Production and characterization of high-titer serum-free cell culture grown hepatitis C virus particles of genotype 1–6

Christian K. Mathiesen a,b, Tanja B. Jensen a,b, Jannick Prentoe a,b, Henrik Krarup c, Alfredo Nicosia d,e,f, Mansun Law g, Jens Bukh a,b, Judith M. Gottwein a,b,n

a Copenhagen Hepatitis C Program (CO-HEP), Department of Infectious Diseases and Clinical Research Centre, Hvidovre Hospital, Kettegaard Allé 30, 2650 Hvidovre, Denmark
b Department of Infectious Diseases, Hvidovre Hospital, Kettegaard Allé 30, 2650 Hvidovre, Denmark
c Department of International Health, Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, Blegdamsvej 3B, 2200 Copenhagen N, Denmark
d Department of Clinical Biochemistry, Section of Molecular Diagnostics, Aalborg University Hospital, Fredrik Bajers Vej 5, 9220 Aalborg, Denmark
e CEINGE, Via Gaeto Salvatore, 486, 80145 Naples, Italy
f Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II, via S. Pansini 5, 80131, Naples, Italy
g Department of Immunology and Microbial Science, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, USA
h Department of Clinical Biochemistry, Section of Molecular Diagnostics, Aalborg University Hospital, Fredrik Bajers Vej 5, 9220 Aalborg, Denmark
i Department of Immunology and Microbial Science, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, USA

Keywords:
Hepatitis C virus
Cell culture system
Serum-free
High-titer
Biophysical characterization
Vaccine development
Receptor blocking
Neutralization
Genotypes
Adenovirus expression medium

A B S T R A C T

Recently, cell culture systems producing hepatitis C virus particles (HCVcc) were developed. Establishment of serum-free culture conditions is expected to facilitate development of a whole-virus inactivated HCV vaccine. We describe generation of genotype 1–6 serum-free HCVcc (sf-HCVcc) from Huh7.5 hepatoma cells cultured in adenovirus expression medium. Compared to HCVcc, sf-HCVcc showed 0.6–2.1 log10 higher infectivity titers (4.7–6.2 log10 Focus Forming Units/mL), possibly due to increased release and specific infectivity of sf-HCVcc. In contrast to HCVcc, sf-HCVcc had a homogeneous single-peak density profile. Entry of sf-HCVcc depended on HCV co-receptors CD81, LDLr, and SR-BI, and clathrin-mediated endocytosis. HCVcc and sf-HCVcc were neutralized similarly by chronic-phase patient sera and by human monoclonal antibodies targeting conformational epitopes. Thus, we developed serum-free culture systems producing high-titer single-density sf-HCVcc, showing similar biological properties as HCVcc. This methodology has the potential to advance HCV vaccine development and to facilitate biophysical studies of HCV.

© 2014 Elsevier Inc. All rights reserved.

Introduction

Hepatitis C virus (HCV) is a major public healthcare burden with 3–4 million new infections occurring each year and more than 150 million individuals estimated to be chronically infected worldwide (Fauvelle et al., 2013). Many of these individuals develop serious chronic liver diseases such as cirrhosis and hepatocellular carcinoma, making HCV the most frequent cause of liver transplantation (Alter and Seeff, 2000).

HCV is an enveloped, positive-stranded RNA virus of the genus Hepacivirus within the Flaviviridae family. Due to a high degree of genetic heterogeneity, HCV has been classified into 6 epidemiologically important genotypes and numerous subtypes, differing in approximately 30% and 20% of their nucleotide and amino acid sequence, respectively (Simmonds et al., 2005; Gottwein and Bukh, 2008). Genotypes show important clinical and biological differences (Amoroso et al., 1998; Cross et al., 2010; Prentoe et al., 2011; Scheel et al., 2012; Sarrazin et al., 2012; Scheel and Rice, 2013). Serotypes have not been defined; however, different genotypes and subtypes show differential sensitivity to neutralizing antibodies found in sera of chronically infected patients and to monoclonal neutralizing antibodies with therapeutic potential.

Abbreviations: AEM, adenovirus expression medium; ApoA, ApoC1, ApoE, apolipoprotein B, CI, C; BSA, bovine serum albumin; HCVcc, cell culture produced hepatitis C virus; FBS, fetal bovine serum; FFU, Focus Forming Units; HCV, hepatitis C virus; HRP, horseradish peroxidase; HVR1, hypervariable region 1; LVP, lipovirus-particle; LDLr, low-density-lipoprotein receptor; IC50, median inhibitory concentration; MOI, multiplicity of infection; NS protein, nonstructural protein; ND, not determinable; BSK, PBS + 1% BSA + 0.2% skim milk; PE, phycoerythrin; RCF, relative centrifugal force; RT, room temperature; SR-BI, scavenger receptor class B type I; sf-HCVcc, serum-free cell culture produced hepatitis C virus; SEM, standard error of the mean; VLDL, very-low-density-lipoprotein.

* Corresponding author at: Copenhagen Hepatitis C Program (CO-HEP), Department of Infectious Diseases, Hvidovre Hospital, Kettegaard Allé 30, 2650 Hvidovre, Denmark.

E-mail address: judith@gottwein.eu (J.M. Gottwein).

http://dx.doi.org/10.1016/j.virol.2014.03.021
0042-6822/© 2014 Elsevier Inc. All rights reserved.
Subsequently, culture systems producing HCV particles (HCVcc) of (Wakita et al., 2005; Lindenbach et al., 2005; Zhong et al., 2005) and Bartenschlager, 2014). Feasible for HCV (Steinmann and Pietschmann, 2013; Lohmann particle-producing cell culture systems, this approach was not (Plotkin, 2008; Plotkin and Plotkin, 2011). Due to a lack of HCV needed to control HCV globally. Most successful antiviral vaccines contraindications (Fauvelle et al., 2013). Thus, an HCV vaccine is the asymptomatic nature of infection, economic constraints and (Sarrazin et al., 2012). Even though promising new compounds for treatment of HCV are being developed and licenced (Sarrazin et al., 2012; Scheel and Rice, 2013), only a minority of HCV-infected individuals is expected to be diagnosed and treated, mainly due to the asymptomatic nature of infection, economic constraints and contraindications (Fauvelle et al., 2013). Thus, an HCV vaccine is needed to control HCV globally. Most successful antiviral vaccines employ inactivated or attenuated whole viral particles as vaccine antigen and depend on the induction of neutralizing antibodies (Plotkin, 2008; Plotkin and Plotkin, 2011). Due to a lack of HCV particle-producing cell culture systems, this approach was not feasible for HCV (Steinmann and Pietschmann, 2013; Lohmann and Bartenschlager, 2014).

Only in 2005, the first HCV cell culture system supporting the full viral life cycle was developed, based on the genotype 2a isolate JH11 and the human hepatoma cell line Huh7 and derived cell lines (Wakita et al., 2005; Lindenbach et al., 2005; Zhong et al., 2005). Subsequently, culture systems producing HCV particles (HCVcc) of the major genotypes were developed using JFH1-based recombinants expressing genotype specific Core, E1, E2, p7 and NS2 (Pietschmann et al., 2006; Gottwein et al., 2007; Yi et al., 2007; Scheel et al., 2008; Jensen et al., 2008; Gottwein et al., 2009). Such particles could serve as antigens in a whole-virus inactivated HCV vaccine primarily aiming at induction of neutralizing antibodies against structural proteins of the major HCV genotypes.

However, HCVcc yields from the developed cell culture systems are relatively low compared to quantities envisioned to be required for vaccine production. Further, as patient derived HCV particles (Nielsen et al., 2006), HCVcc showed a heterogeneous density profile (Lindembach et al., 2005; Lindenbach et al., 2006; Gastaminza et al., 2006; Prentoe et al., 2011), making density-based purification and concentration procedures difficult. Also, cell cultures are typically treated with animal-derived trypsin, and growth medium used for production of HCVcc is typically supplemented with fetal bovine serum (FBS). Vaccine development, as well as other research applications, such as biophysical studies of HCV particle composition, require generation of purified and concentrated HCVcc stocks. This is expected to be facilitated by reducing concentrations of non-HCV proteins such as FBS derived proteins in HCVcc producing cell cultures. Further, use of FBS and animal-derived trypsin increases the risk of contamination with adventitious microbial agents, of relevance for HCV vaccine development (WHO, 1998; Houghton et al., 2013). Thus, development of methods for production of HCVcc under serum-free conditions is a research focus. At the onset of this study it had been demonstrated that Huh7 cells could be cultured in serum-free medium (RPMI 1640 supplemented with Na2SeO3) without previous adaptation for an extended period of time, and that serum-free cell cultures (DMEM supplemented with Na2SeO3 and lipid rich albumin) allowed replication of HCV (Nakabayashi et al., 1982; Abe et al., 2007).

