Scavenger Receptor BI Is a Key Host Factor for Hepatitis C Virus Infection Required for an Entry Step Closely Linked to CD81

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Running title : SR-BI targets an HCV entry step closely linked to CD81

1

FOOTNOTES

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Abbreviations: CLDN1: claudin-1; DC-SIGN: dendritic cell-specific intercellular adhesion molecule 3 grabbing non integrin; HCV: hepatitis C virus; HCVcc: cell culture-derived HCV; HCVpp: HCV pseudotype particles; HDL: high-density lipoprotein; LDL: low-density lipoprotein; siRNA: small interfering RNA; SR-BI: scavenger receptor class B type I

ABSTRACT

4

Hepatitis C virus (HCV) is a major cause of chronic hepatitis worldwide. Scavenger receptor class B type I (SR-BI) has been shown to bind HCV envelope glycoprotein E2, participate in entry of HCV pseudotype particles and modulate HCV infection. However, the functional role of SR-BI for productive HCV infection remains unclear. In this study, we investigated the role of SR-BI as an entry factor for infection of human hepatoma cells using cell culture-derived HCV (HCVcc). Anti-SR-BI antibodies directed against epitopes of the human SR-BI extracellular loop specifically inhibited HCVcc infection in a dose-dependent manner. Down-regulation of SR-BI expression by SR-BI-specific siRNAs markedly reduced the susceptibility of human hepatoma cells to HCVcc infection. Kinetic studies demonstrated that SR-BI acts predominately following binding of HCV at an entry step occurring at a similar time point as CD81-HCV interaction. Although the addition of HDL enhanced the efficiency of HCVcc infection, anti-SR-BI antibodies and SR-BI-specific siRNA efficiently inhibited HCV infection independent of lipoprotein. In conclusion, our data suggest that SR-BI (i) represents a key host factor for HCV entry, (ii) is implicated in the same HCV entry pathway as CD81 and (iii) targets an entry step closely linked to HCV-CD81 interaction.

INTRODUCTION

With an estimated 170 million infected individuals, hepatitis C virus (HCV) has a major impact on public health (1). The liver is the primary target organ of HCV, and the hepatocyte is its primary target cell. Attachment of the virus to the cell surface followed by viral entry is the first step in a cascade of interactions between the virus and the target cell that is required for successful entry into the cell and initiation of infection (2). Using recombinant HCV envelope glycoproteins (3) and HCV pseudotype particles (HCVpp) (4, 5), several cell surface molecules have been identified interacting with HCV during viral binding and entry. These include the tetraspanins CD81 (3) and claudin-1 (6), highly sulfated heparan sulfate (7), the low-density lipoprotein (LDL) receptor (8) and scavenger receptor class B type I (SR-BI) (9).

SR-BI is a 509 amino acid glycoprotein with a large extracellular loop anchored to the plasma membrane at both the N- and C- termini by transmembrane domains with short extensions into the cytoplasm (10). SR-BI is involved in bidirectional cholesterol transport at the cell membrane and can bind both native high-density lipoprotein (HDL) and LDL as well as modified lipoproteins such as oxidized LDL (oxLDL). SR-BI is highly expressed in liver and steroidogenic tissues (10) as well as antigen presenting cells (11). Furthermore, SR-BI and its splicing variant SR-BII, have been found to mediate binding and uptake of a broad range of bacteria into human epithelial cells overexpressing SR-BI and SR-BII (12, 13) suggesting that class B scavenger receptors may serve as pattern recognition receptors for bacteria.

Cross-linking studies using recombinant C-terminally truncated HCV envelope glycoprotein E2 isolated SR-BI as a cellular protein binding envelope glycoprotein E2 (9).

Antibodies directed against cell surface expressed SR-BI partially inhibited cellular binding of recombinant envelope glycoproteins (14) as well as HCVpp entry (15-17). Moreover, it has been shown that physiological SR-BI ligands, such as HDL or oxLDL, can modulate HCV infection either by enhancing or by inhibiting HCVpp entry, respectively (18-20).

Recently, several laboratories succeeded in establishing a model for the efficient production of infectious HCV particles in cell culture (HCVcc) (21-23) now allowing determining the role of cell surface molecules involved in HCV infection. Recent evidence suggests that SR-BI and CD81 may act in a cooperative manner for the initiation of HCVcc infection (24) and that overexpression of SR-BI can modulate HCVcc infection (25). However, the functional role of SR-BI in productive HCV infection still remains elusive. In particular, is unclear whether the impact of SR-BI for HCV entry is of key importance or optional, whether SR-BI and CD81 are involved in the same pathways of HCV entry and which HCV entry step is targeted by SR-BI.

