing concentrations of heparin resulted in a dose-dependent inhibition of binding to Huh7 cells (Fig. 4A). Although HCV particle binding (47% inhibition at 1 U/well and 59% inhibition at 10 U/well, Fig. 4A) was significantly inhibited by heparin, RNA titers in Huh7 cells infected with JFH-1 (the control) and in Huh7 cells infected with heparin-treated virus were similar at 48 h (Fig. 4B and C).

To confirm that HSPG plays a role in adhesion of HCV particles to Huh7 cells, Huh7 cells were pre-treated with different GAG-lyases and incubated with HCV. Viral particle binding and infectivity were assessed by determining HCV RNA replication. Heparinase I pre-treatment of Huh7 cells (degrading heparin and the highly sulfated domains in heparan sulfate) inhibited 71–74% of HCV particle binding (Fig. 5A). Likewise heparinase II pre-treatment (degrading heparin and heparan sulfate) and heparinase III pre-treatment (degrading heparan sulfate) inhibited 51–75% (Fig. 6A) and 60–75% (Fig. 7A) of HCV particle binding, respectively. But there was no significant difference in RNA titer at 48 h in Huh7 cells infected with JFH-1 (the control) and cells pretreated with heparinase I, II and III before JFH-I infection (Figs. 5–7).

Thus, both heparin and heparinase treatments reduced the virus binding to Huh7 cells, suggesting that HCV binds to Huh7 cells via HSPG. However, these treatments did not inhibit HCV infectivity.

Heparinase I Pre-Treatment Plus Anti-CD81 Antibody Post-Treatment Markedly Suppressed the Infectivity of HCV

To confirm that both HSPG and CD81 are needed for HCV particle entry, heparinase I pre-treatment was combined with anti-CD81 antibody post-treatment. HCV particle binding was reduced to the same level as that of heparinase I mono-treatment (Fig. 8A). But after the combination treatment, no HCV RNA replication occurred in Huh7 cells at 48 hr (Fig. 8B and C). Taken together, a functional and cooperative interaction between HSPG and CD81 for HCV entry is suggested.

DISCUSSION

Up to the present, early steps in the HCV life cycle have been difficult to study. In the absence of an effective infectivity assay, surrogate models have been adopted to investigate HCV entry. These include HCV pseudo-particles (HCVpp) derived from vesicular stomatitis

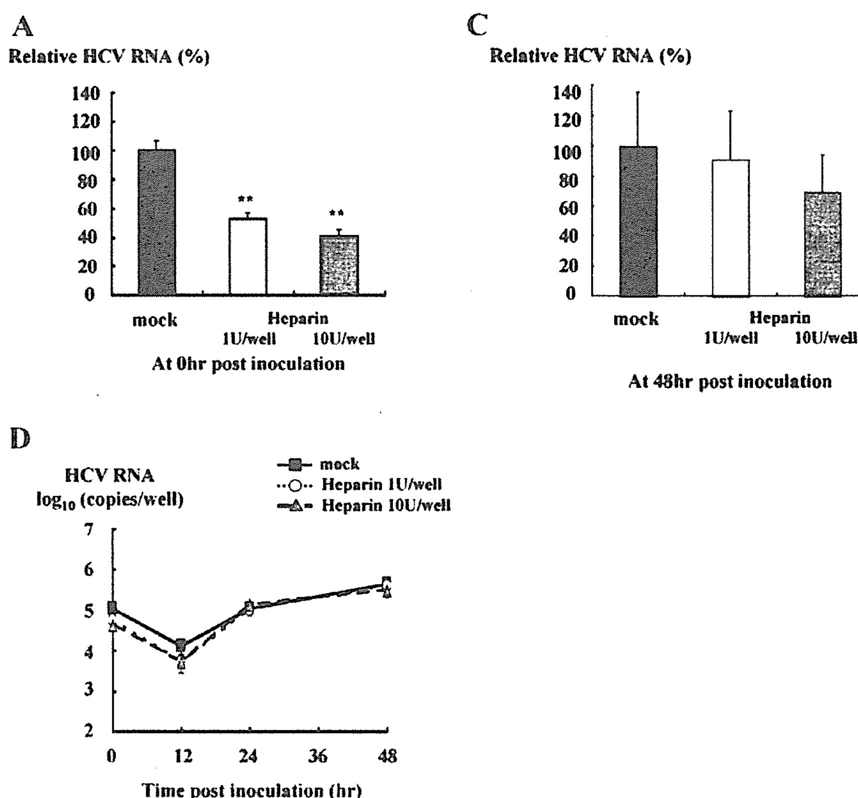

Fig. 4. Inhibition of JFH-1 particles infection using heparin. (**A**) HCV particles pre-treated with PBS (mock; black), or 1 U/well (white), or 10 U/well (grey) of heparin resulted in a dose-dependent inhibition of binding to Huh7 cells. Relative HCV RNA titers are shown. The untreated HCV associated RNA titer (the mock) was arbitrarily set at 100%. (**B**) The RNA titers in Huh7 cells at 0, 12, 24, and 48 hr were determined by RTD-PCR. (**C**) The efficiencies of infection by pre-treatment for HCV particles with heparin were determined 48 hr post-inoculation. The bars represent the mean and standard deviation of the means of six wells for triplicate experiments. Asterisks indicate that heparin treatment significantly inhibited HCV particle binding. $^{**}P < 0.01$.