In this study, we aimed at developing and characterizing serum-free genotype 1–6 HCVcc particles (sf-HCVcc). From infected Huh7.5 cell cultures maintained in adenoivirus expression medium (AEM) without using trypsin, we were able to harvest genotype 1–6 sf-HCVcc containing culture supernatants. The sf-HCVcc particles were characterized by increased infectivity titers and an altered density profile with a single infectivity peak, expected to facilitate purification procedures. However, sf-HCVcc showed similar biological properties as HCVcc regarding routes of viral entry and susceptibility to neutralizing antibodies. Establishment of a robust methodology for generation of high-titer single-density serum-free HCVcc is expected to aid HCV vaccine development.

**Results**

**Huh7.5 cells cultured in serum-free medium yield higher HCV infectivity titers than conventional cultures**

To produce sf-HCVcc, HCV recombinants were cultured in Huh7.5 cells maintained in AEM, a commercially available cell culture medium without animal or human serum as described in “Materials and methods” section. Because AEM cultured Huh7.5 cells did not tolerate detachment, AEM was replaced every 2–3 days without splitting the cells. Thus, animal-derived trypsin was not used during the virus production phase. Cultures handled in this manner became over-confluent but could be maintained for at least 29 days (data not shown).

We tested if AEM cultured cells supported production of sf-HCVcc. Huh7.5 cells cultured in DMEM+10% FBS were initially infected with JFH1-based Core-NS2 recombinants H77(1a), J4(1b), S52(3a) and ED43(4a) (Gottwein et al., 2009) (Fig. 1). When viral infection had spread to ~80% of culture cells, as determined by immunostaining of HCV NS5A antigen, one replicate culture was maintained in AEM and another in DMEM+10% FBS. Similar to previous observations (Gottwein et al., 2009), HCVcc peak supernatant infectivity titers were 3.4–4.2 log10 Focus Forming Units (FFU)/ml, followed by a drop in infectivity titers, when virus induced cell death was observed (Fig. 1) (Gottwein et al., 2007). For sf-HCVcc, peak infectivity titers were higher than for HCVcc, reaching 4.6–5.0 log10 FFU/ml (Fig. 1). Also, relatively high infectivity titers were maintained for a longer period for sf-HCVcc than for HCVcc.

We next aimed at producing sf-HCVcc virus stocks of prototype strains of the six major HCV genotypes and epidemiologically important subtype 1b for further characterization. We infected Huh7.5 cells cultured in DMEM+10% FBS with Core-NS2
recombinants indicated in Fig. 2. DMEM + 10% FBS was replaced by AEM, when 40–80% of culture cells were infected (Fig. 2). From these cultures, high-titer sf-HCVcc virus stocks were harvested at four consecutive time points, before cultures were closed. Peak infectivity titers between 4.7 and 6.2 log_{10} FFU/ml were observed for sf-HCVcc, with sf-J4(1b) and sf-ED43(4a) showing the lowest and sf-SA13(5a) showing the highest titers (Fig. 2 and Table 1). Thus, sf-HCVcc stocks showed 0.6–2.1 log_{10} FFU/mL increased infectivity titers compared to previously described HCVcc reference stocks (Table 1) (Gottwein et al., 2009). HCV RNA and Core titers for sf-HCVcc and HCVcc reference stocks were similar (Table 1). Thus, genotype 1–6 sf-HCVcc showed increased specific infectivities compared to HCVcc reference stocks (Table 1). This increase in specific infectivity was most pronounced for recombinants with comparatively low infectivity titers. Thus, based on RNA titers, specific infectivity was 20-fold increased for sf-J4(1b) and 40-fold increased for sf-ED43(4a) compared to their HCVcc counterparts (Table 1). This resulted in differences in specific infectivity between sf-HCVcc of different genotypes being smaller than those between HCVcc of different genotypes. For sf-HCVcc, based on RNA titers, specific infectivities were between 1/40 FFU/IU and 1/631 FFU/IU, while HCVcc showed specific infectivities between 1/398 FFU/IU and 1/12,589 FFU/IU (Table 1).

Higher infectivity titers of sf-HCVcc might be due to increased viral release and specific infectivity

We next aimed at determining if the observed differences in infectivity titers were due to the fact that DMEM + 10% FBS cultures were split at regular intervals, while AEM cultures were kept over-confluent. Huh7.5 cell cultures were infected with SA13(5a) and (i) maintained in DMEM + 10% FBS and split every 2–3 days, (ii) maintained in DMEM + 10% FBS without splitting, or (iii) maintained in AEM without splitting (Fig. 3A). The DMEM + 10% FBS culture yielded a peak infectivity titer of 5.6 log_{10} FFU/ml, while the AEM culture yielded a peak infectivity titer of 6.1 log_{10} FFU/ml (Fig. 3A). However, the DMEM + 10% FBS culture maintained without splitting reached only 4.6 log_{10} FFU/mL (Fig. 3A). This suggested that the high infectivity titers observed for the AEM cultures were not due to reduced stress related to avoiding cell culture splitting.

We subsequently investigated cell viability and proliferation of cells cultured in AEM versus DMEM + 10% FBS. After 48 h of culture in AEM, when increased infectivity titers were observed, cell viability and proliferation of AEM cultures was similar to that of DMEM + 10% FBS cultures (Fig. 3B). Thus, changes in cell viability or proliferation did not explain the increased infectivity titers observed.

We further investigated, whether sf-HCVcc were more stable than HCVcc, which might contribute to the observed increase in infectivity titers. Up to 5 freeze/thaw cycles did not result in major decrease in infectivity or differences in infectivity for SA13(5a), sf-SA13(5a) or sf-SA13(5a) supplemented with 10% FBS (Fig. 3C). Incubation for 48 h at 4°C resulted in a minor decrease in infectivity of sf-SA13(5a) compared to SA13(5a) and sf-SA13(5a) supplemented with 10% FBS (Fig. 3D). Incubation for 4–48 h at room temperature or 37°C resulted in a gradual decrease in infectivity; this decrease was more pronounced for sf-SA13(5a)
than for SA13(5a), and partially rescued by addition of 10% FBS to sf-SA13(5a) (Fig. 3D). Thus, FBS might result in stabilization of HCVcc. However, apparently, increased infectivity titers were not caused by increased stability of sf-HCVcc.

In order to investigate whether certain steps of the viral life cycle were affected by serum-free culture conditions, we carried out single-cycle virus production assays using CD81-deficient S29 cells, which are derived from Huh7.5 cells (Russell et al., 2008). Following transfection with SA13(5a) HCV RNA, AEM cultures showed a ~1 log decrease in intracellular HCV Core and infectivity titers compared to DMEM + 10% FBS cultures, indicating a decrease in viral replication/translation (Fig. 4A and B). In contrast, AEM and DMEM + 10% FBS cultures showed similar extracellular Core titers, but AEM cultures had a ~1 log increase in extracellular infectivity titers compared to DMEM + 10% FBS cultures. These findings suggest that AEM culture resulted in increased viral release and that sf-HCVcc had increased specific infectivity compared to HCVcc. Furthermore, for HCVcc, the peak extracellular infectivity titer was observed 48 h post transfection followed by a decrease at 72 h post transfection, while for sf-HCVcc high titers were observed at both time points (Fig. 4B). This is in agreement with the prolonged peak of infection observed in Huh7.5 cells (Figs. 1–3).

Infectious sf-HCVcc particles displayed a homogeneous density profile

To investigate their biophysical properties we subjected sf-HCVcc (Fig. 2) to equilibrium buoyant density ultracentrifugation on iodixanol gradients. As described previously for genotypes 2a, 3a, 5a and 6a HCVcc (Lindenbach et al., 2005; Lindenbach et al.,...
Serum-free cultures were infected and maintained as described in “Materials and methods” section (Fig. 2). For sf-HCVcc, supernatant HCV infectivity titers, Core antigen, and RNA titers were determined, and specific infectivities were calculated. Representative peak infectivity titers as well as Core and RNA titers from the same sample are shown. Core-E2 sequences were determined by direct sequence analysis as described in “Materials and methods” section. For sf-J6(2a), sf-S52(3a), sf-ED43(4a), sf-SA13(5a) and sf-HK6a(6a), Core-E2 sequences were identical to the plasmid sequence. The sf-H77(1a) had acquired the previously described amino acid change Y361H, estimated to be present in 50% of viral genomes; this change was also present in the H77(1a) HCVcc stock shown in this table (Gottwein et al., 2009). The sf-J4(1b) had acquired amino acid changes T578A and D584G, estimated to be present in the majority of viral genomes. For HCVcc, characteristics of references stocks are reproduced from Gottwein et al. (2009).