Therefore, in this study, we used the HCVcc system to analyze the functional role of SR-BI for productive HCV infection of human hepatoma cells. Using novel anti-SR-BI antibodies and SR-BI specific siRNAs we demonstrate that SR-BI (i) represents a key host factor for HCV entry, (ii) is most likely implicated in the same HCV entry pathway as CD81 (iii) and mediates an entry step occurring postbinding and closely linked to HCV-CD81 interaction.

MATERIALS AND METHODS

Cells. Human embryonic kidney cells 293T, Chinese hamster ovary cells CHO and Huh7.5 have been described (4, 7, 26, 27). Primary human hepatocytes were isolated and cultured as described (28).

Antibodies. Antibodies directed against the extracellular loop of SR-BI were raised by genetic immunization of Wistar rats and Balb/c mice using a pcDNA-expression vector containing the full-length human SR-BI cDNA (pcDNA SR-BI/CLA-1) (Genovac GmbH, Freiburg, Germany) (29). In brief, animals received two to four injections of 20 μg pcDNA SR-BI intraperitoneally at 2-week intervals. Pre-immune control serum was collected from the same animal bled before immunization. To analyze specificity of the produced anti-SR-BI polyclonal serum, CHO cells were transfected with pcDNA (control vector) or pcDNA SR-BI using liposome-mediated gene transfer (Lipofectamine; Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. CHO cells were then incubated with anti-SR-BI polyclonal serum or pre-immune control serum and analyzed for cell surface SR-BI expression by flow cytometry as described (14). R-phycoerythrin (PE)-conjugated goat anti-rat immunoglobulin G (IgG) antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, Pa.). Due to the small amounts of pre-immune sera from individual animals used for immunization, commercially available non-immune rat serum (PAN Biotech) was used as an additional negative control serum for experiments. Rabbit anti-SR-BI antibody (NB 400-104) was obtained from Novus Biologicals (Littleton, Colo.). This antibody is directed against an epitope within the SR-BI cytoplasmic C-terminal domain (CSPAAKGTVLQEAKL, corresponding to amino acids 496 through 509). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG antibodies were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), mouse anti β-actin

antibody was from Sigma and anti-CD81 (JS-81) from BD Biosciences (Heidelberg, Germany).

RNA interference assay. Commercially available siRNAs pools targeting SR-BI, CD81 and CD13 as well as control non-targeting siRNAs were purchased from Dharmacon (Pierce) and transfected into Huh7.5 cells using DharmaFect[TM] solution (Pierce) following the manufacturer's protocol. Silencing of SR-BI expression was assessed by Western blot and flow cytometry 72 h after transfection as described previously (14). 72 h after transfection, cells were incubated with HCVcc and HCV infection was assessed as described below.

Production of viral stocks and infection assays. Plasmids pJFH1, pFK-Jc1 and pFK-Luc-Jc1 have been described (21, 28, 29) and encode the full-length HCV JFH1 cDNA or the chimeric HCV genome designated Jc1 which consists of J6CF and JFH1 segments. The latter construct (pFK-Luc-Jc1) represents a bicistronic reporter virus carrying a firefly-luciferase reporter gene (30). *In vitro* HCV RNA synthesis (30) and RNA transfection was carried out as described (21, 29). To study the effect of HDL on HCVcc infection, JFH1 HCVcc were also generated in lipoprotein-deficient human serum (LPDS) and HDL (30 µg/mL) (31) was added extemporaneously for infection experiments. Culture supernatants from transfected cells were cleared and concentrated as previously described using Amicon Ultra 15 (Millipore, Billerica, MA) (21) and used directly or were stored at 4°C or –80°C. Viruses were titered by using the limiting dilution assay on Huh7.5 cells with a few minor modifications and $TCID_{50}$ was calculated based on the method described (22). siRNA expressing cells and naïve cells were seeded 24 h prior to infection experiments in 12-well tissue culture plates at a density of 5 x 10^4 cells/well. Cells were preincubated in the presence or absence of anti-SR-BI serum or control serum for 1 h at 37°C and then infected at 37°C for 3 h with JFH1 HCVcc challenge virus titers ranging from 1 x 10^7 to 5 x 10^9 copies/mL or 4 h with Luc-Jc1 HCVcc at low

multiplicity of infection. Alternatively, Huh7.5 were inoculated with a high-titer Luc-Jc1 stock for 1h at 4°C in the presence or absence of anti-SR-BI serum or control serum, heparin, anti-CD81 monoclonal antibodies or concanamycin A (ConA) at concentrations indicated in the text. Subsequently, cells were washed three times with ice cold PBS, supplied with fresh culture fluid pre-warmed to 37°C and supplemented with the respective inhibitors and shifted to 37°C. Finally, 4 h later, cells were washed with pre-warmed PBS, supplied with fresh culture fluid without inhibitors and cultured an additional 48 h at 37°C. Depending on the experiment, cells were then washed with ice-cold PBS and RNA extracted using RNeasy® Mini kit (Qiagen, Hilden, Germany). Alternatively, cells were lysed for luciferase assay as previously described (30). HCV RNA was quantitated using VERSANT HCV-RNA 3.0 Assay (bDNA) (Bayer Corporation Diagnostic, Tarrytown, NY) and/or TaqMan real-time PCR as described (32).