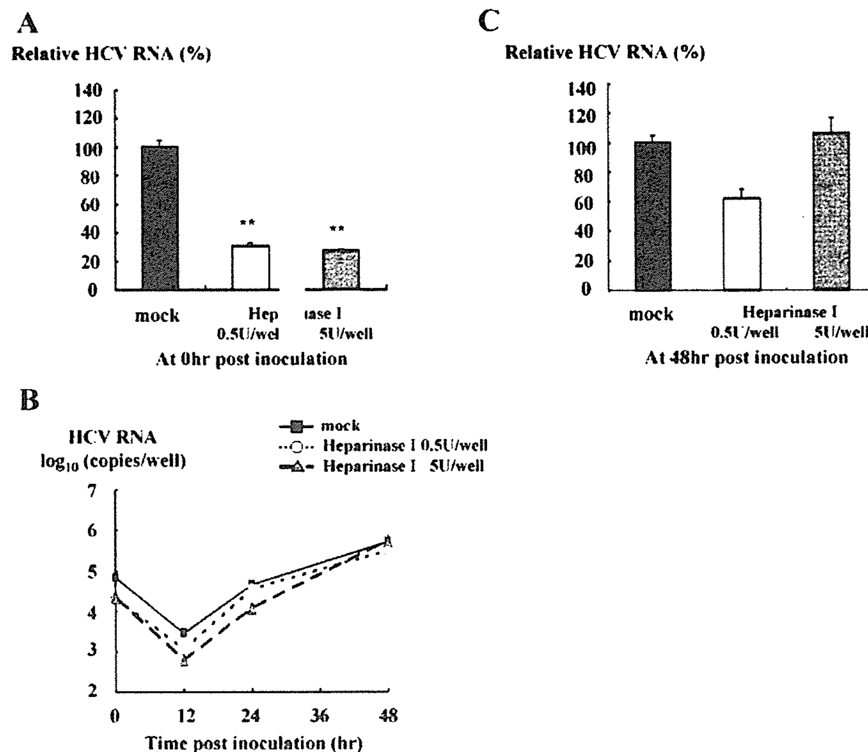

Fig. 5. Heparinase I treatments reduced the virus particles binding to Huh7 cells. Huh7 cells were pre-treated for 1 hr at 37°C with PBS (mock; black), or 0.5 U/well (white), or 5 U/well (grey) of heparinase I, which resulted in a dose-dependent inhibition of binding to Huh7 cells (**A**). Relative HCV RNA titers are shown. The untreated HCV associated RNA titer (the mock) was arbitrarily set at 100%. RNA titers in Huh7 cells at 0, 12, 24, and 48 hr were determined by RTD-PCR (**B**). The efficiencies of infection by pre-treatment for Huh7 cells with heparinase I were determined 48 hr post-inoculation (**C**). Columns and bars represent the mean and standard deviation of the means of six wells for triplicate experiments. Asterisks indicate that heparinase I treatment significantly inhibited HCV particle binding. $^{**}P < 0.01$.

virus or retrovirus incorporating cell surface expressing chimeric or native forms of HCV glycoproteins. Though HCVpp expressing HCV-specific E1 and E2 glycoproteins are infective, it is unclear whether the infectivity of these pseudo-particles reflects that of native HCV. Using a cell–cell fusion assay, it has been reported that the induction of cell fusion requires cell surface expression of both HCV glycoproteins and low pH [Takikawa et al., 2000; Kobayashi et al., 2006]. This experiment suggests that certain cell surface proteins other than human CD81 and some glycosaminoglycans are also involved in HCV-mediated cell fusion.

In the present study, infectious HCV particles produced in cultured cells were used to investigate the early steps of HCV entry. Virus entry was markedly inhibited by anti-CD81 mAb, even of virus already attached to target cells, at a temperature that is nonpermissive for internalization. Thus, HCV adsorption and entry into target cells may be mediated by other cellular factors acting in concert with CD81. CD81 is expressed on most cell types, it is unlikely to be the sole determinant of HCV hepatotropism. Since the hepatoma cell surface has many possible virus-binding sites, a specific interaction with a single high affinity molecule is unlikely to mediate both initial viral attachment and cell entry. This understanding is consistent with the results of binding studies of other viruses [Jackson et al., 1996; Fry et al., 1999].

The main disaccharide of heparin unit is slightly different and its degree of sulfation is higher than that of heparan sulfate (HS) [Hileman et al., 1998]. The degree of sulfation of HS is significantly higher in liver than other tissues [Toida et al., 1997], and is probably responsible for the differential binding of various HS ligands. Our results using a heparin column revealed that infectious HCV particles bind to heparin with low affinity, because only the heparin-bound and eluted with 0.3 M NaCl fraction retained infectivity. Despite the heparin-bound and eluted with 1 M NaCl fraction had a greater affinity for heparin than the heparin-bound and eluted with 0.3 M NaCl fraction, the heparin-bound and eluted with 1M NaCl fraction had no

Fig. 7. Heparinase-III treatments reduced the virus particles binding to Huh7 cells. Huh7 cells were pre-treated for 1 hr at 37°C with PBS (mock; black), or 0.5 U/well (white), or 5 U/well (grey) of heparinase III, which resulted in a dose-dependent inhibition of binding to Huh7 cells (**A**). Relative HCV RNA titers are shown. The untreated HCV associated RNA titer (the mock) was arbitrarily set at 100%. RNA titers in Huh7 cells at 0, 12, 24, and 48 hr were determined by RTD-PCR (**B**). The efficiencies of infection by pre-treatment for Huh7 cells with heparinase III were determined 48hr post inoculation (**C**). Columns and bars represent the mean and standard deviation of the means of six wells for triplicate experiments. Asterisks indicate that heparinase III treatment significantly inhibited HCV particle binding. $^{*}P < 0.05$.