<table>
<thead>
<tr>
<th>Isolate (genotype)</th>
<th>Peak HCV Infectivity titer(b)</th>
<th>Peak HCV RNA titer(c)</th>
<th>Peak HCV Core titer(d)</th>
<th>Specific infectivity(c)</th>
<th>Specific infectivity(c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\log_{10}) FFU/mL</td>
<td>(\log_{10}) IU/mL</td>
<td>(\log_{10}) amol Core/mL</td>
<td>FFU/IU</td>
<td>FFU/amol Core</td>
</tr>
<tr>
<td>sf-HCVcc</td>
<td>HCVcc</td>
<td>sf-HCVcc</td>
<td>HCVcc</td>
<td>sf-HCVcc</td>
<td>HCVcc</td>
</tr>
<tr>
<td>H77(1a)</td>
<td>5.0</td>
<td>4.3</td>
<td>7.6</td>
<td>7.5</td>
<td>1/198</td>
</tr>
<tr>
<td>J4(1b)</td>
<td>4.7</td>
<td>3.2</td>
<td>7.6</td>
<td>7.3</td>
<td>1/631</td>
</tr>
<tr>
<td>J6(2a)</td>
<td>5.6</td>
<td>5.0</td>
<td>7.6</td>
<td>7.6</td>
<td>1/100</td>
</tr>
<tr>
<td>S52(3a)</td>
<td>4.9</td>
<td>4.3</td>
<td>7.4</td>
<td>7.2</td>
<td>1/316</td>
</tr>
<tr>
<td>ED43(4a)</td>
<td>4.7</td>
<td>3.6</td>
<td>7.1</td>
<td>7.6</td>
<td>1/251</td>
</tr>
<tr>
<td>SA13(5a)</td>
<td>6.2</td>
<td>4.1</td>
<td>7.8</td>
<td>7.0</td>
<td>1/40</td>
</tr>
<tr>
<td>HK6a(6a)</td>
<td>5.6</td>
<td>4.0</td>
<td>7.7</td>
<td>7.0</td>
<td>1/126</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(a\) Isolate and genotype of Core-NS2 of the used JFH1-based recombinants is indicated. Recombinants are further described in “Materials and methods” section.

\(b\) For sf-HCVcc, supernatant infectivity titers were determined as FFU/mL by a cell culture-based titration assay as described in “Materials and methods” section. Values are means of three replicates. For HCVcc, values are reproduced from Gottwein et al. (2009).

\(c\) For sf-HCVcc, supernatant RNA titers were determined in the samples, for which infectivity titers are given, as IU/mL by TaqMan PCR as described in “Materials and methods” section. Values are means of two replicates. For HCVcc, values are reproduced from Gottwein et al. (2009).

\(d\) Core titers were determined in the samples, for which infectivity titers are given, as amol/mL using the ARCHITECT HCV Ag assay (Abbott).

\(e\) For sf-HCVcc, specific infectivity was calculated as FFU/IU by dividing supernatant infectivity titers with the corresponding RNA titers. For HCVcc, values were adapted from Gottwein et al. (2009) to FFU/IU by dividing supernatant infectivity titers with the corresponding RNA titers.

\(f\) Specific infectivity was calculated as FFU/amol Core by dividing supernatant infectivity titers with the corresponding Core titers.

\(g\) The peak infectivity titer of this SA13(5a) reference stock was lower than what we typically observe. Typically, peak titers for SA13(5a) are \(\sim 5 \log_{10}\) FFU/mL (Fig. 3A).

Infectious sf-HCVcc particles were apparently associated with ApoE

The altered density profile of sf-HCVcc might indicate an altered association with lipoproteins. To investigate if sf-HCVcc was associated with ApoE, a key component of HCV associated lipoproteins (Popescu and Dubuisson, 2010; Felmee et al., 2013), we carried out neutralization assays using a monoclonal antibody directed against ApoE (Weisgraber et al., 1983; Chang et al., 2007). Due to limited availability of this antibody, we studied only the genotype 5a recombinant. SA13(5a) and sf-SA13(5a) showed a similar concentration-dependent response with median inhibitory concentrations (IC\(50\)) of 1.3 \(\mu\)g/mL for SA13(5a) and 1.1 \(\mu\)g/mL for sf-SA13(5a), and almost complete neutralization achieved at the highest \(a\)-ApoE concentrations (Fig. 6A), suggesting that genotype 5a HCVcc and sf-HCVcc showed similar association with ApoE. These data were confirmed by neutralizing SA13(5a) and sf-SA13 (5a) using anti-Apolipoprotein E antibody (ab24139, abcam). Using this polyclonal rabbit IgG, complete neutralization, as well as 50% neutralization, was observed at similar dilutions for SA13(5a) and sf-SA13(5a) (data not shown).

We further carried out immunoprecipitation of SA13(5a) and sf-SA13(5a) with the ApoE specific monoclonal antibody also used for neutralization experiments in Fig. 6A (Weisgraber et al., 1983; Chang et al., 2007). We observed no major differences in the amount of viral RNA precipitated by this antibody (Fig. 6B). Collectively, these data indicate that HCVcc and sf-HCVcc showed similar association with ApoE.

Establishment of the use of sf-HCVcc in biological assays

To further characterize sf-HCVcc, we aimed at studying routes of sf-HCVcc entry and sf-HCVcc sensitivity to neutralizing antibodies. To avoid potential in vitro association of sf-HCVcc with FBS components such as lipoproteins (Wünschmann et al., 2006), we aimed at replacing DMEM + 10% FBS, typically used in such assays, by AEM during the viral infection step. However, using AEM, in initial experiments with genotypes 5a and 2a viruses, we found greatly reduced infectivity for sf-SA13(5a) and sf-J6(2a). Interestingly, SA13(5a) and J6(2a) infectivity was also reduced when these viruses were diluted in AEM prior to infection (Fig. 7A and data not shown). This loss of infectivity was not due to down-regulation of important HCV co-receptors on AEM cultured Huh7.5 cells, since expression of CD81, LDLr, SR-BI and claudin-1 was similar in Huh7.5 cells cultured for three hours in either DMEM + 10% FBS or AEM, as determined by flow cytometry (Fig. 8).

To determine how infectivity of sf-HCVcc in AEM could be rescued, we diluted sf-SA13(5a) in DMEM + 10% FBS (reference culture), DMEM, AEM, or AEM supplemented with 10% FBS and/or GlutaMax (a glutamine supplement present in DMEM + 10% FBS culture medium but not in AEM). For sf-SA13(5a) diluted in AEM, infectivity was only 12% of infectivity of the reference culture (Fig. 7B). While supplementing AEM with GlutaMax did not influence infectivity, supplementing AEM with 10% FBS and/or GlutaMax increased infectivity of sf-SA13(5a) to 67% and 68% respectively, compared to the reference culture (Fig. 7B). When sf-SA13(5a) was diluted in DMEM without FBS (GlutaMax only), infectivity was only 30% compared to the reference culture (Fig. 7B). Apparently other FBS components than lipoproteins and lipoprotein-associated factors mediated this enhancement.
of infectivity, because supplementing AEM with either VLDL, low density lipoprotein, high density lipoprotein, ApoB, ApoCl, ApoE or water-soluble cholesterol did not rescue infectivity, while lipoprotein deficient FBS partly restored infectivity (data not shown). Furthermore, we observed that sf-SA13(5a) infectivity correlated with the % of FBS in AEM (Fig. 7C). These findings suggested that yet undefined factors in FBS culture medium supplement were crucial for infectivity of HCVcc and sf-HCVcc. Therefore, we investigated if sf-HCVcc was able to associate in vitro with FBS components, leading to alteration of the observed sf-HCVcc density profile. We incubated sf-SA13(5a) with different media and serum concentrations in the absence of cells. Such incubations did not affect the density profile of sf-SA13(5a) (Fig. 9), suggesting that association between sf-HCVcc and serum components did not occur to an extent that influenced the previously observed density profile (Fig. 5). Thus, it was feasible to carry out further biological studies of sf-HCVcc in AEM supplemented with 10% FBS.

**Entry of sf-HCVcc depended on HCV co-receptors CD81, LDLr and SR-BI as well as on clathrin-mediated endocytosis**

To investigate if sf-HCVcc differed from HCVcc regarding entry into the host cell, we first studied HCV co-receptors LDLr and SR-BI, which might interact with lipoprotein components on the IVP (Owen et al., 2009; Thi et al., 2012), as well as CD81, supposed to directly interact with E2 (Pileri et al., 1998; McKeating et al., 2004).
When blocking CD81, for genotype 1–6 HCVcc we observed dilution-dependent blocking for genotype 1–6 HCVcc, maximum blocking values ($B_{\text{max}}$) were between 69% for ED43(4a) and 100% for SA13(5a) at the highest concentrations of α-LDLr, suggesting genotype/isolate-specific differences in dependency on LDLr (Fig. 10, middle column). Genotype 1–6 sf-HCVcc could also be blocked in a concentration-dependent manner, with $B_{\text{max}}$ values between 33% for sf-HK6a(6a) and 74% for sf-H77(1a) at the highest concentrations of α-LDLr. While sf-H77(1a), sf-S52(3a) and sf-ED43(4a) showed similar $B_{\text{max}}$ values as their HCVcc counterparts, sf-J4(1b), sf-J6(2a), sf-SA13(5a) and sf-HK6a(6a) showed 31–55% lower $B_{\text{max}}$ values than their HCVcc counterparts, suggesting that sf-HCVcc of certain genotypes had lower dependency on LDLr than their HCVcc counterparts.