RESULTS

Production of antibodies directed against the extracellular loop of SR-BI expressed on human hepatocytes. To assess the functional role of SR-BI for initiation of HCV infection, we first generated polyclonal anti-SR-BI sera directed against the extracellular loop of SR-BI by genetic immunization. Following completion of immunization, antibodies were selected for their ability to bind to human SR-BI expressed on the cell surface of non-permeabilized transfected CHO cells. As shown in Fig. 1, incubation of CHO cells expressing human SR-BI with rat polyclonal anti-SR-BI antibodies resulted in a specific interaction of this serum with the extracellular ectodomain of SR-BI (Fig. 1). In contrast, no interaction was present in CHO cells transfected with the pcDNA3 control vector and incubated with rat anti-SR-BI serum or in CHO cells transfected with human SR-BI cDNA and incubated with rat pre-immune serum (Fig. 1). To study whether anti-human SR-BI recognizes SR-BI on cells susceptible to HCV infection, human hepatocytes and Huh7.5 hepatoma cells were incubated with the sera and analyzed by flow cytometry. As shown in Fig. 2, incubation of human Huh7.5 cells (Fig. 2A) and human hepatocytes (Fig. 2B) with rat polyclonal anti-SR-BI antibody demonstrated that the antibody recognized SR-BI expressed on HCV target cells including human hepatocytes. In contrast, no interaction could be detected in the mouse cell line Hepa 1.6 (Fig. 2C), confirming the species specificity of the antibody. Similar results were obtained for anti-SR-BI antibodies raised in Balb/c mice (data not shown). Taken together, these data demonstrate that anti-SR-BI sera produced by genetic immunization specifically binds to the ectodomain of human SR-BI expressed on hepatocytes.

Inhibition of HCV infection of different isolates by anti-SR-BI antibodies. To assess the role of SR-BI for HCV infection, we studied JFH1 HCVcc infection of Huh7.5 cells in the presence of anti-SR-BI antibodies directed against epitopes of the SR-BI extracellular loop.

10

Anti-SR-BI polyclonal antibodies markedly inhibited JFH1 HCVcc infection of Huh7.5 cells in a dose-dependent manner (Fig. 3A). Fig. 3A shows that anti-SR-BI serum (rat#4) inhibited JFH1 HCVcc infection by more than 70% (Fig. 3A). In contrast, the control pre-immune serum had no inhibitory effect on JFH1 HCVcc infection (Fig. 5 and 6). Moreover, mouse anti-SR-BI antibodies generated by genetic immunization of Balb/c mice but not mouse control pre-immune serum were able to reduce JFH1 HCVcc infection of Huh7.5 in a similar manner (data not shown). Taken together, the data demonstrate that antibodies directed against the SR-BI ectodomain efficiently inhibit HCV infection.

To confirm that inhibition of JFH1 HCVcc infection was indeed mediated by anti-SR-BI antibodies, we purified IgG from both rat anti-SRBI (rat#4) and control serum. As shown in Fig. 3B, anti-SR-BI IgG (100 μg/mL) markedly inhibited JFH1 HCVcc infection of Huh7.5 cells in a similar manner as anti-SR-BI serum (Fig. 3B). In contrast, control IgG (100 μg/mL) purified from pre-immune serum did not inhibit JFH1 HCVcc infection (Fig. 3B). These data clearly demonstrate that the inhibitory effect of anti-SR-BI serum is mediated by anti-SR-BI antibodies and not by other substances present in the serum (such as oxidized lipoproteins potentially interfering with SR-BI function).

To study whether anti-SR-BI-mediated inhibition of HCV infection applies also to other isolates than JFH1, we performed similar experiments using chimeric J6/CF-JFH1 firefly luciferase reporter virus Luc-Jc1 (30). Fig. 3C shows that, similar as for JFH1 HCVcc, both rat (rat#4) and mouse anti-SR-BI antibodies (data not shown) reduced the chimeric reporter virus infectivity in a dose-dependent manner (Fig. 3C) whereas the control pre-immune sera had no inhibitory effect (Fig. 3C and data not shown). Interestingly, we observed variations between the inhibitory effect of anti-SR-BI sera from different rats ranging from 70%-90%. Strongest inhibition was obtained with anti-SR-BI serum from rat#5 (see Fig. 5

and 6), demonstrating over 90% inhibition of HCVcc infection, both with JFH1 isolate and Luc-Jc1 chimera.