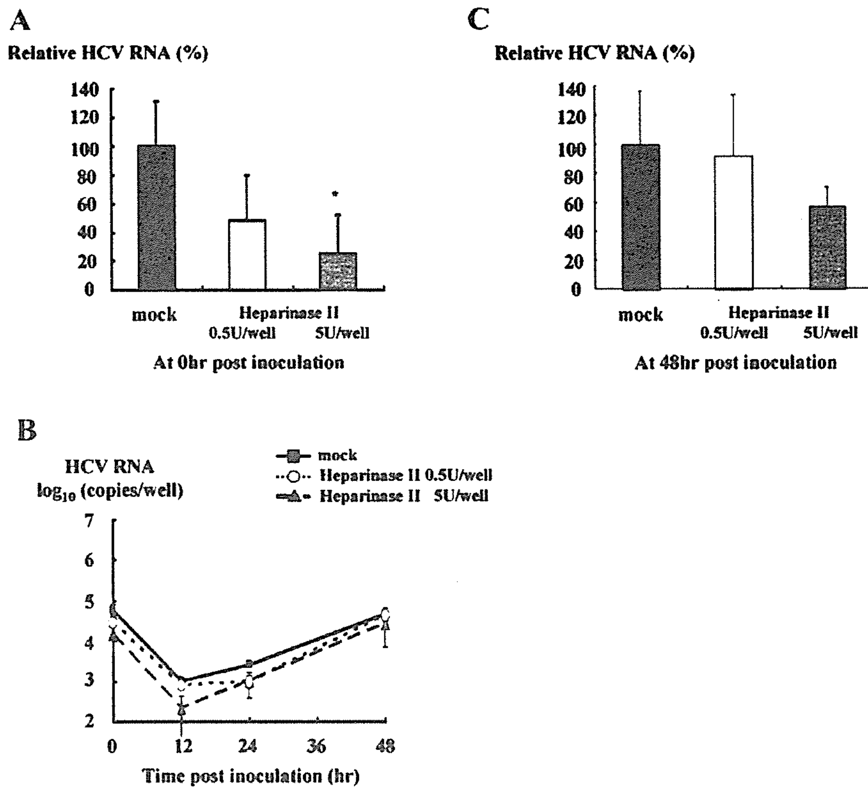

Fig. 6. Heparinase II treatments reduced the virus particles binding to Huh7 cells. Huh7 cells were pre-treated for 1 hr at 37°C with PBS (mock; black), or 0.5 U/well (white), or 5 U/well (grey) of heparinase II, which resulted in a dose-dependent inhibition of binding to Huh7 cells (A). Relative HCV RNA titers are shown. The untreated HCV associated RNA titer (the mock) was arbitrarily set at 100%. RNA titers in Huh7 cells at 0, 12, 24, and 48 hr were determined by RTD-PCR (B). The efficiencies of infection by pre-treatment for Huh7 cells with heparinase II were determined 48 hr post inoculation (C). Columns and bars represent the mean and standard deviation of the means of six wells for triplicate experiments. Asterisks indicate that heparinase II treatment significantly inhibited HCV particle binding. $^{*}P < 0.05$.

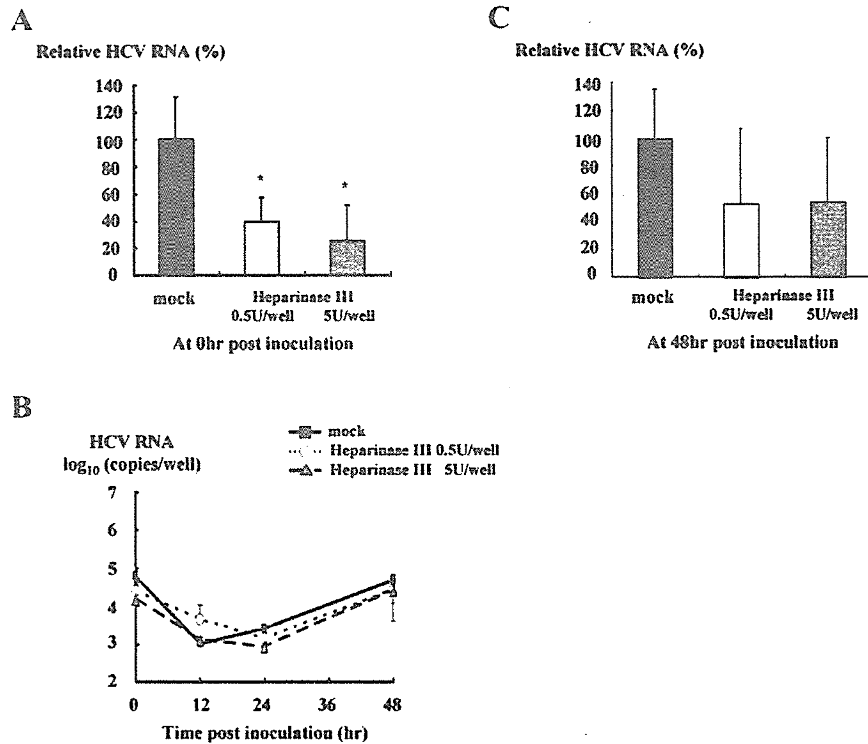

Fig. 7.