Blocking of SR-BI had only limited effect on entry of J4(1b) and HK6a(6a) HCVcc with $B_{\text{max}}$ values of $\sim$100% were observed at the highest α-LDLr concentrations; for these viruses, similar blocking rates were previously observed (Gottwein et al., 2009). Genotype 1–6 sf-HCVcc showed similar concentration-dependent sensitivity towards CD81 blocking as their HCVcc counterparts (Fig. 10, left column).

When blocking LDLr, we found concentration-dependent blocking for genotype 1–6 HCVcc. $B_{\text{max}}$ values were between 69% for ED43(4a) and 100% for SA13(5a) at the highest concentrations of α-LDLr, suggesting genotype/isolate-specific differences in dependency on LDLr (Fig. 10, middle column). Genotype 1–6 sf-HCVcc could also be blocked in a concentration-dependent manner, with $B_{\text{max}}$ values between 33% for sf-HK6a(6a) and 74% for sf-H77(1a) at the highest concentrations of α-LDLr. While sf-H77(1a), sf-S52(3a) and sf-ED43(4a) showed similar $B_{\text{max}}$ values as their HCVcc counterparts, sf-J4(1b), sf-J6(2a), sf-SA13(5a) and sf-HK6a(6a) showed 31–55% lower $B_{\text{max}}$ values than their HCVcc counterparts, suggesting that sf-HCVcc of certain genotypes had lower dependency on LDLr than their HCVcc counterparts.

Blocking of SR-BI had only limited effect on entry of J4(1b) and HK6a(6a) HCVcc with $B_{\text{max}}$ values $<50\%$ (Fig. 10, right column). For HCVcc of other genotypes we observed concentration-dependent blocking with $B_{\text{max}}$ values between 51% for J6(2a) and 80% for H77(1a) at the highest concentrations of α-SR-BI. Thus, sensitivity to SR-BI blocking apparently depended on the genotype/isolate. Blocking of SR-BI also had limited effect on entry of sf-J4(1b) and sf-HK6a(6a). Entry of sf-HCVcc of other genotypes was blocked in a concentration-dependent manner, with $B_{\text{max}}$ values between 60% for sf-SA13(5a) and 86% for sf-H77(1a). $B_{\text{max}}$ values were similar between HCVcc and sf-HCVcc of the same genotype. Thus, overall, HCVcc and sf-HCVcc of the same genotype showed similar sensitivity to SR-BI blocking.

Finally, we studied dependency of genotype 1–6 sf-HCVcc on clathrin-mediated endocytosis. When pre-treating cells with chlorpromazine, we observed concentration-dependent blocking rates of up to 93%, suggesting that both sf-HCVcc and HCVcc depended on clathrin-mediated endocytosis (Fig. 11A–C). We were not able to achieve 100% blocking for any of the recombinants at 10 μg/ml chlorpromazine, the highest concentration not resulting in cytotoxic effects (Fig. 11H). Interestingly, most sf-HCVcc were slightly more sensitive to chlorpromazine treatment than their HCVcc counterparts. This difference was greatest for J4(1b), ED43(4a) and HK6a(6a). For J6(2a) and SA13(5a) no obvious difference was observed, while H77(1a) and S52(3a) showed relatively small differences. This suggested that dependency on clathrin-mediated endocytosis might be slightly greater for sf-HCVcc of most genotypes/isolates than for HCVcc.

In conclusion, these data suggest that, overall, entry of HCVcc and sf-HCVcc relied on CD81, LDLr and SR-BI HCV co-receptors as well as on clathrin-mediated endocytosis, with exception of genotype 1b and 6a particles, which could not be blocked by α-SR-BI. However, we detected minor differences for recombinants of different HCV genotypes and for HCVcc versus sf-HCVcc regarding dependency on certain receptors and clathrin-mediated endocytosis.

**Fig. 4. Serum-free culture decreased viral replication/translation but enhanced viral release and specific infectivity. S29 cells were transfected with SA13(5a) as well as positive control (J6(2a)) and negative control (J6(2a)-GND) HCV RNA transcripts as described in “Materials and methods” section. (A) Intracellular (black bars) and extracellular (gray bars) Core levels were determined 24, 48 and 72 h post transfection. Core levels were normalized to intracellular Core levels measured 4 h post transfection. (B) Intracellular (black bars) and extracellular (gray bars) infectivity titters were determined 24, 48 and 72 h post transfection. Intracellular infectivity titters are shown as the means (FFU/mL) of three replicates with SEM. Extracellular infectivity titters are shown as the means (FFU/mL) of three replicates with SEM. The lower limits of detection are indicated by y-axis breaks.

Chronic-phase patient sera and monoclonal antibodies against conformational epitopes in E1E2 and E2 neutralized sf-HCVcc

To investigate if there were differences between HCVcc and sf-HCVcc in sensitivity to neutralizing antibodies, we first did neutralization of genotype 1–6 viruses using serum from genotype 1a infected Patient H, taken 29 years after acute infection (H06 (Scheel et al., 2008)). For HCVcc, as previously described, S52(3a) was the least sensitive to neutralization with H06 (Fig. 12D) (Scheel et al., 2008; Gottwein et al., 2009; Prentoe et al., 2011). For HCVcc of other genotypes we observed dilution-dependent neutralization with IC$_{50}$ values ranging from 1/1,436 to 1/233,209 and relatively high neutralization rates by high concentrations of H06 serum (Fig. 12). For genotype 1–6 sf-HCVcc, we found similar neutralization patterns as for their HCVcc counterparts. For all sf-HCVcc except S52(3a) we observed dilution dependent neutralization with IC$_{50}$ values ranging from 1/605 to 1/156,666 and relatively high neutralization rates by high concentrations of H06 serum (Fig. 12). Thus, sf-HCVcc particles showed similar susceptibility to neutralizing antibodies in chronic phase patient serum as HCVcc.

To confirm these observations, we next neutralized SA13(5a) and sf-SA13(5a) with a genotype 5a chronic-phase patient serum (SA3 (Jensen et al., 2008)). These viruses showed similar neutralization profiles, with IC$_{50}$ values of 1/928 for SA13(5a) and 1/654 for sf-SA13(5a) as well as high neutralization rates by high concentrations of SA3 serum (Fig. 13A).

Finally, we tested a panel of monoclonal antibodies (AR1B and AR2A-5A) targeting defined conformational epitopes in E1E2 and E2 neutralized sf-HCVcc. The lower limits of detection are indicated by y-axis breaks.
sensitivity to neutralizing antibodies as HCVcc. In addition, these findings suggest that sf-HCVcc and HCVcc do not show major differences regarding conformation of E1 and E2.

Discussion

In this study, we describe the generation and characterization of genotype 1–6 serum-free HCVcc particles, using AEM to culture infected Huh7.5 hepatoma cells. Compared to HCVcc, sf-HCVcc showed similar biological properties but increased infectivity titers and a homogenous single-peak density profile. These unique characteristics, as well as the reduced concentration of non-HCV proteins in serum-free culture supernatants, are expected to facilitate generation of purified and concentrated virus stocks, required for vaccine development and biophysical studies of HCV particle composition (Gastaminza et al., 2010; Merz et al., 2010; Catanese et al., 2013; Akazawa et al., 2013). Further, the developed serum-free culture conditions might reduce the risk of contamination with adventitious microbial
Fig. 6. HCVcc and sf-HCVcc showed similar association with ApoE. (A) Monoclonal α-ApoE antibody (1D7) and control mouse IgG1 (1D1) were diluted in DMEM + 10% FBS to the indicated concentrations. SA13(5a) (black circles) and sf-SA13(5a) (gray squares) were diluted in DMEM + 10% FBS and incubated with dilutions of α-ApoE or mouse IgG1 for 30 min at 37 °C. The virus–antibody mixes were added to Huh7.5 cells, plated the previous day in poly-α-lysine coated 96 well plates. After 3 h of incubation, virus–antibody mixes were removed and DMEM + 10% FBS was added. Cells were fixed 48 h post infection and stained, and the number of single HCV NS5A positive cells per well was determined by automated counting as described in “Materials and methods” section. The % neutralization was calculated by relating counts of experimental wells to the mean count of six replicate wells with untreated control virus. Data points are means of three replicates with SEM (error bars). Following logarithmic transformation of X-values, variable-slope sigmoidal dose–response curves were fitted \[
Y = \text{Bottom} + \left(\frac{\text{Top} - \text{Bottom}}{1 + 10^{\log_{10}(EC_{50}/C_0)} \text{HillSlope}}\right)
\]. \text{Bottom} was constrained to 0. \text{Top} was constrained to 100. (B) Immunoprecipitation was carried out on 10^6 IU HCV RNA of SA13(5a) (black bars) and sf-SA13(5a) (gray bars), using monoclonal α-ApoE (1D7) and control mouse IgG1 (1D1) as described in “Materials and methods” section. Amounts of HCV RNA (IU) were determined in the immunoprecipitated fractions using TaqMan PCR as described in “Materials and methods” section. RNA titers are shown as the mean of two replicates with SEM.

