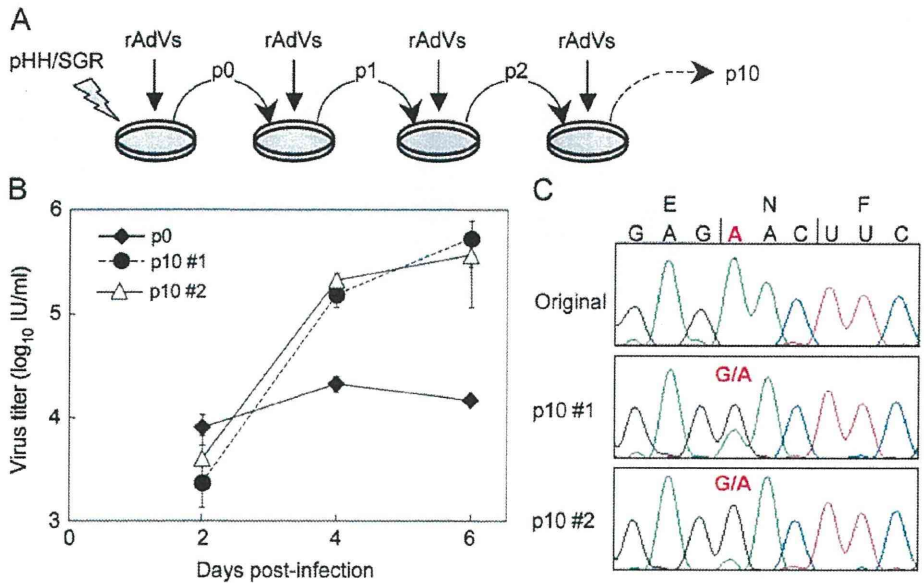

Fig. 6. Genotypic changes in HCVtcp following blind passage. (A) Experimental procedure for blind passage of HCVtcp. Huh7.5.1 cells were transfected with pHH/SGR and were doubly infected with AxCANCre and AxCALNLH-CNS2. Culture fluids were collected and were inoculated into cells infected with AxCANCre and AxCALNLH-CNS2. These procedures were repeated 10 times with two independent samples (#1 and #2). (B) Growth curves of HCVtcp p0 and p10 on Huh7.5.1 cells expressing core-NS2. Cells were infected with HCVtcp at an MOI of 0.05, and medium was collected at the indicated time points and subjected to titration. (C) Nucleotide sequences of original and blind-passaged replicons from HCVtcp. Nucleotides of mutated position are shown in red and bold.

The impact of the N1586D mutation on production of intra- and intergenotypic HCVtcp chimeras was also investigated. The N1586D mutation in the replicon enhanced the production of chimeric HCVtcp by providing core-p7 from all strains examined, although not statistically significant in THpa, and Con1 strains (Fig. 7D). Finally, to determine whether the N1586D mutation was responsible for enhancing HCVcc production, this mutation was introduced into pHHJFH1, which carries the full-length wild-type JFH-1 cDNA (Masaki et al., 2010), yielding pHHJFH1N1586D. The virus titer obtained from cells transfected with the pHHJFH1N1586D was significantly higher than that of WT (Fig. 7E), thus demonstrating that the N1586D mutation enhances yields of HCVcc, in addition to HCVtcp.

Discussion

Single-round infectious viral particles generated by *trans*-packaging systems are considered to be valuable tools for studying virus life cycles, particularly the steps related to entry into target cells, assembly and release of infectious particles. However, limited HCV strains have been applied for the efficient production of HCVtcp to date. In this study, we improved the HCVtcp system in order to enhance the productivity of infectious particles. Production of chimeric HCVtcp by providing genotype 1b-derived core-p7, in addition to intragenotypic viral proteins, was also confirmed. Furthermore, we exploited the system to investigate genetic changes during serial passage of target cells and identified a novel cell culture-adaptive mutation in NS3, which also contributes to enhance the productivity of HCVtcp.

HCVpp (Bartosch et al., 2003a; Hsu et al., 2003) has proven to be a valuable surrogate system by which the study of viral and cellular determinants of the viral entry pathway is possible. Early steps of HCV infection, including the role of HCV glycoprotein heterodimers, receptor binding, internalization and pH-dependent endosomal fusion, have been at least in part mimicked by HCVpp (Lavie et al., 2007). However, as HCVpp is generated in non-hepatic cells such as the human embryo kidney cells 293T, it is likely that the cell-derived component(s) of HCVpp differ from those of HCVcc. Hepatocytes play a role in maintaining lipid homeostasis in the body by assembling and secreting lipoproteins, including VLDL. It is highly likely that HCV exploits lipid synthesis pathways, as there is a tight link between virion formation and VLDL synthesis. Down-regulation of ApoE considerably reduces HCV production (Benga et al., 2010; Chang et al., 2007; Hishiki et al., 2010; Jiang and Luo, 2009; Owen et al., 2009). Infectivity of HCVcc is also neutralized by anti-ApoE antibodies (Chang et al., 2007). These data suggest that ApoE is important for HCV infectivity. Furthermore, Niemann-Pick C1-like 1 (NPC1L1), involving cholesterol uptake receptor, was recently identified as a host factor for HCV entry (Sainz et al., 2012). Knockdown of NPC1L1 had no effect on the entry of HCVpp whereas HCVcc entry was impaired, possibly due to different cholesterol content of these particles. Here, we found that the anti-ApoE antibody neutralized infection by HCVtcp and HCVcc, but not by HCVpp (Fig. 4A and C), thus suggesting that biogenesis and/or secretion pathways of VLDL are involved in HCVtcp similarly to HCVcc, but not in HCVpp.