Silencing of SR-BI expression results in markedly reduced susceptibility to HCV infection. To further investigate the role of SR-BI in HCVcc infection, we silenced SR-BI expression in Huh7.5 cells using siRNAs targeting SR-BI expression. Immunoblot analysis of transfected cells shows that SR-BI specific siRNA reproducibly down-regulates SR-BI expression whereas β-actin expression was not affected (Fig. 4A). In contrast, a pool of negative control siRNA as well as siRNA targeting CD81 or CD13 did not significantly modulate SR-BI expression, confirming the specificity of the siRNA used (Fig. 4A). Down-regulation of SR-BI expressed on the cell surface of Huh7.5 cells by SR-BI specific siRNA was also confirmed by flow cytometry (difference in mean fluorescence intensity (ΔMFI) of SR-BI siRNA treated cells = 13.98 vs ΔMFI of naïve cells = 148.31). Importantly, down-regulation of SR-BI cell surface expression strongly reduced the susceptibility of human hepatoma cells to infection with HCV (Fig. 4B). As shown in Fig. 4B, siRNA targeting SR-BI or CD81 markedly inhibited JFH1 HCVcc infection of Huh7.5 cells as compared to cells without silenced cell surface molecules (Fig. 4B). In contrast, pools of negative control siRNA as well as CD13 specific siRNA did not significantly reduce JFH1 HCVcc infection (Fig. 4B). Taken together, reduced susceptibility to HCV infection by specific silencing of SR-BI expression clearly demonstrates that SR-BI plays a key role for the establishment of HCV infection of human hepatoma cells.

Impact of lipoproteins for SR-BI-mediated HCV infection. To investigate the impact of lipoproteins on SR-BI mediated HCV infection, we determined the ability of anti-SR-BI antibodies to inhibit JFH1 HCVcc infection in the absence of HDL, a physiological SR-BI ligand that has been shown to enhance HCVcc infection of human hepatoma cells (33). To

study the role of HDL during inhibition experiments, HCVcc were generated in medium supplemented with lipoprotein-free serum (LPDS) and HDL was added extemporaneously for infection experiments. Fig. 5A shows that rat anti-SR-BI serum (rat#5) inhibited JFH1 HCVcc infection of Huh7.5 cells in the absence of HDL (Fig. 5A). Interestingly, whereas HDL was able to enhance JFH1 HCVcc infection in control cells and control serum preincubated cells, no such effect was observed in the presence of anti-SR-BI antibodies in concentrations blocking HCVcc infection (Fig. 5A). These results suggest that these antibodies may block both HCV interaction with SR-BI and HDL-mediated enhancing effect on HCVcc infection. To study whether HDL-dependent enhancement of HCVcc infection was dependent on the level of input virus, we repeated experiments using different JFH1 HCVcc preparations with challenge virus titers ranging from 1×10^7 copies/mL to 5×10^9 copies/mL resulting in similar observations (data not shown). Furthermore, the effects of HDL on HCVcc infection was confirmed by titration experiments using anti-SR-BI antibodies: as shown in Fig. 5A, the enhancing effect of HDL on HCVcc infection appeared to be restored when anti-SR-BI antibodies were used at decreasing concentrations (Fig. 5A). In addition, the role of HDL on JFH1 HCVcc infection was also studied in siRNA-transfected Huh7.5 cells. As shown in Fig. 5B, a minor enhancing effect of HDL was detected in Huh7.5 cells transfected with siRNA targeting SR-BI, suggesting that a low level of SR-BI may still be available for HCV/HDL interplay on these cells. In cells with silenced CD81 expression, no marked enhancing effect of HDL on JFH1 HCVcc infection was observed (Fig. 5B).