Fig. 7. FBS enhances infectivity of both HCVcc and sf-HCVcc. (A–C) Huh7.5 cells seeded in poly-α-lysine coated 96-well plates the previous day, were incubated with (A) SA13 (5a) and sf-SA13(5a) diluted in DMEM + 10% FBS (black bars) or AEM (gray bars), (B) sf-SA13(5a) diluted in different media with supplements as indicated or (C) sf-SA13(5a) diluted in AEM supplemented with different concentrations of FBS. % FBS in growth medium indicates the final FBS concentration. (A–C) Cells were incubated with virus mixes for 3 h. After incubation, fresh DMEM + 10% FBS was added to all wells. Cells were incubated for 48 h before they were fixed, stained and the number of single HCV NS5A positive cells per well was determined by automated counting as described in “Materials and methods” section. Error bars represent SEM of triplicates. For (B), the mean infectivity (HCV NS5A positive cells/well) of triplicate wells of the reference culture (DMEM + 10% FBS, black bar) was set to 100%. The number of HCV NS5A positive cells/experimental well was related to this mean to calculate % infectivity relative to the reference culture.

Efficient production of HCVcc has primarily been achieved in the continuous hepatoma cell line Huh7 and derived cell lines, such as Huh7.5 cells (Steinmann and Pietschmann, 2013; Lohmann and Bartenschlager, 2014). Due to their increased permissiveness to infection with recombinant HCV, Huh7.5 cells were previously used for cell culture adaptation and growth of HCV genotype 1–6 recombinants used in this study (Lindenbach et al., 2005; Gottwein et al., 2007; Scheel et al., 2008; Jensen et al., 2008; Gottwein et al., 2009). According to WHO recommendations, a wide range of continuous cell lines are now considered as suitable substrates for production of various medicinal substances if certain requirements are met (WHO, 1998). These requirements include use of well-characterized cell banks, use of suitable manufacturing procedures aiming at a high degree of purification of the end product, and thorough characterization of the end product (WHO, 1998). Thus, Huh7.5 cells could potentially be characterized to comply with these recommendations, allowing their use for vaccine development. Huh7 derived cell lines have typically been subjected to long-term passage using serum-containing growth medium and animal-derived trypsin. According to WHO recommendations, animal derived products should be reduced or eliminated from cell cultures used for production of medicinal substances due to risk of contamination with adventitious microbial agents (WHO, 1998). In this study, we describe a method for production of sf-HCVcc, thus avoiding the use of trypsin and bovine serum during the virus production phase. To further reduce presence of animal-derived components in sf-HCVcc producing cell culture, it might be possible to culture Huh7.5 cells in serum-free medium (Nakabayashi et al., 1982), prior to sf-HCVcc production. Alternatively, based on recently generated knowledge on host-factors required for HCV infection, it might be possible to engineer cell lines already approved for vaccine development with susceptibility to HCV infection (Narbus et al., 2011; Costa et al., 2012; Kambara et al., 2012; Vogt et al., 2013; Sourisseau et al., 2013; Frentzen et al., 2014). However, this might be a cumbersome process and might require re-approval of the modified cell-line. Of note, most genotype 1–6 recombinants used in this study (Lindenbach et al., 2005; Gottwein et al., 2007; Scheel et al., 2008; Jensen et al., 2008; Gottwein et al., 2009) contained adaptive mutations conferring efficient growth in Huh7.5 cells; for HK6a(6a) cell culture adaptive mutations localized to the envelope proteins. In addition, in this study H77(1a) and J4(1b) polyclonal virus stocks

![Figure 8](image_url)

**Fig. 8.** Huh7.5 cells cultured in DMEM + 10% FBS or AEM showed similar surface expression of HCV co-receptors. Huh7.5 cells were incubated for 3 h in DMEM + 10% FBS or AEM and subsequently prepared for flow cytometry analysis as described in "Materials and methods" section. Cell surface expression of HCV co-receptors was determined using antibodies against (A) CD81, (B) LDLr, (C) SR-BI and (D) claudin-1 as described in "Materials and methods" section. Phycoerythrin (PE) signals were recorded on a BD FACSCalibur flow cytometer. Histograms show the co-receptor surface expression in cells cultured in DMEM + 10% FBS (dark blue) or AEM (light blue) compared to unstained cells (black and gray, respectively).
had acquired putative cell culture adaptive mutations in the envelope proteins. In future studies, it will be of relevance to develop a panel of genotype 1–6 recombinants without envelope mutations, thus not differing from naturally occurring isolates. Recently, proof-of-concept for immunogenicity of genotype 2a HCVcc was obtained, since immunization of mice resulted in induction of HCV neutralizing antibodies (Akazawa et al., 2013; Houghton et al., 2013; Gottwein and Bukh, 2013). This underlines the potential of inactivated HCVcc particles as future vaccine antigens. However, HCVcc used for immunizations were grown in cell culture medium supplemented with 2% FBS (Akazawa et al., 2013), even though the authors had previously reported development of serum-free cultures for genotype 2a recombinants JFH1 and J6/JFH1, using growth medium DMEM/F-12 supplemented with Insulin–Transferrin–Selenium-X (Akazawa et al., 2011). In contrast to our study, infectivity titers and specific infectivity of 2a virus from such serum-free cultures were apparently equal to or lower than titers of viruses from serum-supplemented cultures (Akazawa et al., 2011). In addition, serum-free 2a HCVcc showed a similar density profile as 2a HCVcc derived from serum-containing cell culture, following sucrose gradient ultracentrifugation (Akazawa et al., 2011). These differences between previously produced serum-free 2a HCVcc (Akazawa et al., 2011) and sf-HCVcc described in this study are most likely due to the different culture media used and/or other differences in experimental conditions.

We describe establishment of serum-free cell cultures producing HCV particles of prototype strains of genotypes 1–6 with favorable biophysical and biological characteristics (Figs. 2 and 5 and Table 1) (Gottwein et al., 2009; Bukh et al., 2010). Supernatant infectivity titers of sf-HCVcc were 0.6–2.1 log_{10} FFU/ml higher than titers of HCVcc (Table 1 and Fig. 2) (Gottwein et al., 2009). Of the panel of previously developed HCVcc recombinants (Gottwein et al., 2009), SA13(5a) showed the highest infectivity titers (~5 log_{10} FFU/ml). Infectivity titers of >6 log_{10} FFU/ml, as observed for sf-SA13(5a) (Table 1 and Fig. 2F and 3A), are among the highest infectivity titers reported to date for cell culture grown HCV (Russell et al., 2008; Dhillon and Witteveeld, 2010; Pokrovskii et al., 2011; Liu et al., 2012; Jiang and Luo, 2012; Horwitz et al., 2013). Furthermore, recombinants with relatively low infectivity titers, such as J4(1b) and ED43(4a) (Scheel et al., 2008; Gottwein et al., 2009), yielded significantly increased infectivity titers, when grown under serum-free conditions (Table 1 and Fig. 2B and E). Genotype 1b is considered to be the most prevalent genotype worldwide and in certain countries, such as Egypt, genotype 4a has a prevalence of up to 15%; thus genotype 4a sf-HCVcc might prove to be an important antigen for vaccine trials (Abdo and Lee, 2004; Simmonds et al., 2005). The reason why serum-free culture conditions resulted in increased infectivity titers remains to be fully elucidated. Our studies indicated that increased infectivity in AEM cultures was not due to (i) avoiding stress related to cell splitting (Fig. 3A), (ii) changes in Huh7.5 cell viability or proliferation (Fig. 3B) or (iii) increased stability of sf-HCVcc (Fig. 3C and D). While in S29 cells, serum-free culture conditions resulted in reduction of viral replication/translation, they increased viral release and specific infectivity, possibly contributing to the higher infectivity titers observed (Fig. 4).

Specific infectivities were generally higher for sf-HCVcc than for HCVcc as observed in both Huh7.5 cell cultures (Table 1) and S29 cell cultures (Fig. 4). This is in line with previous reports that HCVcc fractions with the highest specific infectivity had a buoyant density between 1.09 and 1.10 g/ml (Lindenbach et al., 2005), similar to the density of the majority of infectious sf-HCVcc particles (Fig. 5). Higher specific infectivity might be due to absence of serum, which might have non-specific neutralizing or inhibitory activity; alternatively, less immature viral particles might be produced using the developed culture conditions.