We also observed that infectivity of HCVtcp and HCVcc is more efficiently neutralized by the anti-CD81 antibody, as compared to that of HCVpp (Fig. 4B and D). It has recently been reported that E2 of HCVcc contained both high-mannose-type and complex-type glycans, whereas most of the glycans on HCVpp-associated E2 were complex-type, which is matured by Golgi enzymes (Vieyres et al., 2010). Mutational analysis of the N-linked glycosylation sites in E1/E2 demonstrated that several glycans on E2 may affect the sensitivity of HCVpp against antibody neutralization, as well as access of CD81 to its binding site on E2 (Helle et al., 2010). The differences in sensitivity between HCVtcp and HCVpp to neutralization by anti-CD81 antibody observed here may be due to differences in carbohydrate composition of HCV glycoproteins during expression and processing of E1/E2 in cells and morphogenesis of HCVtcp and HCVpp.

By analyzing the various replicons for *trans*-packaging, we observed the highest production of HCVtcp with replicons from pHH/SGR, which lacked sequences not essential for RNA

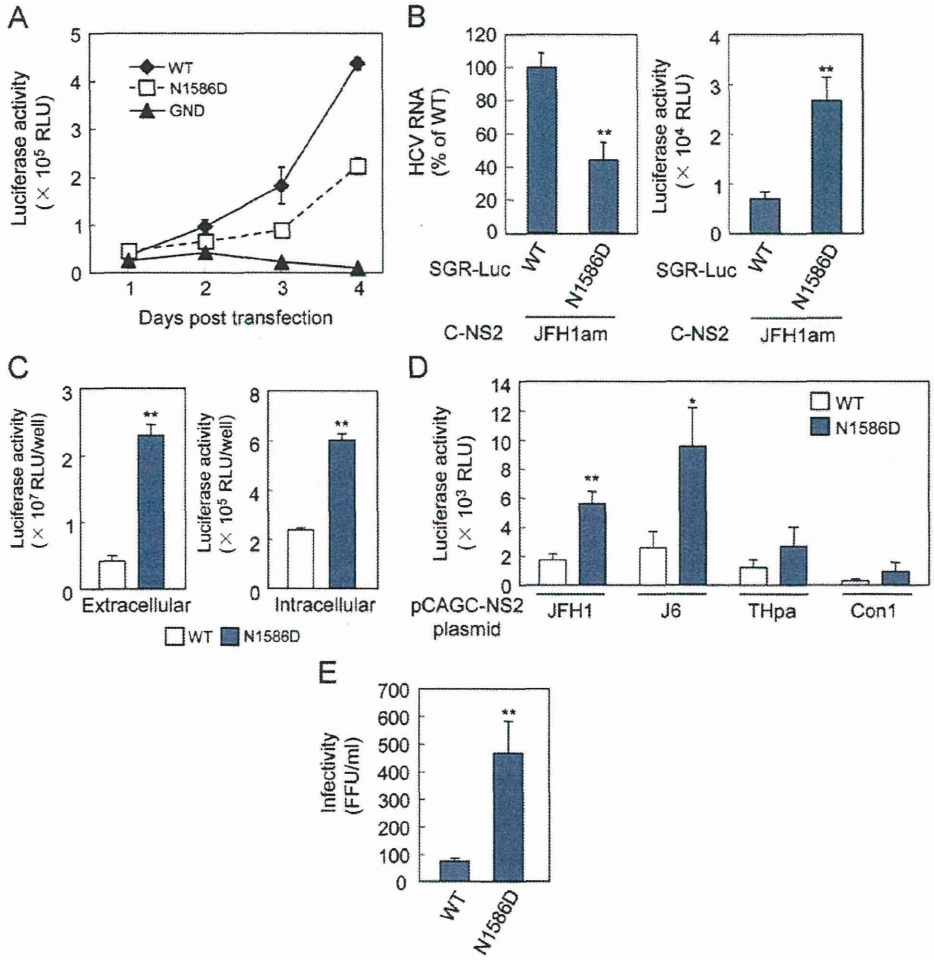

Fig. 7. Effects of N1586D mutation on RNA replication and production of HCVtcp or HCVcc. (A) RNA replication of replicons in cells transfected with pHH/SGR-Luc (WT) or N1586D mutant. Luciferase activities at 1 to 4 day post-transfection were determined. (B) Relative levels of HCV RNA in the supernatants from cells transfected with pHH/SGR-Luc (WT) or N1586D mutant plasmid along with pCAGC-NS2/JFH1am were shown in the left panel. Luciferase activities in cells inoculated with supernatants from cells transfected with indicated plasmids at 4 day post-transfection were shown in the right panel. (C) Luciferase activity in cells inoculated with supernatant and cell lysates from Huh7-25 cells transfected with pHH/SGR-Luc (WT) or N1586D mutant plasmid along with pCAGC-NS2/JFH1am at 5 day post-transfection. (D) Luciferase activity in cells inoculated with culture supernatant from cells transfected with pHH/SGR-Luc (WT) or N1586D mutant plasmid along with indicated core-NS2 plasmids at 4 day post-transfection. (E) Infectivity of supernatant from cells transfected with pHH/JFH1 (WT) or its derivative plasmid containing N1586D mutation at 6 day post-transfection. Statistical differences between WT and N1586D were evaluated using Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.005$ vs. WT.

replication, while less efficient productivity was observed from pHH/SGR-Luc, pHH/SGR-C177, pHH/SGR-C191 and pHH/SGR-C-p7/am (Fig. 2C). Differences in the replication efficiency of the replicon do not appear to be a major determinant for HCVtcp productivity, at least in the present settings, as all replicon constructs except pHH/SGR-Luc replicated at similar levels, as confirmed by Western blotting (Fig. 2B). Although the shorter viral genome sequence may offer advantages over the longer sequence, further investigation is required in order to understand the molecular mechanisms underlying viral genome packaging. By comparing pHH/SGR vs. pHH/SGR-C177, pHH/SGR-C191 and pHH/SGR-C-p7/am, it is likely that the expression of the structural protein in *cis* does not increase HCVtcp production when sufficient amounts of structural proteins are supplied in *trans*.