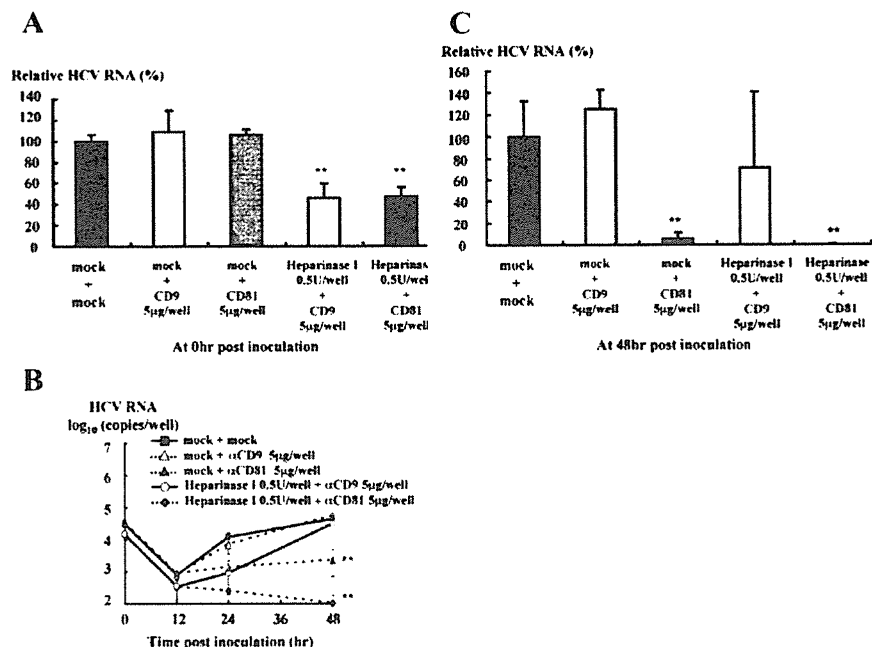

Fig. 8. Interaction between HSPG and CD81 required for HCV entry. Huh7 cells infected with HCV with PBS (mock) were used as controls (black squares). Huh7 cells infected with HCV, and then treated at 4°C for 1 hr with 5 μg/well of anti-CD9 antibody (white triangles). Huh7 cells infected with HCV, and then treated at 4°C for 1 hr with 5 μg/well of anti-CD81 antibody (grey triangles). Huh7 cells were pre-treated with 0.5 U/well of heparinase I, infected with HCV, and then treated with 5 μg/well anti-CD9 antibody (white circles). Huh7 cells were pre-treated with 0.5 U/well of heparinase I, infected with HCV, and then treated with 5 μg/well anti-CD81 antibody (black diamonds). When Huh7 cells treated with heparinase I, it resulted in inhibition of binding to Huh7 cells (**A**). Relative HCV RNA titers are shown. The untreated HCV associated RNA titer (the mock) was arbitrarily set at 100%. RNA titers in Huh7 cells at 0, 12, 24, and 48 hr were determined by RTD-PCR (**B**). The efficiencies of infection by each treatments for Huh7 cells was determined 48 hr post-inoculation (**C**). Columns and bars represent the mean and standard deviation of the means of six wells for triplicate experiments. Asterisks indicate heparinase I pre-treatment plus anti-CD81 antibody post-treatment markedly blocked HCV particle adsorption and infection. $^{**}P < 0.01$.

capability of productive infection. One possible explanation is that the heparin-bound and eluted with 1 M NaCl fraction had been composed of incomplete or damaged virus particles that were less infectious. Circulating virus particles might be caught by cell surface HS and transferred from low-affinity binding sites on GAGs to high-affinity receptors that mediate viral internalization. For example, cell surface HSPG has been shown to mediate a major proportion of picornavirus adhesion to target cells whereas an integrin family receptor has been shown to be the principal mediator of viral internalization [Jackson et al., 1996]. In the present study, the marked and specific inhibition of HCV attachment was observed both by heparin for HCV particles (59% inhibition at 10 U/well, Fig. 4A and 90% inhibition at 50 U/well, data not shown) and by heparinase for Huh7 cells. However, HCV infection was partially inhibited and not significantly both treatments (p values were more than 0.05). When inoculating with different virus doses (5×10^6 copies/well) which preincubated with larger amount of heparin (50 U/well), the results were not significantly different from the present data (data not shown). Furthermore, to exclude the possibility that virus spread due to secondary infections covered up the initial differences both by heparin and heparinase treatments, anti-CD81 antibody was applied at the point of 12 and 24 hpi subsequent to virus entry. But HCV RNA titer in the infected cells was not significantly different between mock and anti-CD81 antibody treated groups (data not shown). From our results, heparin and heparinase diminished HCV adsorption with less affect for infectivity. This may suggest that the heparin and heparinase treatments only block attachment of defective particles that do not contribute to the infection. Alternatively, it is conceivable that the limited inhibitory effect measured in the binding assay may be poorly resolved at a subsequent time points when variability of the assay is generally higher (Fig. 4A and C) thus possibly masking subtle differences. Taken together, HSPG may play an important role for initial binding but may be also involved in this step other cellular molecules (e.g. LDL receptor, SR-BI). Koutsodakis and colleagues reported a dose dependent inhibition of infection using cell-culture derived recombinant HCV particles by heparin and pre-treatment to cells by GAG-lyases [Koutsoudakis et al., 2006]. These discrepancies may be due to using the different virus systems and experimental conditions. The JFH1-luciferase chimeric viruses have less infectivity than wild-type JFH1 and the time of inoculation was shorter than ours (1 and 3 hr, respectively). In the present study anti-CD81 antibody markedly reduced infectivity, although heparin and heparinase did not block infectivity using the same amounts of virus inoculum. It is thus considered that CD81 may be an important determinant of HCV infection.

The large number of binding sites and the low binding affinity indicated that virus–HSPG interaction was not

ligand-receptor binding in the usual sense. HSPG may capture the virions and concentrate them on the cell surface, then the bound HCV particles move to the further stages of infection by CD81 which triggers virus-dependent activation of signaling that leads to viral internalization by clathrin-mediated endocytosis. No productive infection was detected in the Huh7 cells with both heparinase -I and anti-CD81 antibody treatments. The mechanism of this synergistic effect remains unknown, and thus further investigation is required.