Furthermore, in our serum-free cultures, supernatants with high infectivity titers could typically be harvested for a prolonged period of time compared to DMEM + 10% FBS cultures (Figs. 1–4). For example, for SA13(5a), high-titer supernatants could typically be harvested at 2–3 subsequent time points in DMEM + 10% FBS cultures, whereas in serum-free cultures, high-titer supernatants could be harvested at 4–6 subsequent time points (Figs. 2F and 3A). This further increased the yield of infectious virus that could be harvested from serum-free cultures.

In contrast to HCVcc, sf-HCVcc displayed a homogeneous density distribution with a single peak of infectious virus at densities of ~1.10 g/ml, following iodixanol gradient ultracentrifugation (Fig. 5). We believe that the density profile of sf-HCVcc might allow more effective density-based purification and concentration using ultracentrifugation and gel chromatography, since a single fraction, containing the majority of infectious virus, could be collected. Density changes were previously observed for HCVcc without hypervariable region 1 (HVR1) (Bankwitz et al., 2010; Prentoe et al., 2011), HCVcc with a specific E2 mutation (Zhong et al., 2006; Grove et al., 2008; Gastaminza et al., 2010) and for HCV recovered from HCVcc-infected chimpanzees and uPA-SCID mice engrafted with human liver cells (Lindenbach et al., 2006). These density changes were suggested to be due to differences in lipoprotein association (Lindenbach et al., 2006; Zhong et al., 2006; Grove et al., 2008; Bankwitz et al., 2010; Prentoe et al., 2011). We showed that sf-SA13(5a) could be neutralized as efficiently as its HCVcc counterpart by a monoclonal α-ApoE antibody and polyclonal α-ApoE IgG (Fig. 6A and data not shown). Further, immunoprecipitation of sf-SA13(5a) and SA13(5a) showed similar efficacy (Fig. 6B). These data indicate that HCVcc and sf-HCVcc do not show major differences in association to ApoE, and thus possibly to lipoproteins (Yamamoto et al., 2011). Therefore, further studies will be required to elucidate the cause for the observed density shift (Fig. 5). Preliminary studies indicated a decrease in intracellular lipid content in serum-free
cultures (data not shown). However, determination of expression levels of genes involved in lipid production or of lipid/lipoprotein composition of HCVcc versus sf-HCVcc (Merz et al., 2010) was considered outside the scope of this study.

Compared to HCVcc, HCVcc without HVR1, displaying a similar density distribution as sf-HCVcc, were less susceptible to blocking of SR-BI and more susceptible to neutralizing antibodies (Bankwitz et al., 2010; Prentoe et al., 2011; Prentoe et al., 2014). Furthermore,
previously described serum-free HCVcc were more susceptible to blocking of CD81 and SR-BI and to neutralization by a monoclonal antibody targeting E2 (AP33) (Akazawa et al., 2011). Of note, in this study biological assays were carried out in AEM supplemented with FBS, which was required for viral infection, but apparently did not alter composition of sf-HCVcc LVP (Figs. 7 and 9). When blocking CD81 and SR-BI, we did not observe major differences between sf-HCVcc and HCVcc (Fig. 10). Even though sf-HCVcc of certain genotypes showed slightly lower dependency on LDLr and slightly higher dependency on clathrin mediated endocytosis than their HCVcc counterparts (Figs. 10 and 11), overall our findings suggested that sf-HCVcc relied on similar routes of entry as HCVcc. In the future, it will be of interest to further investigate the small differences observed for LDLr usage and dependency on clathrin mediated endocytosis using different blocking antibodies and alternative methods of inhibition such as RNA interference.

We further confirmed previous results showing that HCVcc of genotype 1–6 showed similar dependency on CD81 (Gottwein et al., 2009). Previously, we reported that blocking SR-BI had a similar effect on genotype 1–6 HCVcc entry (Gottwein et al., 2009). However, in this study and another recent study by our group (Prentoe et al., 2014), using a different blocking antibody, we...
found differential sensitivity of genotype 1–6 HCVcc to SR-BI blocking. Single E2 mutations in culture adapted JFH1(2a) were reported to cause reduced dependency on SR-BI (Grove et al., 2008; Dhillon and Witteveldt, 2010). Further studies are required to elucidate if the E2 mutations present in HK6a(6a) and J4(1b) HCVcc virus stocks mediated reduced dependency on SR-BI. For genotype 1–6 HCVcc, we also observed small but consistent differences regarding dependency on LDLr (Fig. 10). Whereas dependency on LDLr for entry has been shown for JFH1(2a) (Owen et al., 2009; Albecka et al., 2012) and was recently shown for H77(1a), J6(2a) and S52(3a) HCVcc (Prentoe et al., 2014), this study is the first to show dependency on LDLr for J4(1b), ED43(4a), SA13(5a) and HK6a(6a) HCVcc. In future studies, also involving recombinants of additional isolates of each genotype, it will be of interest to investigate if different genotypes, subtypes or isolates differ regarding receptor usage.

Compared to HCVcc, sf-HCVcc showed similar sensitivity to neutralization by chronic phase patient sera and human monoclonal antibodies targeting conformational epitopes in E1E2 and E2 (Figs. 12 and 13). These results suggest that sf-HCVcc resemble

Fig. 12. HCVcc and sf-HCVcc show similar sensitivity to neutralization with genotype 1a chronic-phase patient serum. Genotype 1a serum H06 was diluted in DMEM + 10% FBS as indicated. HCVcc (black circles) were diluted in DMEM + 10% FBS and sf-HCVcc (gray squares) were diluted in AEM + 10% FBS, mixed with H06 serum dilutions and incubated 1 h at 37°C. Virus-serum mixes were added to HuH7.5 cells, plated the previous day onto poly-D-lysine coated 96 well plates. After 6 h incubation, virus-serum mixes were removed and DMEM + 10% FBS was added. Cells were fixed 48 h post infection and stained, and the number of single HCV NS5A positive cells per well was determined by automated counting as described in “Materials and methods” section. The HCV Core-E2 sequences of all virus stocks used were determined by direct sequencing. Sequences were identical for HCVcc and sf-HCVcc of the same recombinant. Compared to the plasmid sequence, H77(1a) viruses had acquired amino acid change E348S and J4(1b) had acquired amino acid change V710L, both estimated to be present in the majority of viral genomes. The % neutralization was calculated by relating counts of experimental wells to the mean count of six replicate wells with untreated control virus. Data points are means of three replicates with SEM (error bars). Following logarithmic transformation of X-values, variable-slope sigmoidal dose–response curves were fitted \[ Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log_{10}\text{EC}_{50}/\text{C}_{0})\text{HillSlope}}} \] “Bottom” was constrained to “0” for all curves. “Top” was constrained to “100” for all curves in all panels except D; for these curves, median inhibitory concentrations (IC_{50}) were calculated (black for HCVcc and gray for sf-HCVcc). ND, not determinable.
HCVcc regarding epitope exposure and conformation, of importance for vaccine development using sf-HCVcc as antigen.

In conclusion, we have established a method allowing for robust production of genotype 1–6 sf-HCVcc with favorable biological and biophysical characteristics. Serum-free culture apparently reduced viral replication/translation but enhanced viral release and specificity. Sf-HCVcc had increased infectivity titers compared to HCVcc, and compared to serum-free HCVcc reported previously, thus contributing to an increased yield of infectious virus from infected cell cultures. Furthermore, sf-HCVcc displayed a homogeneous density distribution. Together with a reduced concentration of non-HCV proteins in supernatants from serum-free cultures, these features are expected to facilitate viral purification and concentration required for vaccine production and morphological analysis of HCV particles. Biologically, sf-HCVcc particles resembled their HCVcc counterparts regarding association to ApoE, routes of viral entry and sensitivity to neutralizing antibodies. Thus, sf-HCVcc particles could prove important as antigens in a prophylactic HCV vaccine against all six epidemiologically important HCV genotypes. To this aim future studies are required, focusing on establishment of large-scale sf-HCVcc production as well as efficient purification, concentration and inactivation. Finally, it will be of great interest to test immunogenicity of genotype 1–6 sf-HCVcc in small animal models.