Blind passage of HCVtcp in packaging cells infected with rAdVs providing core-NS2 enabled us to identify a novel culture-adaptive mutation in NS3. The N-terminal third of NS3 forms a serine protease, together with NS4A, and its C-terminal two-thirds exhibits RNA helicase and RNA-stimulated NTPase activities. In addition, similarly to flaviviruses (Kummerer and Rice, 2002; Liu et al., 2002), it is now apparent that HCV NS3 is also involved in viral morphogenesis (Han et al., 2009; Ma et al., 2008), although its precise role and underlying molecular mechanism(s) have not fully been elucidated. Two cell-culture adaptive NS3 mutations which are involved in HCV assembly have been identified. The Q1251L mutation in helicase subdomain 1 resulted in approximately 30-fold higher production of HCV without affecting NS3 enzymatic activities (Ma et al., 2008). The M1290K adaptive mutation was also located in subdomain 1 of the NS3 helicase (Han et al., 2009). The N1586D mutation identified here was located in subdomain 3 of helicase. Analogous to Q1251L and M1290K, the N1586D mutation enhanced the infectious viral assembly by increasing specific infectivity without affecting the efficiency of viral RNA replication. Considering the possibility that NS3 plays a role in linking between the viral replicase and assembly sites (Jones et al., 2011), it is likely that NS3 helicase is one of the determinants for interaction with the structural proteins. Our results, together with earlier studies, suggest that chimeric and defective mutations as well as supplying the viral components in *trans*, function as selective pressures in virion assembly.

In summary, we have established a plasmid-based reverse genetics for efficient production of HCVtcp with structural

proteins from various strains. Single-round infectious HCVtcp can complement the HCVcc and HCVpp systems as a valuable tool for the study of HCV life cycles.

Materials and methods

Cells

Huh7 derivative cell line Huh7.5.1 and Huh7-25 were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with nonessential amino acids, 100 U of penicillin/mL, 100 μg of streptomycin/mL, and 10% fetal bovine serum at 37 °C in a 5% CO_2 incubator.

Plasmids

Plasmids pHHJFH1, pHH/SGR-Luc, pHH/SGR-Luc/GND and pCAG/C-NS2 were as described previously (Masaki et al., 2010). In this study, plasmid pCAG/C-NS2 was designated as pCAGC-NS2/JFH. The plasmid pCAGC-NS2/JFHam having adaptive mutations in E2 (N417S), p7 (N765D), and NS2 (Q1012R) in pCAGC-NS2/JFH was constructed by oligonucleotide-directed mutagenesis. These mutations were also introduced in pHHJFH1, resulting in pHHJFH1am. To generate core-NS2 expression plasmids with different strains of HCV, the cDNA coding core to the first transmembrane region of NS2 (33 amino acids) in pCAGC-NS2/JFH was replaced with the corresponding sequence of the J6 (Lindenbach et al., 2005), H77c (Yanagi et al., 1997), THpa (Shirakura et al., personal communication) and Con1 (Koch and Bartenschlager, 1999) strains. The THpa sequence contained the P to A mutation at 328 aa at E1 in the original TH strain. To generate pHH/SGR, pHH/SGR-Luc was digested with MluI and PmeI, followed by Klenow enzyme treatment and self-ligation to delete the luciferase coding sequence. To generate pHH/SGR-C177, pHH/SGR-C191 and pHH/SGR-C-p7/am, cDNA coding the partial core and luciferase in pHH/SGR-Luc were replaced with coding sequences for mature core (177aa), full-length core (191aa) or core-p7 polyprotein containing adaptive mutations in E2 and p7, respectively. The selected NS3 mutation (N1586D) was introduced into pHH/SGR-Luc and pHHJFH1 by oligonucleotide-directed mutagenesis.

Generation of viruses

HCVcc and HCVtcp were generated as described previously (Masaki et al., 2010). For the production of HCVpp-2a, plasmid pcDNAdeltaC-E1-E2(JFH1)am having adaptive mutations in E2 (N417S) in pcDNAdeltaC-E1-E2(JFH1) (Akazawa et al., 2007) was constructed by oligonucleotide-directed mutagenesis. Murine leukemia virus pseudotypes with VSV G glycoprotein expressing luciferase reporter (VSVpp) were generated in accordance with previously described methods (Akazawa et al., 2007; Bartosch et al., 2003a).

Luciferase assay

Huh7.5.1 cells were seeded onto a 24-well plate at a density of 3×10^4 cells/well 24 h prior to inoculation with reporter viruses. Cells were incubated for 72 h, followed by lysis with 100 μL of lysis buffer. Luciferase activity of the cells was determined using a luciferase assay system (Promega, Madison, WI). All luciferase assays were performed in triplicate.