SR-BI mediates an HCV entry step occurring postbinding and closely linked to CD81.
Kinetic studies using chimeric JFH1 firefly luciferase reporter virus have demonstrated that glycosaminoglycans predominantly act at the stage of HCV attachment to target cells, whereas CD81 mediates HCV infection at a step post binding (30). To map the step targeted by SR-BI during HCV entry, we investigated the inhibitory capacity of anti-SR-BI serum

13

(rat#5) and corresponding purified IgG when administered during and/or after virus binding in side-by-side experiments using heparin – a structural and functional homologue of highly sulfated heparan sulfate and anti-CD81 antibody. Luc-Jc1 HCVcc binding to Huh7.5 cells was performed for 1 h at 4°C in the presence or absence of inhibitors. Under these conditions, virus attaches to the cells but does not efficiently enter, thus permitting synchronous infection when the inoculum is removed and cells are shifted to 37°C. Therefore, subsequent to virus attachment, unbound virus was washed away, cells were shifted to 37°C to allow entry to proceed and inhibitors or control medium were added for 4 h (Fig. 6A). Fig. 6B shows that rat anti-SR-BI serum as well as purified anti-SR-BI IgG were able to inhibit Luc-Jc1 HCVcc infection when added following binding of the virus to the target cell (Fig. 6B). The control serum only had no significant effect on Luc-Jc1 HCVcc infection (Fig. 6B). In contrast, heparin – a homologue of highly sulphated heparan sulfate, inhibited Luc-Jc1 HCVcc infection only when it was present during virus binding but not when added postbinding (Fig. 6B). To further characterize the entry step mediated by SR-BI, anti-SR-BI and anti-CD81 antibodies were added in side-by-side experiments every 20 min for up to 120 min after viral binding (Fig. 7A). Fig. 7B shows that rat anti-SR-BI serum was able to inhibit Luc-Jc1 HCVcc infection even when added up to 60 min post HCVcc binding (Fig. 7B). These data clearly indicate that SR-BI is involved in an entry step occurring postbinding. Since an almost identical kinetics of inhibition of HCV infection was observed for anti-CD81 antibody assessed in side-by-side experiments (Fig. 7B), it is likely that the entry steps mediated by SR-BI and CD81 occur during a similar time point and are closely linked to each other. To further address the contribution and interplay of CD81 and SR-BI in HCV entry, we added anti-CD81 and anti-SR-BI IgG simoultaneously prior to Luc-Jc1 HCVcc infection. Fig. 8B shows that blocking both CD81 and SR-BI inhibited Luc-Jc1 HCVcc infection more potently than blocking of each receptor alone (Fig. 8A and B). This effect was not observed when

control IgG were used in combination with anti-CD81 monoclonal antibody (Fig. 8B). Taken together, our results suggest that SR-BI and CD81 may act in concert to mediate HCV entry.

DISCUSSION

Using an infectious HCV tissue culture system, we demonstrate that SR-BI (i) represents a key host factor for HCV entry, (ii) is implicated in the same HCV entry pathway as CD81 and (iii) targets an entry step occurring postbinding closely linked to CD81.

SR-BI delivers HDL cholesteryl ester to the liver and steroidogenic tissues by a process termed the 'selective uptake pathway' (34-37). This process differs markedly from that of the classic clathrin coated pit-mediated LDL receptor endocytic pathway, in which the entire lipoprotein is internalized and degraded (38). In the selective uptake pathway, SR-BI binds HDL and the core cholesteryl ester are delivered to the plasma membrane without the endocytosis of the entire HDL particle. SR-BI mediated selective HDL cholesteryl ester uptake is a two-step process: the first step involves lipoprotein binding to the extracellular domain of SR-BI and the second step consists in the selective transfer of lipid to the plasma membrane (39, 40).

The marked inhibition of HCV infection of two different isolates (JFH1 and Jc1) by anti-SR-BI antibodies and siRNAs suggests that SR-BI plays a key role for establishment of HCV infection. These results extend recently obtained evidence suggesting that SR-Bs modulate HCV infection (24, 25). Extending previous studies, we demonstrate that inhibition of HCVcc infection by anti-SR-BI antibodies or SR-BI specific siRNA was not dependent on the presence of lipoproteins in the tissue culture medium suggesting that SR-BI can mediate HCV entry independent from an interaction of HCV or SR-BI with HDL. Lavillette *et al.* demonstrated that silencing SR-BI expression markedly reduced HCVpp entry independent of HDL (17), whereas Voisset et al. demonstrated that SR-BI silencing only reduced the HDL-mediated enhancement of HCVpp entry (19). Using the HCVcc model system and transfected

siRNAs we now demonstrate that silencing of SR-BI expression resulted in a marked down-regulation of susceptibility to HCV infection independent of the presence of lipoproteins, although HDL was able to enhance HCV infection. In our hands, the use of an optimal siRNA delivery system was crucial for the study of HCV infection. Whereas recombinant lentiviral vectors were characterized by interference with HCV infection unrelated to the expressed siRNA (data not shown), the transfection of *in vitro* transcribed SR-BI siRNAs specifically resulted in down-regulation of HCV infection. The specific effect of SR-BI siRNAs is demonstrated by the lack of inhibitory effects of various control siRNAs including siRNAs targeting another protein expressed on the cell surface of hepatoma cells (CD13).