In summary, the results of this study support and extend the hypothesis that HSPG is responsible for targeting and binding HCV to liver cells. Infectious HCV particles bind to HSPG, however, most cell-bound viruses do not infect. Moreover, though anti-CD81 antibody did not inhibit virus binding to the cell surface, CD81 appears to play an important role as a co-receptor of virus entry. Inhibitors of both HSPG and CD81 or HSPG–CD81 interaction may provide a novel treatment of HCV infection.

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Upregulation of Indoleamine 2,3-Dioxygenase in Hepatitis C Virus Infection[▽]

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Indoleamine 2,3-dioxygenase (IDO) is induced by proinflammatory cytokines and by CTLA-4-expressing T cells and constitutes an important mediator of peripheral immune tolerance. In chronic hepatitis C, we found upregulation of IDO expression in the liver and an increased serum kynurenine/tryptophan ratio (a reflection of IDO activity). Huh7 cells supporting hepatitis C virus (HCV) replication expressed higher levels of IDO mRNA than noninfected cells when stimulated with gamma interferon or when cocultured with activated T cells. In infected chimpanzees, hepatic IDO expression decreased in animals that cured the infection, while it remained high in those that progressed to chronicity. For both patients and chimpanzees, hepatic expression of IDO and CTLA-4 correlated directly. Induction of IDO may dampen T-cell reactivity to viral antigens in chronic HCV infection.

Indoleamine 2,3-dioxygenase (IDO) mediates conversion of tryptophan to catabolites collectively known as kynurenines (22). This enzyme is expressed by both epithelial and dendritic cells induced by proinflammatory cytokines, including gamma interferon (IFN-γ) and tumor necrosis factor alpha (20, 25). Also, engagement of CTLA-4 with CD80/CD86 on the membrane of dendritic cells stimulates IDO transcriptional expression and activity (4, 9, 19). Increased IDO activity provokes tolerogenicity of antigen-presenting cells and deprives T cells of tryptophan, leading to proliferation arrest and T-cell apoptosis (15). Kynurenine, on the other hand, has been shown to act as an immunoregulatory molecule that mediates immunosuppressive effects in the tissue microenvironment (7, 22, 26). IDO activity contributes to maternal tolerance in pregnancy (21), control of allograft rejection (9), and protection against autoimmunity (8).

Chronic infection caused by hepatitis C virus (HCV) is characterized by weak T-cell responses, recognizing very few epitopes. In contrast, viral clearance after acute infection or after interferon therapy is associated with the presence of a robust and polyclonal T-cell reaction (2, 3, 6, 10, 14, 18, 23, 24). Thus, HCV has developed efficient means to escape T-cell immunity, thus causing a high rate of chronic infections. The molecular mechanisms that are responsible for immune tolerance to HCV antigens remains ill understood. Since IDO activity may dampen T-cell reactivity and can contribute to tolerogenicity of dendritic cells (17), we have analyzed IDO expression by quantitative real-time PCR using β-actin gene expression as an endogenous control (12, 13) (IDO sense primer, TGGCACACGCTATGGAAAAC; antisense, ATGCATCCCAGAACTAGACG; β-actin sense primer, AGCCTCGCCTTTGCCGA; antisense, CTGGTGCCTGGGGCG) in liver samples from patients with chronic hepatitis C (CHC), subjects with sustained virological response (SVR) after interferon therapy, and patients with other forms of chronic liver inflammation (chronic hepatitis B and steatohepatitis) and in normal liver samples (Table 1, cohort 1). IDO mRNA levels were significantly higher in the CHC group than in the other groups. Patients with other forms of liver disease had values higher than those for normal livers but lower than the CHC values (Fig. 1A). Subjects with SVR showed values similar to those for controls.

As an index of IDO activity, we measured the serum kynurenine/tryptophan ratio (KTR) for equivalent groups of patients and for healthy controls (Table 1, cohort 2). KTR was determined by high-performance liquid chromatography (27). We found that KTR was significantly higher for the CHC group than for the other groups, which did not show significant differences among them (Fig. 1B). Since both IDO mRNA levels and serum KTRs are significantly higher for CHC than in other forms of liver disease (see Fig. 1A and B), it seems possible that HCV might be especially efficient at facilitating IDO overexpression in an inflamed milieu.

To determine whether HCV replication may enhance IDO expression in response to proinflammatory cytokines, we stimulated with IFN-γ (100 U/ml; R&D Systems, Minneapolis, MN), for 16, 24, and 40 h, Huh7 cells containing the full-length HCV replicon (Huh7-Core-3′) (12, 16), Huh7 cells producing JFH1-HCV viral particles (28), and control cells. JFH1-Huh7 cells were used at 30 to 35 days postin-

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TABLE 1. Characteristics of patient cohorts

Variable[a]	Value for patient group			
	Normal liver	Chronic hepatitis C	Sustained virological response	Miscellaneous liver diseases
Amt of aspartate aminotransferase (IU/liter)				
Cohort 1	14.7 ± 5	42.1 ± 28	10.8 ± 2	35.4 ± 24
Cohort 2		46.2 ± 28.3	13.8 ± 5	67.0 ± 55
Amt of alanine aminotransferase (IU/liter)				
Cohort 1	19.3 ± 9	78.8 ± 74	10.7 ± 2	53.1 ± 31
Cohort 2		36.5 ± 17.9	15.4 ± 5	50.3 ± 44.5
Viral load (mean, IU/ml)				
Cohort 1		6.5×10^7	0	
Cohort 2		1.1×10^8	0	
No. of samples with viral genotype (1/non-1/not determined)				
Cohort 1		15/5/4		
Cohort 2		12/4/3		
Liver biopsy (Knodel's score) inflammatory activity				
Cohort 1		4.8 ± 1.8	2.3 ± 1.0	5.6 ± 2.5
Cohort 2		5.4 ± 2.2	0.25 ± 0.5	5.4 ± 2.5
Fibrosis score				
Cohort 1		0.5 ± 0.8	0.5 ± 0.5	1.5 ± 1.5
Cohort 2		1.4 ± 1.5	0.25 ± 0.5	1.1 ± 1.2