Quantification of HCV infectivity and HCV RNA

To determine the titers of HCVtcp and HCVcc, Huh7.5.1 cell monolayers prepared in multi-well plates were incubated with dilutions of samples and then replaced with media containing 10% FBS and 0.8% carboxymethyl cellulose. Following incubation for 72 h, monolayers were fixed and immunostained with rabbit polyclonal anti-NS5A antibody, followed by Alexa Fluor 488-conjugated anti-rabbit secondary antibody (Invitrogen), and stained foci or individual cells were counted and used to calculate a titer of focus-forming units (FFU)/mL for spreading infections or infectious units (IU)/mL for non-spreading infections. For intracellular infectivity, the cell pellet was resuspended in culture media, and cells were lysed by four freeze–thaw cycles. Cell debris was pelleted by centrifugation for 5 min at 4000 rpm. Supernatant was collected and used for titration. To determine the amount of HCV RNA in culture supernatants, RNA was extracted from 140 μL of culture medium by QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA) and treated with DNase (TURBO DNase; Ambion, Austin, TX) at 37 °C for 1 h. Extracted RNA was further purified by using an RNeasy Mini Kit, which includes RNase-free DNase digestion (QIAGEN). Copy numbers of HCV RNA were determined by real-time quantitative reverse transcription-PCR as described previously (Wakita et al., 2005).

Antibodies

Mouse monoclonal antibodies against actin (AC-15) and CD81 (JS-81) were obtained from Sigma (St. Louis, MO) and BD Biosciences (Franklin Lakes, NJ), respectively. Goat polyclonal antibody to ApoE (LV1479433) was obtained from Millipore (Tokyo, Japan). Anti-NS5A and anti-NS5B antibodies were rabbit polyclonal antibody against synthetic peptides.

Neutralization assay

For neutralization experiments with anti-CD81 antibody, Huh7.5.1 cells were incubated with dilutions of anti-CD81 antibody for 1 h at 37 °C. Cells were then infected with viruses for 5 h at 37 °C. For neutralization experiments with anti-ApoE antibody, viruses were incubated with various concentrations of anti-ApoE antibody at room temperature for 1 h and cells were infected with viruses for 5 h at 37 °C. Following infection, supernatant was removed and cells were incubated with culture medium, and luciferase activity was determined at 3 day post-infection for HCVtcp and pseudotyped viruses. For neutralization experiments with HCVcc generated with pHHJFH1am, a multiplicity of infection (MOI) of 0.05 was used for inoculation, and intracellular core protein levels were monitored by ELISA (Ortho Clinical Diagnostics) at 24 h post-infection.

Immunoblotting

Transfected cells were washed with PBS and incubated with lysis buffer (50 mM Tris–HCl, pH 7.4, 300 mM NaCl, 1% triton X-100). Lysates were then sonicated for 5 min and were added to the same volume of SDS sample buffer. Protein samples were boiled for 10 min, separated by SDS-PAGE, and transferred to PVDF membrane. After blocking, membranes were probed with first antibodies, followed by incubation with peroxidase-conjugated secondary antibody. Antigen–antibody complexes were visualized using an enhanced chemiluminescence detection system (Super Signal West Pico Chemiluminescent Substrate; PIERCE, Rockford, IL), in accordance with the manufacturer's protocols.

Generation of recombinant adenoviruses

rAdV, AxCANCre, expressing Cre recombinase tagged with nuclear localization signal under CAG promoter was prepared as described previously (Baba et al., 2005). The target rAdV AxCALNLH-CNS2 expressing HCV core-NS2 polyprotein with adaptive mutations in E2, p7 and NS2 was generated as follows. Cosmid pAxCALNLwit2 is identical to pAxCALNLw (Sato et al., 1998), except that both the terminal sequences of the rAdV genome are derived from pAxCAwit2 (Fukuda et al., 2006). The core-NS2 fragment obtained from pCAGC-NS2/JFH1am by StuI-EcoRI digestion and subsequent Klenow treatment was inserted into the SwaI site of pAxCALNLwit2. The resultant cosmid pAxCALNLH-CN2it2 was digested with PacI and transfected into 293 cells to generate rAdV AxCALNLH-CNS2.

Preparation of packaging cells for HCVtcp

Huh7.5.1 cells were coinfected with AxCANCre at an MOI of 1 and AxCALNLH-CNS2 at an MOI of 3 for expression of JFH-1 core-NS2 polyprotein containing the adaptive mutations in E2, p7 and NS2.

RNA preparation, RT-PCR and sequencing

Total cellular RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA), and subjected to reverse transcription with random hexamer and Superscript III reverse transcriptase (Invitrogen). Three fragments of HCV cDNAs that cover the entire HCV subgenomic replicon genome, were amplified by nested PCR with TaKaRa Ex Taq polymerase (Takara, Shiga, Japan). Amplified products were separated by agarose gel electrophoresis, and were used for direct DNA sequencing.

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Specific interaction of the envelope glycoproteins E1 and E2 with liver heparan sulfate involved in the tissue tropismatic infection by hepatitis C virus

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Abstract The first step in the process of infections by the hepatitis C virus (HCV) is attachment to the host cell, which is assumed to be mediated by interaction of the envelope glycoproteins E1 and E2 with cell surface glycosaminoglycans. In this study, a variety of glycosaminoglycans, heparan sulfate (HS) from various bovine tissues as well as chondroitin sulfate (CS)/dermatan sulfate from bovine liver, were used to examine the direct interaction with recombinant E1 and E2 proteins. Intriguingly, among HS preparations from various bovine tissues, only liver HS strongly bound to both E1 and E2. Since HS from liver, which is the target tissue of HCV, contains highly sulfated structures compared to HS from other tissues, the present results suggest that HS-proteoglycan on the liver cell surface appears to be one of the molecules that define the liver-specific tissue tropism of HCV infection. The interaction assay with chemically modified heparin derivatives provided evidence that the binding of the viral proteins to heparin/HS is not only mediated by simple ionic interactions, but that the 6-*O*-sulfation and *N*-sulfation are important. Heparin oligosaccharides equal to or larger than 10-mer were required to inhibit the binding. Notably, a highly sulfated CS-E preparation from squid cartilage also strongly interacted with both viral proteins and inhibited the entry of pseudotype HCV into the target cells, suggesting that the highly sulfated CS-E might be useful as an anti-HCV drug.