Since the presence of HDL did not inhibit but rather enhanced HCV infection, it is unlikely that HCV and HDL compete for the SR-BI HDL binding domain. The highly reproducible enhancement of HCV infection by HDL may point to a more efficient interaction of SR-BI with HCV e. g. as a result of a conformational change induced by HDL. These findings are in line with a previous study demonstrating that HDL is a serum factor that attenuates neutralization by antiviral antibodies of HCVpp or HCVcc (33). The authors hypothesized that HDL may stimulate cell entry of viral particles by accelerating their endocytosis (33). In contrast to results of ectodomain blocking by anti-SR-BI, HDL appeared to slightly enhance HCVcc infection in cells with silenced SR-BI. This may not be unexpected since down-regulation by siRNA, in contrast to ectodomain blocking, most likely leaves some SR-BI accessible for HDL/HCV interplay.

Furthermore, we demonstrate that in target cells with silenced CD81 expression, HDL appeared not to markedly enhance HCVcc infection (Fig. 5B). These findings strongly suggest that the HDL/SR-BI-HCV interaction acts in concert with CD81 within the same entry pathway and does not represent another or redundant route of cell entry. This conclusion

is further supported by an additive inhibitory effect of anti-SR-BI and anti-CD81 antibodies on Luc-Jc1 HCVcc infection (Fig. 8B) confirming previous results obtained for JFH1 (24).

Using an HCVcc-based kinetic entry assay (Fig. 6 and 7), we mapped the HCV entry step targeted by SR-BI. As shown in Fig. 6 and 7, anti-SR-BI IgG markedly inhibited HCVcc infection when added up to 60 min postbinding of attached virus. These data for the first time directly demonstrate that SR-BI predominantly mediates an HCV entry step occurring postbinding of HCV to the hepatocyte cell surface membrane. These findings confirm the hypothesis raised by Hahn and colleagues based on experiments using oxidized lipoproteins as SR-BI ligands (20). In contrast to anti-SR-BI and anti-CD81 antibodies, heparin – a homologue of highly sulfated heparan sulfate- was able to inhibit HCVcc infection only when added prior to HCV binding. Taken together, these data suggest that glycosaminoglycans such as highly sulfated heparan sulfate act predominantly at the stage of viral attachment, whereas SR-BI and CD81 mediate entry steps occurring postbinding. Subsequent steps in HCV entry are most likely mediated by claudin-1, a recently discovered co-host factor for HCV infection (6).

Kinetic studies using anti-SR-BI and CD81 antibodies in side-by-side experiments demonstrated that SR-BI is required for an entry step occurring at a similar time point as CD81-HCV interaction. Although the magnitude of antibody-mediated inhibition of HCVcc infection was different, the kinetics of inhibition of HCV infection by anti-SR-BI and anti-CD81 antibodies was remarkably similar (Fig. 6). Both anti-SR-BI and anti-CD81 antibodies were able to inhibit HCV infection when added up to 60 min postbinding and lost their ability to inhibit HCV infection when added 80 min postbinding. The rate of loss of CD81 antibody HCVcc inhibition overtime in our study appeared to be different than the one observed for HCVpp in previously published studies (6, 41). This may be either due to experimental

18

differences or be a difference between the behaviors of HCVpp and HCVcc. Taken together, our results demonstrate that the entry steps mediated by SR-BI and CD81 occur during a similar time frame and are closely linked to each other.

SR-BI and its splicing variant SR-BII contain an identical extracellular domain. SR-BII is encoded by an alternatively spliced mRNA from the SR-BI gene and differs from SR-BI only in the carboxy-terminal cytoplasmic tail, which, as shown previously, must contain a signal that confers predominant intracellular expression and rapid endocytosis of HDL (42). Scavenger receptor BII, which is expressed at low levels in the liver compared with SR-BI (43), mediates rapid HDL endocytosis through a clathrin-dependent, caveolae-independent pathway (42) but is inefficient compared with SR-BI in HDL cholesteryl ester selective uptake (44). Since our tools (anti-SR-BI antibody and SR-BI siRNAs) may also target SR-BII, we cannot completely exclude a role for SR-BII in HCV infection as most recently shown by other investigators (25).

In conclusion, our results demonstrate that SR-BI plays a key role for the establishment of HCV infection mediating HCV infection during an entry step occurring postbinding closely linked to the interaction of HCV with CD81. The functional mapping of SR-BI-HCV interaction and its impact for HCV entry has important implications for the understanding of the very first steps of HCV infection and the development of novel antiviral strategies targeting HCV entry.