[a] Cohort 1, liver tissues from normal liver, n = 13 (samples obtained at surgery of liver metastasis or cholelithiasis); miscellaneous liver diseases, n = 23 (of whom 11 were chronic hepatitis B patients and 12 were steatohepatitis patients); chronic hepatitis C, n = 24 (of whom 11 were naive and 13 were nonresponders to pegylated IFN-α2 plus ribavirin); sustained virological response, n = 11. Cohort 2, serum samples from healthy subjects, n = 14; miscellaneous liver diseases, n = 17 (of whom 6 were chronic hepatitis B patients and 11 steatohepatitis patients); chronic hepatitis C, n = 19 (of whom 7 were naive and 12 nonresponders to pegylated IFN-α2 plus ribavirin); sustained virological response, n = 19. Miscellaneous liver diseases and chronic hepatitis C patients did not differ in terms of aspartate aminotransferase/alanine aminotransferase levels and histological grading. The study was approved by the local ethical committee.

fection, when about 50 to 60% of cells were positive for the HCV core protein, as determined by immunofluorescence. As shown in Fig. 2A and B, both Huh7-Core-3′ cells and Huh7 cells producing JFH1 generated increased amounts of IDO mRNA in response to IFN-γ at all time points compared to control Huh7 cells. These findings indicate that HCV replication sensitizes the cells to produce IDO at high levels in response to IFN-γ, a proinflammatory cytokine that is upregulated in the livers of patients with CHC (1). IDO upregulation in response to IFN-γ does not affect the replicative activity of HCV in the infected cells, since treatment of the cells with IFN-γ plus an IDO inhibitor (1-methyl tryptophan) or plus kynurenine did not provoke changes in HCV-RNA levels in the infected cells with respect to those observed with treatment of the cells with IFN-γ alone (data not shown). It seems, therefore, that IDO upregulation may represent a strategy of HCV to escape T-cell immunity rather than a mechanism directly influencing HCV replication.

Our data suggest that one of the strategies used by HCV to

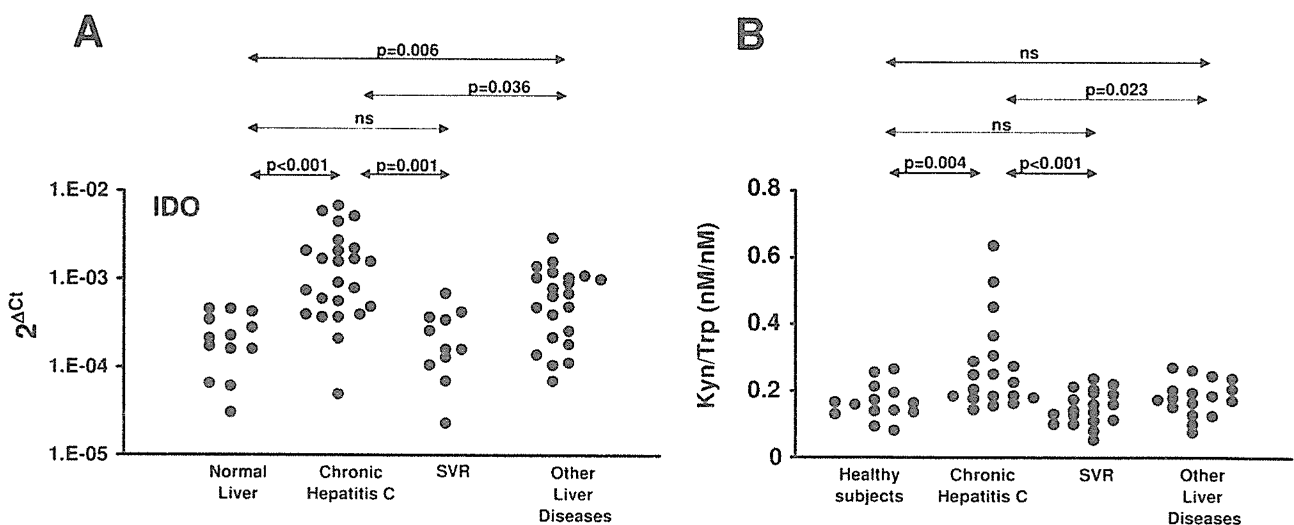

FIG. 1. IDO and HCV infection. (A) Real-time PCR quantitation of IDO mRNA in liver samples from normal livers, from patients with chronic hepatitis C (CHC), from patients with CHC who cleared the virus after interferon therapy (SVR), or from a miscellaneous group of patients with liver disorders unrelated to HCV. Results are normalized with β-actin. (B) Kynurenine/tryptophan ratio in serum samples from individuals belonging to groups equivalent to those shown in panel A. Statistical analyses were performed using nonparametric Kruskal-Wallis and Mann-Whitney U tests. ns, not significant.