Keywords Heparan sulfate · Chondroitin sulfate · Hepatitis C virus · Glycosaminoglycan · Tissue tropism

The contributions of Fumi Kobayashi and Shuhei Yamada should be considered equal.

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Abbreviations

2AB	2-aminobenzamide
CDNA	completely desulfated and *N*-acetylated heparin
CDNS	completely desulfated and *N*-sulfated heparin
NDNA	*N*-desulfated and *N*-acetylated heparin
2ODS	2-*O*-desulfated heparin
6ODS	6-*O*-desulfated heparin
CS	chondroitin sulfate
DS	dermatan sulfate
ELISA	enzyme-linked immunosorbent assay
FGF	fibroblast growth factor
GAG	glycosaminoglycan
HCV	hepatitis C virus

ΔHexA	4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid
HPLC	high performance liquid chromatography
HS	heparan sulfate
PG	proteoglycan
VSV	vesicular stomatitis virus
NS	2-*N*-sulfate
2S	2-*O*-sulfate
4S	4-*O*-sulfate
6S	6-*O*-sulfate

Introduction

Hepatitis C virus (HCV) is classified in the genus *Hepacivirus* within the family *Flaviviridae*, which includes classical flaviviruses (ex. yellow fever, dengue and tickborne encephalitis viruses) and animal pestiviruses (ex. bovine viral diarrhoea virus). Nearly 170 million people worldwide are infected with HCV [1]. Chronic HCV infections can lead to liver cirrhosis and hepatocellular carcinoma [2]. However, no specific antiviral drug is available for treatment. Therefore, a better understanding of the mechanism of HCV infection and the development of an effective anti-HCV drug are high priority tasks in medical and pharmaceutical communities.

The first step in the HCV infection process is the attachment of the virus to the host cell, which is assumed to be mediated by interactions of the envelope glycoproteins E1 and E2 with heparan sulfate (HS)-proteoglycan (PG) [3, 4] and a low-density lipoprotein receptor [5, 6]. Subsequently, specific binding between the viral glycoproteins and entry receptor proteins induces receptor-mediated endocytosis and the ingress of HCV particles across the plasma membrane of cells. There are several candidate receptor proteins on the host cell including a member of the tetraspanin protein family, CD81 [7], the scavenger receptor BI [8], and the tight-junction proteins human claudin-1 [9] and occludin [10]. Little is known about how these factors co-ordinate to facilitate the actual viral entry process. One current model predicts a multistep process that includes attachment, receptor binding, post-binding association with tight-junction proteins, and then internalization by endocytosis, which is followed by a pH-dependent step that results in the fusion of membranes and the release of the viral RNA into the cytoplasm of the host cells [11]. Several studies have demonstrated the role of glycosaminoglycans (GAGs) in the HCV adsorption and the binding of the E2 protein [3, 12, 13]. Recently, the interaction of glypican-3, a cell surface HS-PG, with CD81 in the liver has been demonstrated [14], suggesting the HCV particles to be transferred from the cell surface HS to CD81. However, little is known about the structural features of GAGs required for the binding of HCV to host cells.

GAGs are linear polymers composed of alternating amino sugar and hexuronic acid residues and distributed as side chains of PGs in the extracellular matrix or at the cell surface of animal tissues. GAG chains play important roles in various biological functions such as cell proliferation, differentiation, migration, tissue morphogenesis, organogenesis, infection, and wound repair [15–17] by interacting with bioactive molecules. Major GAGs include chondroitin sulfate/dermatan sulfate (CS/DS) and HS/heparin. Although the polysaccharide backbones of these GAGs are simple, repetitive linear chains, these structures acquire a considerable degree of variability by extensive modifications involving sulfation and uronate epimerization, which are the basis for a wide variety of their biological activities [17–19].

Many bacteria, parasites, and viruses exploit cell surface GAGs as receptors [17, 20, 21]. Among several GAG types present in animal cells, HS has been the most studied, and demonstrated to associate with various pathogens including dengue virus, herpes simplex virus type 1, human papillomavirus, and HCV [22–25]. Most interactions between adherent microorganisms and cell surface GAGs are considered to be nonspecific and ionic because of the high charge density of GAGs due to a cluster of sulfate groups. However, in some cases, unique sugar sequences in GAG chains appear to be involved in microbial adherence [20].

Detailed investigations of GAG structure not only should provide a better understanding of the mechanism of HCV attachment, but may also lead to the potential application of GAG as an anti-HCV drug. In the present study, specific binding of the E1 and E2 proteins to the HS from liver among the HS preparations from various bovine tissues was demonstrated, which revealed the tissue tropism of HCV infection. To characterize the structure of GAG chains involved in the HCV infection process, the sulfation and chain length required for binding to the E1 and E2 proteins were studied using heparin oligosaccharides. Moreover, the inhibition of HCV infectivity by highly sulfated CS/DS preparations was unveiled.