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ACKNOWLEDGEMENTS

FIGURE LEGENDS

Fig. 1. Production of antibodies directed against the human SR-BI ectodomain by genetic immunization. Specific binding of rat anti-human SR-BI serum to SR-BI expressed in CHO cells. Anti-SR-BI polyclonal serum directed against the SR-BI ectodomain loop was raised by genetic immunization of Wistar rats using a plasmid harboring human SR-BI cDNA. CHO cells were transfected with pcDNA-SR-BI (pCDhSR-BI) or control vector (pcDNA). Flow cytometry of SR-BI or control transfected non permeabilized CHO cells incubated with rat anti-human SR-BI polyclonal serum and PE-conjugated anti-rat IgG demonstrated specific interaction of anti-SR-BI antibodies with human SR-BI. In contrast, no interaction was present in CHO cells transfected with control vector and incubated with anti-SR-BI serum.

Fig. 2. Interaction of anti-SR-BI antibodies with the SR-BI ectodomain on human hepatocytes and Huh7.5 hepatoma cells. Cell surface expression of SR-BI was determined by flow cytometry using rat anti-human SR-BI serum or control pre-immune serum as described in Fig. 1. Histograms corresponding to cell surface expression of the respective cell surface molecules (open curves) are overlaid with histograms of cells incubated with the appropriate isotype control (gray shaded curves). In contrast to absent interaction on murine Hepa 1.6 hepatoma cells, rat anti-human SR-BI serum specifically detected SR-BI on the cell surface of human hepatoma Huh7.5 cells and human primary hepatocytes.

Fig. 3. Inhibition of HCV infection by anti-SR-BI antibodies. (A) Inhibition of JFH1 HCVcc infection by rat polyclonal anti-SR-BI antiserum. Huh7.5 cells were preincubated for 1 h at 37°C with various dilutions of rat anti-SR-BI or control serum before infection with JFH1 HCVcc for 3 h at 37°C. HCV infection was assessed by HCV RNA quantitation in

lysates of infected Huh7.5 cells 72 h post-infection. Total RNA was isolated and HCV RNA was quantified as described in Materials and Methods. Results are expressed as mean % HCVcc infectivity in the absence of antibody (mean ± SD; n = 4). (B) Inhibition of JFH1 HCVcc infection by purified rat anti-SR-BI IgG. Huh7.5 cells were preincubated for 1 h at 37°C with 100 µg/mL of IgG isolated from rat anti-SR-BI or control serum before infection with JFH1 HCVcc. Results are expressed as % HCVcc infectivity in the absence of antibody (mean ± SD; n = 4). (C) Inhibition of Luc-Jc1 HCVcc infection by anti-SR-BI. Huh7.5 cells were preincubated for 1 h at 37°C with various dilutions of rat anti-SR-BI serum or control serum before infection with Luc-Jc1 HCVcc for 4 h at 37°C. HCV infection was assessed by measurement of luciferase activity 48 h post-infection in lysates of infected cells. Results are expressed as mean Log_{10} RLU/well (mean ± SD; n = 4).

Fig. 4. Silencing of SR-BI expression results in reduced susceptibility to HCV infection. (A) Western blot analysis of siRNA mediated down-regulation of SR-BI expression in Huh7.5 cells. Lysates of control naïve Huh7.5 (Ctrl), Huh7.5 cells expressing control siRNA (Ctrl siRNA) or siRNA targeting SR-BI, CD81 or CD13 were subjected to SDS-PAGE. Immunoblotting was performed using rabbit anti-SR-BI antibodies (1/4000) and HRP-conjugated anti-rabbit antibodies (1/1000) or mouse anti-β-actin monoclonal antibody (1/5000) and HRP-conjugated anti-mouse antibodies (1/1000). The presence or absence of SR-BI and β-actin is indicated on the right, and molecular weight (MW) markers (kDa) are indicated on the left. (B) Susceptibility to HCVcc infection is reduced in SR-BI specific siRNA expressing Huh7.5 cells. Control naïve Huh 7.5 (Ctrl), Huh7.5 cells expressing control siRNA (Ctrl siRNA) or siRNA targeting SR-BI, CD81 or CD13 were incubated with JFH1 HCVcc. Total RNA was extracted 72 h post-infection and HCV RNA was quantified. Data are expressed as % HCVcc infectivity of naïve control cells (mean ± SD; n = 4).