Materials and methods

Huh7.5 cell culture and infection with HCV recombinants

Human hepatoma Huh7.5 cells were cultured in culture flasks (Nunc) in DMEM (Gibco/Invitrogen) supplemented with 10% fetal bovine serum (FBS, Sigma), penicillin 100 U/mL and streptomycin 100 µg/mL (Gibco/Invitrogen), referred to as DMEM+10% FBS. Cells were kept sub-confluent and split every 2–3 days. For splitting, cells were washed in PBS (Invitrogen) and detached using trypsin (Sigma-Aldrich). For serum-free cultures, cells were plated in DMEM+10% FBS. When cells were 80% confluent, DMEM+10% FBS was removed, cells were washed in PBS and detached using trypsin (Sigma-Aldrich). For serum-free cultures, cells were plated in DMEM+10% FBS. When cells were 80% confluent, DMEM+10% FBS was removed, cells were washed in PBS and detached using trypsin (Sigma-Aldrich). For serum-free cultures, cells were plated in DMEM+10% FBS. When cells were 80% confluent, DMEM+10% FBS was removed, cells were washed in PBS and detached using trypsin (Sigma-Aldrich).
Generation of HCVcc virus stocks

For generation of HCVcc and sf-HCVcc virus stocks, HuH7.5 cells cultured in DMEM + 10% FBS at 80% confluency were infected at a multiplicity of infection (MOI) of 0.003 using 1st or 2nd viral passage stocks of the following HCV intra- and intergenotypic recombinants: H77C/JFH1.A1247L (referred to as H77(1a)), J4/JFH1.F236L.A1247L (J4(1b)), J6/JFH1 (J6(2a)), S52/JFH1.T827A.K1398Q (S52(3a)), ED43/JFH1.T827A.T977S (ED43(4a)), SA13/JFH1.A1022G.K1119R (SA13(5a)), and HK6a/JFH1.F355S.N417Y (HK6a(6a)) (Lindenbach et al., 2005; Gottwein et al., 2007; Scheel et al., 2008; Jensen et al., 2008; Gottwein et al., 2009). The % infection was monitored by HCV-specific immunostaining as described below. For generation of HCVcc virus stocks, cells were maintained in DMEM + 10% FBS; supernatants were harvested every 2–3 days, when cells were split, until % of infected cells declined (Gottwein et al., 2007), as detected by immunostaining. High-titer stocks, collected at the peak of viral infection, were used for further experiments. Stocks with relatively low peak titers were concentrated using Amicon 100 kDa centrifugation filters (Millipore). For generation of sf-HCVcc virus stocks, DMEM + 10% FBS cell cultures with 40–80% HCV infected cells were washed with PBS and AEM was added. Cells were maintained in AEM, and supernatants were harvested every 2–3 days, when AEM was exchanged, for up to 29 days. Supernatants were sterile filtered and stored at −80 °C. The HCV Core-E2 sequences of all virus stocks used for further experiments were determined by direct sequencing (described below). Sequences were identical to the plasmid legends.

Evaluation of HCV infected cell cultures

Spread of HCV recombinants in cell cultures was monitored by HCV NSSA immunostaining. Cells plated onto chamber slides (Nunc) the previous day were fixed for 10 min in ice-cold acetone (Sigma-Aldrich) and washed twice with PBS and twice with PBS + 0.1% Tween-20 (Sigma-Aldrich). Cells were stained for HCV NSSA using primary anti-NSSA antibody 9E10 (Lindenbach et al., 2005) at 1:1000 dilution in PBS + 1% bovine serum albumin (BSA, Roche Applied Science) + 0.2% skim milk (PBS/BSK) at two hours at room temperature. Cells were washed twice with PBS and twice with PBS + 0.1% Tween-20 and stained using secondary antibody Alexa Fluor 594-conjugated goat anti-mouse IgG (H+L) (Invitrogen) at 1:500 dilution and Hoechst 33342 (Invitrogen) at 1:1000 dilution in PBS + 0.1% Tween-20. Cells were washed twice in PBS, before being covered by Fluoromount-G (SouthernBiotech) and a cover-slip.

Culture supernatant infectivity titers were determined as FFU/mL. HuH7.5 cells, plated at 6000 cells/well onto poly-l-lysine coated 96-well plates (Nunc) the day before, were infected with serially-diluted supernatants (lowest dilution 1:2). Forty-eight hours after infection, cells were fixed in ice-cold methanol and washed twice with PBS + 0.1% Tween-20 before being incubated with 3% H2O2 for five minutes at room temperature. Cells were washed twice with PBS + 0.1% Tween-20 and HCV NSSA was immunostained with primary anti-NSSA antibody 9E10 at 1:1000 dilution in PBS/BSK at 4 °C. The next day, cells were washed twice with PBS + 0.1% Tween-20 and stained using secondary antibody ECL anti-mouse IgG horseradish peroxidase (HRP)-linked whole antibody (GE Healthcare Amersham) at 1:300 in PBS + 0.1% Tween-20 for 30 min at room temperature before being visualized by 30 min incubation at room temperature with a DAB substrate kit (Dako). FFU were counted automatically using an ImmunoSpot series 5 UV analyzer (CTL Europe GmbH) with the customized software as described previously (Gottwein et al., 2010). Lower limit of detection was calculated for each 96-well plate as the mean of at least 6 negative wells plus 3 standard deviations plus 3. Upper limit of detection was set to 200 FFU/well as this was within the linear range of test dilution series and comparable with manual determinations (Scheel et al., 2011).

For determination of HCV RNA titers in culture supernatant, RNA was extracted from 200 μL supernatant using the Total Nucleic Acid Isolation Kit (Roche Applied Science); titers were determined by TaqMan real-time PCR as previously described (Gottwein et al., 2007). HCV Core titers in culture supernatant were determined using the ARCHITECT HCV Ag assay (Abbott).

Direct sequencing of cell culture-derived HCV

HCV RNA was purified from 200 μL cell culture supernatant using the High Pure Viral Nucleic Acid Kit (Roche Applied Science). Overall, reverse transcription, 1st round PCR and 2nd round nested PCR were carried out as previously described (Gottwein et al., 2007). Primers used to generate cDNA and PCR amplicons spanning the Core-E2 region have been previously reported for H77(1a) and ED43(4a) (Scheel et al., 2008); J4(1b) and HK6a(6a) (Gottwein et al., 2009), J6(2a) and S52(3a) (Gottwein et al., 2007); as well as SA13(5a) (Jensen et al., 2008). Direct sequencing of amplicons was carried out by Macrogen Europe.

Single-cycle virus production assay in S29 cells

Overall, S29 cell experiments were carried out as previously described (Serre et al., 2013). Briefly, 400,000 CD81-deficient S29 cells (Russell et al., 2008) were plated in 6-well plates 24 h before transfection. In vitro HCV RNA transcripts of SA13(5a) (Jensen et al., 2008) as well as of positive control (J6(2a)) and of negative control (J6(2a)-GND) (Lindenbach et al., 2005) were generated using T7 RNA polymerase (Promega) for 2 h at 37 °C. DNAse treated using DNA RQ1 DNAse (Promega) and purified using RNasey kit (Qiagen). HCV RNA transcripts (2.5 μg) were mixed with 5 μL Lipofectamine 2000 (Invitrogen) in 500 μL serum-free Opti-MEM (Gibco/Invitrogen). S29 cells were incubated with transfection complexes for 4 h in Opti-MEM. Following transfection, Opti-MEM was replaced by either DMEM + 10% FBS or AEM. S29 cells were collected at 4, 24, 48 and 72 h post transfection and prepared for determination of intracellular HCV Core and infectivity titers as previously described (Serre et al., 2013). Culture supernatants were collected at 24, 48 and 72 h post transfection for determination of extracellular HCV Core and infectivity titers. Infectivity titers were determined as described above, while Core titers were determined using the ARCHITECT HCV Ag assay (Abbott).

Equilibrium density gradient ultracentrifugation

Semi-continuous 10–40% iodixanol gradients were prepared by layering 2.5 mL of 40%, 30%, 20% and 10% OptiPrep (iodixanol; Sigma-Aldrich) on top of each other as described previously (Prentoe et al., 2011). HCVcc containing supernatants were either loaded directly on top of the gradient, or concentrated using Amicon 100 kDa centrifugation filters (Millipore). For generation of sf-HCVcc virus stocks, cells were maintained in DMEM + 10% FBS or AEM. S29 cells were plated in 4, 24, 48 and 72 h post transfection and prepared for determination of intracellular HCV Core and infectivity titers as previously described (Serre et al., 2013). Culture supernatants were collected at 24, 48 and 72 h post transfection for determination of extracellular HCV Core and infectivity titers. Infectivity titers were determined as described above, while Core titers were determined using the ARCHITECT HCV Ag assay (Abbott).
described above. Iodixanol containing fractions were diluted to contain \( \leq 10\% \) iodixanol before titration.