Materials and methods

Materials Chondroitinase ABC from *Proteus vulgaris*, standard unsaturated disaccharides, CS-C and CS-D from shark cartilage, CS-E from squid cartilage, chemically modified heparin derivatives (*CDNS*, completely desulfated and *N*-sulfated heparin; *CDNA*, completely desulfated and *N*-acetylated heparin; *NDNA*, *N*-desulfated and *N*-acetylated heparin), and HS from bovine kidney were obtained from Seikagaku Corp., Tokyo, Japan. 2-*O*-Desulfated heparin (*2ODS*) and 6-*O*-desulfated heparin (*6ODS*) derivatives were kindly provided by

Prof. Masayuki Ishihara (National Defense Medical College, Tokorozawa, Japan) [26, 27]. HS preparations from bovine intestine, aorta, and lung were kindly provided by Keiichi Yoshida (Seikagaku Corp., Tokyo, Japan) [28]. HS and CS/DS from bovine liver were prepared as described previously [29]. Recombinant heparinases I and III from *Flavobacterium heparinum* were from IBEX Technologies, Montreal, Canada. Anti-myc antibody, anti-V5 antibody, and ECL anti-mouse IgG horseradish peroxidase-linked whole antibody (from sheep) were obtained from Invitrogen Co., Carlsbad, CA. Phosphatase-labeled anti-mouse IgG+IgM (H+L) antibody was from Kirkegaard & Perry Laboratories, Inc., Geithersburg, MA. A soluble form of the recombinant envelope glycoprotein E1 of genotype 1b (comprising amino acids 192–340) with a V5 and His6 tag fusion protein or E2 of genotype 1b (comprising amino acids 384–711) with a myc and His6 tag fusion protein was generated using a baculovirus/HighFive cell system at 27 °C with Sf-900 II SFM insect cell medium (GIBCO) containing 10 % (v/v) fetal bovine serum (FBS). The expressed proteins were purified using a QIAexpress Protein Purification System (QIAGEN), following a protocol provided by the manufacturer. Concentration of the purified E1 and E2 proteins was estimated on the basis of silver staining using bovine serum albumin as the protein standard.

Analysis of the disaccharide composition of various GAGs An aliquot of the GAG samples was digested with chondroitinase ABC or a mixture of recombinant heparinases I and III as described previously [30, 31]. Each digest was labeled with 2-aminobenzamide (2AB) [32], and excess 2AB reagents were removed by extraction with chloroform [33]. The 2AB-labeled digest was analyzed by anion-exchange HPLC on a PA-03 silica column (YMC Co., Kyoto, Japan) [32]. Identification and quantification of the resulting disaccharides were achieved by comparison with the elution positions of authentic unsaturated disaccharides.

Western blotting The purified E1 and E2 proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5 % polyacrylamide gels (Ready Gels J, Bio-Rad laboratories Inc., Tokyo, Japan), and transferred to a hydrophobic polyvinylidene difluoride membrane (GE Healthcare, Buckinghamshire, UK). The recombinant E1 and E2 proteins were detected with anti-V5 and anti-myc mouse monoclonal IgG antibodies (diluted 1:1,000 in 25 mM Tris-buffered saline containing 2 % blocking reagent), respectively, using Amersham ECL Advance reagents (GE Healthcare).

Enzyme-linked immunosorbent assay (ELISA) GAG preparations (250 μg) were biotinylated using EZ-Link Biotin-LC-Hydrazide as recommended by the manufacturer or Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific Inc., Rockford, IL) [34]. Excess reagent was removed by dialysis against distilled water. ELISA plates (Thermo Fisher Scientific Inc.) were coated with 0.5 or 1 μg of biotinylated GAG per well at 4 °C overnight and then incubated with blocking buffer, 3 % bovine serum albumin in phosphate-buffered saline (PBS), for 1 h at room temperature. The recombinant E1 or E2 protein was added and incubated for 1 h at 37 °C. Since the molecular weight of E1 protein is approximately one sixth of that of E2 protein, the amount of the latter used was six times that of the former for the incubation to perform the assays at a similar molar concentration. After washing, the bound protein was detected by the addition of anti-V5 or anti-myc antibody (diluted 1:200 in PBS for 1 h at 37 °C) for the detection of E1 and E2, respectively, and then alkaline phosphatase-conjugated anti-mouse IgG/IgM secondary antibody (diluted 1:3,000 in Tris-buffered saline for 1 h at 37 °C). *p*-Nitrophenyl phosphate was used as the substrate for alkaline phosphatase.

For inhibition experiments, the recombinant E1 or E2 protein was preincubated for 30 min at room temperature with inhibitors (CS-E, CS-D, heparin, or heparin oligosaccharides) before being added to the biotinylated heparin-coated plate. After washing, the bound protein was detected using anti-V5 or anti-myc antibody and alkaline phosphatase-conjugated anti-mouse IgG/IgM secondary antibody as described above.

Effects of GAGs on infectivity of pseudotype vesicular stomatitis virus (VSV) possessing HCV envelope proteins (HCVpv) The construction of HCVpv and infection experiments were carried out as reported previously [35]. Briefly, HEK293T cells were transfected with an expression plasmid encoding the E1 and E2 proteins and incubated for 24 h at 37 °C. To incorporate these proteins into VSV, the cells were then infected with a VSVG-complemented pseudotype virus, in which the G envelope gene was replaced with the luciferase gene [35]. After 2 h of incubation at 37 °C, the cells were extensively washed four times with DMEM and harvested after incubation for 24 h at 37 °C. The HCVpv secreted in the conditioned medium of the infected cells was used for the infection experiment.