Fig. 5. SR-BI-mediated HCV infection is not dependent on the presence of lipoproteins.
(A) Anti-SR-BI inhibits HCVcc infection in the absence of lipoproteins. Huh7.5 cells were preincubated for 1 h at 37°C with rat anti-SR-BI serum or control serum (diluted 1/20, 1/100 and 1/500) before infection with JFH1 HCVcc generated in LPDS-medium in the presence (black bars) or absence (open bars) of HDL (30 µg/mL). Total RNA was isolated 72 h post-infection and HCV RNA was quantified. Results are expressed as % HCVcc infectivity in the absence of antibody (mean ± SD; n= 4). (B) Reduced susceptibility to HCVcc infection in SR-BI-specific siRNA expressing Huh7.5 cells is independent of lipoproteins. Control naïve Huh7.5 cells (Ctrl), Huh7.5 cells expressing control siRNA (Ctrl siRNA) or siRNA targeting SR-BI, CD81 or CD13 were incubated with JFH1 HCVcc generated in LPDS-medium in the presence (black bars) or absence (open bars) of HDL (30 µg/mL). Total RNA was extracted 72 h post-infection and HCV RNA was quantified. Data are expressed as mean % HCVcc infectivity of naïve control cells (mean ± SD; n = 4).

Fig. 6. SR-BI mediates is involved in an HCV entry step occurring postbinding of virions. (A) Schematic drawing of the experimental setup. Inhibition of Luc-Jc1 HCVcc entry into Huh7.5 cells by rat anti-SR-BI serum or control serum (1/200), anti-SR BI or control IgG (100 µg/mL), anti-CD81 monoclonal antibody (10 µg/mL), heparin (250 µg/mL) or ConA (25 nM) was compared using two different protocols. Virus binding to target cells was performed in the presence (protocol I) or absence (protocol II) of compounds. Subsequently, in both protocols, cells were washed, supplemented with fresh medium containing the given inhibitors and shifted to 37°C to allow entry to proceed. Four hours later, cells were again washed and supplied with medium without inhibitors or antibodies. Dashed lines indicate the time intervals where inhibitors or antibodies were present. Luciferase activity was determined 48 h later and is expressed relative to control infections performed in the same way but without addition of inhibitor. (B) Kinetics of HCVcc entry into human hepatoma cells. The

efficiency of infection using the protocols depicted in panel A (protocol I: open bars; protocol II: black bars) was measured as described in (A). Results are expressed as % Luc-Jc1 HCVcc infectivity in the absence of inhibitory compound or antibody (mean ± SD; n = 4).

Fig. 7. SR-BI mediates an HCV entry step closely linked to CD81. (A) Schematic drawing of the experimental setup. Inhibition of Luc-Jc1 HCVcc entry into Huh7.5 cells by rat anti-SR-BI serum or control serum (1/200) as well as anti-CD81 monoclonal antibody (10 µg/mL) was performed as described in legend to Fig. 6 but inhibitors were added every 20 min for 120 min after viral binding. Dashed lines indicate the time intervals where inhibitors are present. Luciferase activity was determined 48 h later and is expressed relative to control infections performed in the same way but without addition of inhibitor. (B) Kinetics of HCVcc entry into human hepatoma cells. The efficiency of infection using rat anti-SR-BI serum (●), control serum (○) or anti-CD81 antibody (■) was measured by luciferase assay 48 h later. Results are expressed as % Luc-Jc1 HCVcc infectivity in the absence of antibody (mean ± SD; n = 4).

Fig. 8. SR-BI and CD81 act in concert to mediate HCV entry. (A) Dose-dependent inhibition of Luc-Jc1 HCVcc infectivity by anti-SR-BI and anti-CD81 antibodies. Huh7.5 cells were preincubated for 1 h at 37°C with anti-CD81 monoclonal antibody (0.01 and 0.02 µg/mL), rat anti-SR-BI IgG (0.4 and 0.8 µg/mL) or control IgG (0.4 and 0.8 µg/mL) before infection with Luc-Jc1 HCVcc for 4 h at 37°C. HCV infection was assessed by measurement of luciferase activity 48 h post-infection. Data are expressed as % Luc-Jc1 HCVcc infectivity in the absence of antibody (mean ± SD; n = 4). (B) Additive effect of anti-SR-BI and anti-CD81 antibodies in inhibition of HCVcc entry. Huh7.5 cells were preincubated for 1 h at 37°C with rat anti-SR-BI IgG (0.8 µg/mL) or control IgG (0.8 µg/mL) either alone or in combination with anti-CD81 monoclonal antibody (0.02 µg/mL) before infection with Luc-

Jc1 HCVcc for 4 h at 37°C. HCV infection was assessed as described in (A). Data are expressed as % Luc-Jc1 HCVcc infectivity in the absence of antibody (mean ± SD; n = 4).