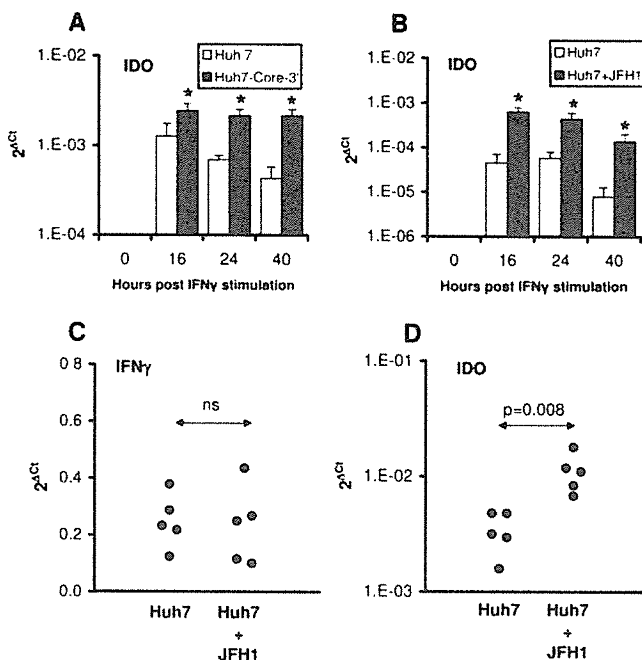

FIG. 2. Induction of IDO mRNA expression by HCV replication. Quantitation of IDO mRNA levels by real-time PCR in Huh7 cells with or without HCV replicon (Huh7-Core-3') (A) or JFH1 virus (B), treated with 100 U/ml of IFN-γ for 0, 16, 24, or 40 h. Results are expressed as the mean ± standard deviations of one representative experiment out of three experiments performed in sextuplicate. (C and D) IDO and IFN-γ mRNA levels measured by real-time PCR in $CD4^+$ $CD25^-$ cells cocultured for 1 day with Huh7 cells with or without HCV-JFH1 in the presence of Dynabeads CD3/CD28 T-cell expander. All results are normalized with β-actin. Statistical analyses were performed using the nonparametric Mann-Whitney U test. (*, $P < 0.01$; Huh7-core 3' versus Huh7 or Huh7 plus JFH1 versus Huh7). ns, not significant.

resist immune attack is by promoting IDO expression when infected hepatocytes interact with effector T cells producing IFN-γ. To test this hypothesis, Huh7 cells infected with JFH1 and control Huh7 cells were cocultured with 1.2×10^5 $CD4^+$ $CD25^-$ cells from a healthy subject (using the negative fraction of the CD4+CD25+ Regulatory T Cell Isolation kit; Miltenyi Biotec, Bergisch Gladbach, Germany) in the presence of the Dynabeads CD3/CD28 T-cell expander (Dynal biotech, Oslo, Norway) to activate T cells. After 1 day, the coculture was collected and both IFN-γ mRNA and IDO mRNA were determined by quantitative real-time PCR (IFN-γ sense primer, CTCTGCATCGTTTTGGGTTC; antisense, GCGTTGGAC ATTCAAGTCAG). As shown in Fig. 2C and D, the induction of IFN-γ was similar in cocultures containing control and infected Huh7 cells, but the expression of IDO was significantly higher in HCV-infected cultures. Since IDO levels were about 100-fold higher in coculture experiments than in experiments using exogenous IFN-γ, whether other factors apart from IFN-γ, such as cell contact, might be involved in this high IDO upregulation was studied. Thus, when supernatant from activated $CD4^+$ $CD25^-$ T cells was added to infected or noninfected Huh7 cells, differences in IDO upregulation were not observed (data not shown). It appears, therefore, that IDO induction is mainly facilitated by cell contact between infected cells and activated T lymphocytes. Whether IDO induction in livers with CHC takes place in infected hepatocytes and/or in inflammatory mononuclear cells has not been analyzed in the present work. However, our data for HCV-infected hepatoma cells suggest that hepatocytes are at least partially responsible for the elevated hepatic levels of IDO found in CHC.

There is an intricate cross talk between IDO and CTLA-4 (17). It has been shown that tryptophan depletion together with the presence of kynurenines promotes the expression of inhibitory molecules, such as CTLA-4 and Foxp3, in T cells (5). On the other hand, CTLA-4 stimulates IDO expression and IDO activity in antigen-presenting cells, inducing tolerogenic dendritic cells (17). Thus, we investigated whether IDO expression in the liver might correlate with the abundance of CTLA-4 mRNA in this organ. By using quantitative real-time PCR (CTLA-4 sense primer, TCATGTACCCACCGCCATAC; antisense, TAGACCCCTGTTGTAAGAGG), we found that CTLA-4 mRNA levels were increased in liver biopsy samples from HCV-infected patients over those in normal hepatic tissue or in samples from patients with SVR or other forms of liver disease (Fig. 3A). A significant direct correlation was found between IDO mRNA levels and CTLA-4 mRNA levels in liver tissue from HCV-infected patients ($r = 0.52$; $P < 0.01$) (Fig. 3B).

Liver biopsies are not routinely performed for patients with acute hepatitis C. Thus, in order to investigate the role of hepatic IDO expression in the evolution of HCV infection, we analyzed serial liver biopsy samples obtained from six chimpanzees after they were infected with 25 50% chimpanzee infectious doses of the HCV 1b J4 virus stock (Robert H. Purcell, NIAID, NIH, Bethesda, MD). This study was approved by independent ethical committees in accordance with international regulations (International Animal Care and Use Committee). As shown in Fig. 3C, hepatic IDO mRNA declined after an initial peak and remained low during evolution in animals that cleared the virus, while in the chimpanzees that evolved to chronicity, the initial peak of IDO expression was lower but the levels remained elevated during evolution. Thus, both in chimpanzees and in humans, chronic HCV infection is associated with persistently high IDO expression in the liver. An initial short-lived upregulation of IDO in the animals that cleared the virus might be secondary to the induction of a potent and efficient immune response. In fact, an early and transient upsurge of IDO might take place in association with activation of dendritic cells and T-cell immunity (11), while persistent IDO overexpression may favor tolerance (17). Our findings for acute infection in chimpanzees lend support to this contention.