HCVpv was preincubated with various concentrations of CS-E, CS-D, HS, or heparin (0, 5, and 50 μg/ml) at 37 °C for 1 h and inoculated into the culture medium of Huh7 cells. After incubation for 1 h at 37 °C, the cells were washed with DMEM containing 10 % FBS three times and incubated at 37 °C for 24 h and the luciferase activity was measured.

Results

Characterization of GAGs in bovine liver tissue Hepatocytes are the main target cells of HCV. GAGs in liver tissue may contain unique structures required for the attachment of

HCV. To characterize the structural features in detail, GAGs were extracted from bovine liver, and the proportion of HS and CS/DS in GAGs derived from bovine liver was quantified to be 65 % and 35 %, respectively. HS was the major component in the GAG preparation from liver tissue. Their disaccharide composition was analyzed and the data are summarized in Tables 1 and 2. The major disaccharide unit in bovine liver HS was the trisulfated disaccharide ΔHexA (2S)-GlcN(NS, 6S) (43 %). The proportion of highly sulfated HS disaccharides (di- and trisulfated disaccharides) in bovine liver HS was 57 % (Table 1), whereas that in bovine aorta, lung, intestine, or kidney was 10 %, 21 %, 26 %, or 19 %, respectively [28], indicating the bovine liver HS to be more highly sulfated than HS from other bovine organs. The major disaccharide unit in CS/DS from bovine liver was ΔHexA-GalNAc(4S) (71 %), followed by the 4- and 6-*O*-disulfated disaccharide ΔHexA-GalNAc(4S, 6S) (25 %) (Table 2). The proportion of highly sulfated CS/DS disaccharides (di- and trisulfated disaccharides) found in the bovine liver (25 %) was significantly higher, compared with that of CS/DS preparations from bovine lung (13 %), trachea (0 %), and heart (15 %) [37, 38].

Interaction of the recombinant E1 and E2 proteins with GAGs derived from bovine liver The direct interaction of various GAGs with the recombinant E1 and E2 proteins was analyzed. HS from bovine liver, kidney, intestine, aorta, and lung as well as CS/DS from bovine liver were biotinylated and immobilized on a streptavidin-coated plate for ELISA. The recombinant proteins were produced by insect High Five cells and detected by Western blotting (Supplementary Data 1). Although their expected sizes were 10 and 40 kDa, respectively, E2 protein was larger than expected, consistent with a previous study indicating the posttranslational modification of the proteins [39]. Only bovine liver HS bound to both E1 and E2 proteins (172 % and 123 %, respectively, compared to the binding to heparin) (Fig. 1) in a concentration-dependent manner (data not shown). The interaction was confirmed using the BIAcore system. E1 and E2 proteins were individually injected at different concentrations onto the surface of the bovine liver HS-immobilized sensor chip. Overlaid sensorgrams are shown in Supplementary Data 2. Both E1 and E2 proteins bound to the bovine liver HS preparation in a concentration-dependent manner. In contrast, no significant binding of E1 or E2 to HS from other tissues or bovine liver CS/DS was observed (Fig. 1), indicating that the E1 and E2 proteins interact specifically with bovine liver HS, which appears to play the major role in the binding of HCV to liver cells, in consistent with the tissue tropism of the infection of HCV.

Determination of the sulfation structure required for the binding to E1 and E2 proteins To study whether the binding of the E1 and E2 proteins to heparin requires structurally defined HS oligosaccharides, the size effect of heparin oligosaccharides (ranging from di- to dodecasaccharides and polysaccharides) on the binding of E1 or E2 to immobilized heparin was analyzed. Although the reactivity of the E1 and E2 proteins with the immobilized heparin was strongly inhibited by free heparin polysaccharide chains, heparin oligosaccharides did not exhibit as much inhibitory activity as heparin polysaccharides. However, the 10-mer and 12-mer forms showed some inhibition (Fig. 2), indicating the minimum length required for the inhibition to be 10-mer. This result is consistent with a report that HCV pseudoparticles required heparin oligosaccharides of at least 10-mer for binding [40].

The structure required for the binding was characterized further. Chemically modified heparin derivatives were used to analyze the direct interaction. Both the E1 and E2 proteins bound strongly to *2ODS* (84 % and 58 %, respectively, compared to the binding to heparin) and moderately to *6ODS* (38 % and 23 %) and *NDNA* (34 % and 19 %), whereas no significant interaction of either protein with *CDNS* or *CDNA* was observed (Fig. 3), indicating that sulfation at the C2 (amino group) and C6 positions of GlcN residues is more important for the interaction than sulfation at the C2 position of uronic acid residues.

Table 1 Disaccharide composition of HS derived from various tissues (%)

	ΔHexA-GlcNAc	ΔHexA-GlcN(NS)	ΔHexA-GlcNAc(6S)	ΔHexA (2S)-GlcNAc	ΔHexA-GlcN(NS, 6S)	ΔHexA(2S)-GlcN(NS)	ΔHexA(2S)-GlcNAc(6S)	ΔHexA(2S)-GlcN(NS, 6S)	S/unit[b]	Ref. No.
Bovine Aorta	63	20	6	1	2	5	ND	3	0.50	28
Bovine Lung	45	16	17	1	9	8	ND	4	0.80	28
Bovine Intestine	45	19	9	1	8	11	ND	7	0.88	28
Bovine Kidney	53	16	11	1	6	7	ND	6	0.72	28
Bovine Liver	20	17	6	ND[a]	8	3	3	36	1.80	–
Human Liver	37	15	10	1	8	6	1	22	1.22	36