Receptor-, endocytosis- and neutralization assays

For receptor blocking assays we used Purified Mouse Anti-Human CD81 primary antibody (JS-81) and Purified Mouse IgG1κ isoform control (MOPC-21) (both BD Biosciences); Purified Goat Anti-human LDLr polyclonal antibody (AP2148) and Normal Goat IgG control (AB108C) (both R&D Systems); Anti-SR-BI primary antibody (C16–71) and control antibody (D) were previously described (Catanean et al., 2007). For HCVcc neutralization, we used chronic-phase serum from patient H taken 29 years after acute infection (H06 (Scheel et al., 2008)) and chronic-phase serum from a genotype 5a infected patient (SA3 (Jensen et al., 2008)) as well as a panel of monoclonal antibodies AR1B and AR2A-5A, which were previously described (Giang et al., 2012; Law et al., 2008). For ApoE neutralization we used a mouse monoclonal primary antibody (1D7) blocking the ApoE receptor binding site, and mouse IgG1κ (1D1) control antibody previously described (Weisgraber et al., 1983). For inhibition of clathrin-mediated endocytosis, we used chlorpromazine hydrochloride (Calbiochem).

Huh7.5 cells were plated at 7000 cells/well onto poly-o-lysine coated 96-well plates. On the following day, for receptor-blocking assays, antibodies were diluted in DMEM + 10% FBS as specified and added to cells for 1 h. For chlorpromazine assays, chlorpromazine was diluted in DMEM + 10% FBS as specified and added to cells for 30 min. HCVcc was diluted in DMEM + 10% FBS, whereas sf-HCVcc was diluted in AEM with FBS concentration adjusted to 10%. Virus dilutions were added to the cells incubated with blocking antibodies or chlorpromazine. Cell cultures were incubated for an additional 6 h.

For HCV neutralization assays, chronic-phase HCV sera, or AR1B and AR2A-5A monoclonal antibodies were diluted in DMEM + 10% FBS as specified and mixed with either HCVcc or sf-HCVcc diluted as for receptor blocking assays. Patient serum—virus or antibody—virus mixes were incubated for 1 h, before being added to cells. Cell cultures were incubated for 6 h. For ApoE neutralization, 1D7 and 1D1 monoclonal antibodies were diluted in DMEM + 10% FBS as specified and mixed with either HCVcc or sf-HCVcc, diluted in DMEM + 10% FBS. Antibody—virus mixes were incubated for 30 min, before being added onto cells. Cell cultures were incubated for 3 h.

For blocking and neutralization assays, after 3 or 6 h incubation as indicated above, the cells were washed in PBS and DMEM + 10% FBS was added to all cultures. Cells were incubated and fixed 48 h post infection in ice-cold methanol and HRP-stained for HCV NS5A as described above. Single HCV NS5A positive cells were counted automatically using an ImmunoSpot series 5 UV analyzer (CTL Europe GmbH) with customized software as described previously (Scheel et al., 2011; Gottwein et al., 2011). The % blocking and neutralization were calculated by relating counts of experimental wells to the mean count of six replicate wells with untreated control virus. For receptor blocking and neutralization assays, following logarithmic transformation of X-values, variable-slope sigmoidal dose—response curves \( Y = Bottom + \text{Top} - \text{Bottom}/[1 + 10^{\log 10IC_{50} - X\times \text{slopeslope}}] \) were fitted to the data using GraphPad Prism 6.0. For receptor blocking and neutralization assays “Bottom” was constrained to “0”.

For neutralization assays, “Top” was constrained to “100”, when appropriate, as indicated in Figure legends, and median inhibitory concentrations \( IC_{50} \) were calculated using GraphPad Prism 6.0. For receptor blocking assays, maximum blocking rates \( (B_{\text{max}}) \), the \( Y \) values at the top plateaus of the fitted curves, were calculated using GraphPad Prism 6.0.

HCV immunoprecipitation using anti-ApoE antibody

Immunoprecipitation was done using the ApoE-specific antibody 1D7 and isotype-matched control antibody 1D1 as previously described (Prentoe et al., 2014). Briefly, 50 μl of magnetic-bead slurry was washed in antibody binding buffer (immunoprecipitation kit; Dynabeads Protein G; 100.070D; Invitrogen) and incubated on a shaker with 5 μg antibody in 50 μl antibody binding buffer for 20 min at room temperature. The beads were subsequently washed twice in washing buffer and incubated with 10⁵ IU of the virus in 200 μl of DMEM + 10% FBS on a shaker for 1 h at room temperature. The beads were removed and washed three times in 200 μl of washing buffer prior to elution in 50 μl according to the manufacturer’s instructions. HCV RNA was extracted from the complete eluate and measured in duplicates as previously described (Gottwein et al., 2007).

Cell viability and proliferation assays

For determination of Huh7.5 cell viability in DMEM + 10% FBS versus AEM, we plated 6000 cells per well of poly-o-lysine coated 96-well plates in DMEM + 10% FBS. The following day, medium was removed and cells were incubated in DMEM + 10% FBS or AEM for 48 h. Then, cell viability was determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer’s instructions. The % viability was calculated by relating absorbance at 490 nm determined for 10 AEM cultures to the mean absorbance of 10 replicate DMEM + 10% FBS cultures.

For determination of chlorpromazine cytotoxicity, chlorpromazine was diluted in DMEM + 10% FBS as specified and then added to 6000 Huh7.5 cells/well, plated the previous day in poly-o-lysine coated 96-well plates. Cells were incubated for 6 h before chlorpromazine was removed and DMEM + 10% FBS was added. Cell viability was determined 6 and 48 h post-treatment using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit. The % viability was calculated by relating absorbance at 490 nm determined for chlorpromazine treated cultures to the mean absorbance of at least three replicate untreated cultures.

For determination of Huh7.5 cell proliferation in DMEM + 10% FBS versus AEM, we used the BrDU cell proliferation kit (Millipore). Cells were plated in poly-o-lysine coated 96-well plates at 2000 cells/well in DMEM + 10% FBS according to the manufacturer’s instructions. The following day, medium was removed and cells were incubated in DMEM + 10% FBS or AEM for 48 h. Then, cell proliferation was determined using the BrDU cell proliferation kit according to the manufacturer’s instructions. The % proliferation was calculated by relating absorbance at 450 nm determined for 10 replicate AEM cultures to the mean absorbance of 10 replicate DMEM + 10% FBS cultures.

Flow cytometry

For surface staining of HCV co-receptors we used Phycoerythrin (PE) Mouse Anti-Human CD81 primary antibody (BD Biosciences, JS-81), Anti-mouse LDL R-Phycoerythrin primary antibody (R&D systems, 263123), Purified Mouse Anti-Human CLA-1 (SR-BI) primary antibody (BD Transduction Laboratories, 25/CLA-1) with PE Goat Anti-Mouse Ig secondary antibody (BD Biosciences, polyclonal 550589) and Anti-human Claudin-1 primary antibody (R&D systems, 421203) with PE Goat Anti-Rat Ig secondary antibody (BD Biosciences, polyclonal 550767). Cells were detached by treatment with a 10 mM solution of EDTA in PBS for 10 min at 37 °C. The cells were washed in PBS and resuspended in FACS buffer (PBS + 1% FBS) and 2.5 × 10⁵ cells/well were plated in a V-bottom 96-well plate. Cells were stained protected from light at 4 °C for 1 h with
either α-CD81 (25 μL/well according to the manufacturer’s instructions), α-LDLR (10 μg/mL in FACS buffer), α-SR-BI (5 μg/mL in FACS buffer) or α-Claudin-1 (5 μg/mL in FACS buffer). Total volume in all wells was adjusted to 50 μL using FACS buffer. After incubation, cells were washed in FACS buffer. SR-BI- and claudin-1-stained cells were stained protected from light at room temperature for 20 min with secondary antibodies α-mouse Ig (4 μg/mL in FACS buffer) or α-rat Ig (2 μg/mL in FACS buffer). Cells were washed in FACS buffer and fixed protected from light at room temperature for 15 min using CellFix (BD Biosciences). Cells were washed and resuspended in PBS before they were analyzed on a BD FACSCalibur flow cytometer using CellQuest Pro. Data analysis was done using FlowJo flow cytometry analysis software.

Acknowledgments

We are grateful to Lubna Ghanem for technical assistance, to Lotte Mikkelsen and Anna-Louise Sørensen for general laboratory support, to Andrea Galli and Stéphanie Serre for scientific discussions, and to Jens Ole Nielsen, Bjarne Ørskov Lindhardt, Ove Andersen and Kristian Schenning (all Copenhagen University Hospital, Hvidovre) for their support of the project, as well as to Robert Milne and Anna Toma (Ottawa Heart Institute, Canada), Suzanne U. Emerson and Robert H. Purcell (National Institutes of Health, US) and Charles Rice (Rockefeller University, US) for providing reagents.

This work was supported by A.P. Møller and Chastine Mathiesen et al. / Virology 458-459 (2014) 190–208

References

Akazawa, D., Moriyama, M., Yokokawa, H., Omi, N., Watanabe, N., Date, T., et al., 2010. Hepatitis C virus hypervariable region 1 modulates receptor interactions, conceals the CD81 binding site, and protects conserved neutralizing epitopes. J. Virol. 84, 5751–5763.