In parallel to IDO results, for chimps that cured the infection, CTLA-4 expression in the liver showed an initial peak and then remained stable at very low levels during the evolution of the disease (Fig. 3D). In contrast, for chimps that became chronic carriers, expression of CTLA-4 showed little change during the early phase of infection but tended to persist above basal values along the course of the infection (Fig. 3D). As with humans, we found a significant direct correlation between IDO and CTLA-4 mRNA values in the liver (Fig. 3E).

In summary, we show upregulation of IDO in the livers of patients and chimpanzees with chronic hepatitis C. This finding is associated, and correlates, with overexpression of CTLA-4 in liver tissue. Our data indicate that HCV infection facilitates

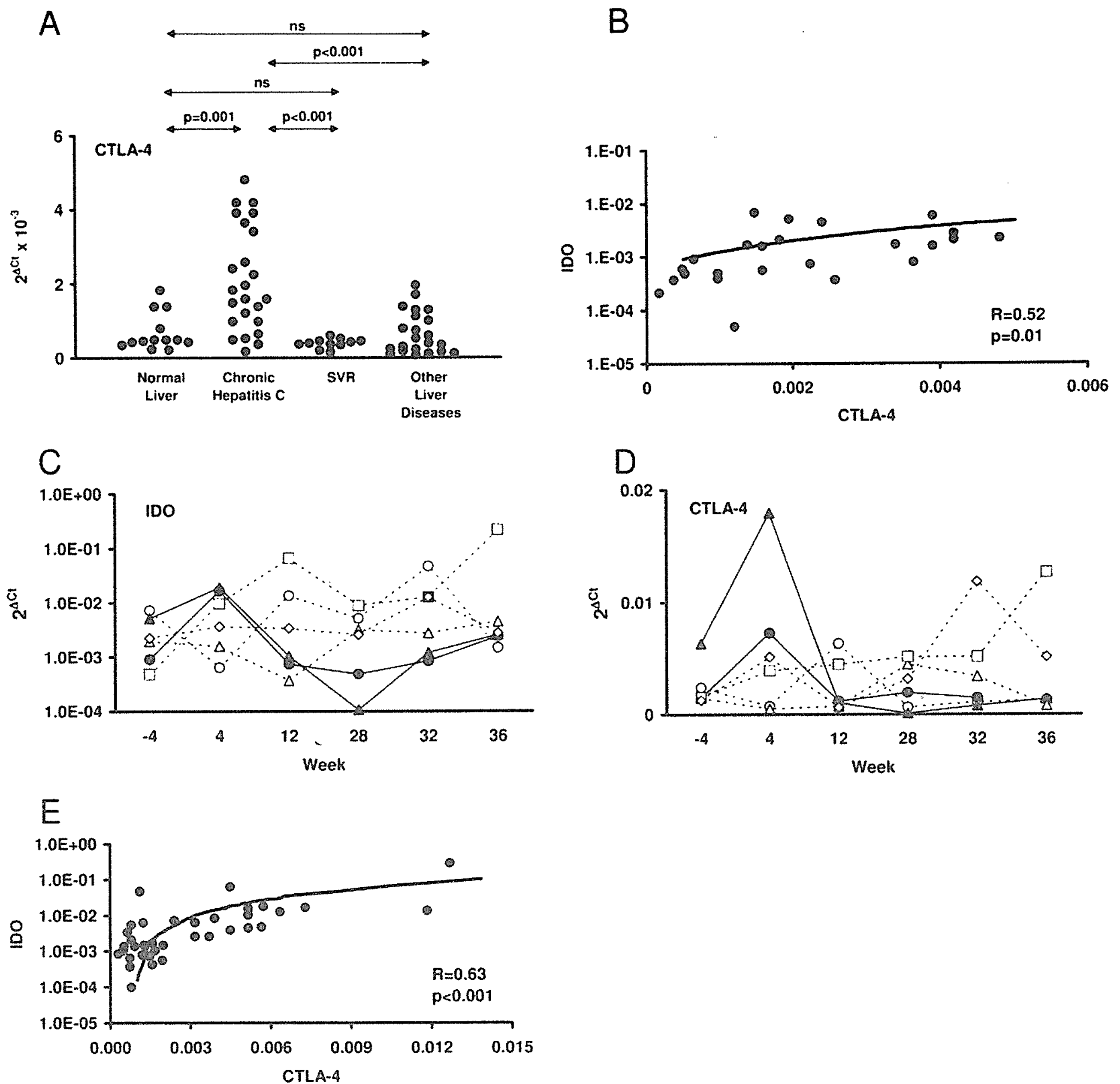

FIG. 3. CTLA-4 and HCV infection. (A) Real-time PCR quantitation of CTLA-4 mRNA in samples from normal livers or from livers from patients with CHC, from patients with CHC who cleared the virus after interferon therapy (SVR), or from a miscellaneous group of patients with liver disorders unrelated to HCV. Statistical analyses were performed using nonparametric Kruskal-Wallis and Mann-Whitney U tests. ns, not significant. (B) Correlation between mRNA levels of IDO and CTLA-4 in liver samples from CHC patients. (C and D) Real-time PCR quantitation of IDO and CTLA-4 mRNA levels in liver samples from chimpanzees obtained at different time points before and after infection with infective HCV inocula, with 0 being the week of infection. Solid lines, chimpanzees who cleared HCV infection; dotted lines, chimpanzees who did not clear HCV infection. (E) Correlation between mRNA levels of IDO and CTLA-4 in liver samples from the chimpanzees described above. Results in panels A, C, and D are normalized with β-actin.

the induction of IDO in response to proinflammatory cytokines and activated T cells. This may constitute an efficient strategy of the virus to escape T-cell immunity. Our findings point to novel targets for therapeutic intervention.

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