[a] *ND* not detected

[b] *S/unit* the number of sulfate groups per disaccharide unit

Table 2 Disaccharide composition of bovine liver CS/DS

CS/DS disaccharide	Proportion (%)
ΔHexA-GalNAc	ND[a]
ΔHexA-GalNAc(6S)	4
ΔHexA-GalNAc(4S)	71
ΔHexA(2S)-GalNAc(6S)	ND
ΔHexA(2S)-GalNAc(4S)	ND
ΔHexA-GalNAc(4S, 6S)	25
ΔHexA(2S)-GalNAc(4S, 6S)	ND
S/unit[b]	1.25

[a] *ND* not detected

[b] *S/unit* the number of sulfate groups per disaccharide unit

Effect of highly sulfated CS preparations on the infectivity of pesudotype HCV Since a highly sulfated structure is required for interaction with the E1 and E2 proteins, some CS preparations derived from marine animals, which are more highly sulfated than those from mammalian tissue, may bind the proteins. To investigate the potential of such highly sulfated CS from marine animals to inhibit the entry of HCV into host cells, effects on the infection by pesudotype HCV (HCVpv) of Huh7 cells were examined. Highly sulfated CS (CS-D and CS-E), heparin, and bovine liver HS preparations showed dose-dependent inhibition of HCVpv infection, whereas no significant effect was observed on the addition of low sulfated HS from bovine kidney (Fig. 4), indicating that highly sulfated CS and HS/heparin can inhibit the infection of Huh7 cells by HCVpv.

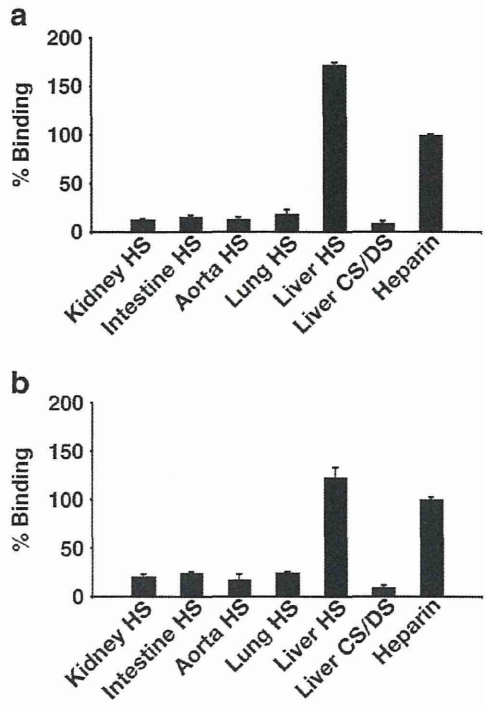

Fig. 1 Interaction of the recombinant E1 or E2 protein with GAGs from various bovine tissues. ELISA plates were coated with 1 μg/well of biotinylated GAGs from various bovine tissues or porcine intestinal heparin as described under "Materials and methods". Recombinant E1 (**a**) or E2 (**b**) protein (3 or 18 μg, respectively) was added and incubated for 1 h at 37 °C. After a wash with PBS/0.05 % Tween 20, the bound E1 and E2 proteins were detected using monoclonal anti-V5 and anti-myc antibodies, respectively, and then alkaline phosphatase-conjugated anti-mouse IgG/IgM secondary antibody. Data are shown as a percentage of the binding of the E1 or E2 protein to heparin. Values represent the mean±standard deviation (SD) (n=2)

Interaction of the recombinant E1 and E2 proteins with highly sulfated CS preparations To examine whether the highly sulfated CS preparations (CS-D and CS-E) bind directly to the recombinant E1 and E2 proteins, the interaction of the proteins with immobilized CS-D and CS-E was examined. Both the E1 and E2 proteins bound strongly to CS-E (69 % and 85 %, respectively, compared to heparin), but very weakly to CS-D (4 % and 19 %, respectively) (Fig. 5). These results may reflect the difference in their total negative charge as represented by the number of sulfate groups per disaccharide unit. CS-D and CS-E contain 1.2 and 1.6 sulfate groups per disaccharide [41], respectively. To further characterize the binding of the recombinant E1 and E2 proteins to highly sulfated CS, the inhibitory effect of CS-E and CS-D on the binding of the E1 and E2 proteins to immobilized heparin was examined. The binding to E1 or E2 was weakly inhibited by CS-D and CS-E or CS-D, respectively (11 % and 22 % or 31 % inhibition at 10 μg/well, respectively, compared to the binding in the absence of inhibitors), whereas the binding to E2 was strongly inhibited by CS-E (54 % inhibition at 5 μg/well, compared to the binding in the absence of inhibitors) (Fig. 6), supporting the higher affinity of CS-E than CS-D for the E1 and E2 proteins.

Discussion

Several lines of evidence have demonstrated that GAGs play an important role in the attachment of HCV to host cells [3, 12, 13, 40]. Among GAGs, heparin has been well studied for its interaction with the envelope glycoproteins E1 and E2 [3, 40]. Heparin is distributed in the cytoplasmic granules of mast cells *in vivo* and more highly sulfated than HS, which is ubiquitous on the cell surface [42]. HS at the cell surface in human liver was predicted to interact with the HCV envelope proteins based on reports by Barth *et al.* [3, 40, 43]. However, no direct interaction between the viral proteins and GAGs derived from liver tissue has been shown. In this study, we characterized the disaccharide composition of the GAGs derived from bovine liver and demonstrated the direct interaction of the highly sulfated HS from bovine liver with both E1 and E2 proteins for the first time, although Barth *et al.* have demonstrated inhibition of the cellular binding of E1 and E2 proteins to human