C-type lectin LSECtin interacts with DC-SIGNR and is involved in hepatitis C virus binding

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Abstract Hepatitis C virus (HCV) is a major cause of liver disease. However, the detailed mechanism underlying hepatocyte infection with HCV is not yet completely understood. We previously identified a novel C-type lectin—LSECtin predominantly expressed on liver sinusoidal endothelial cells. Here we demonstrate that LSECtin can interact with two HCV receptors, DC-SIGNR and CD81, through its central ectodomain. Furthermore, cells expressing LSECtin specifically can be attached by the naturally occurring HCV in the sera of infected individuals. This binding was found to be mediated by the HCV E2 glycoprotein and could be efficiently inhibited by EGTA but not by mannan treatment. The present study suggests that LSECtin interaction with DC-SIGNR might contribute to HCV binding to liver sinusoidal endothelial cells.

Keywords HCV E2 glycoprotein · HCV receptor · Hepatitis · Lectin · Liver sinusoidal endothelial cell

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Introduction

HCV, a positive single-stranded RNA virus in the Flaviviridae family, is the major causative agent of non-A, non-B hepatitis in humans and has infected ~170 million people worldwide [1–3]. The HCV structural protein includes two envelope glycoproteins E1 and E2; these glycoproteins form a heterodimer and mediate viral binding and entry into host cells [4, 5]. To infect a cell, HCV must first attach itself to the cell surface by interaction with specific cell surface receptors, and consequently induce conformational changes in the glycoproteins E1 and E2 and fusion of the viral and cellular membranes [2, 4, 5].

By using surrogate models such as the truncated soluble E2 glycoprotein and HCV pseudoparticles, several potential HCV receptors have been identified, including the human tetraspanin CD81, scavenger receptor class B type 1/2 (SR-B1/2), and low density lipoprotein receptor (LDL-R) [5–8]. Since liver is the major target organ of HCV infection, the expression pattern of HCV receptors has been suggested to be liver specific or dominant within the liver. In addition to cause an infection, HCV can use multiple attachment factors and receptors in parallel or in succession [4]. Dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN; also termed CD209) and DC-SIGN related (DC-SIGNR; also known as L-SIGN and CD209L) are two members of the C-type lectin family; these bind to viral glycoproteins such as E2 in HCV [9–12].

Recently, we have identified a novel C-type lectin predominantly expressed on liver sinusoidal endothelial cells (LSEC) and denominated it as liver sinusoidal endothelial cell C-type lectin (LSECtin) [13]. LSECtin shares 32% and 31% amino acid sequence identities with DC-SIGNR and DC-SIGN, respectively, and has been shown to bind in vitro with mannose, GlcNAc, and fucose. Interestingly, LSECtin can be colocalized with DC-SIGNR on the LSECs [13]. Recent studies showed that LSECtin could bind to Ebola virus surface glycoprotein, filovirus glycoproteins, and the spike protein of SARS coronavirus [14–16]. In addition, LSECtin was also demonstrated to be expressed in human peripheral blood and thymic dendritic cells isolated ex vivo. LSECtin expression confers Ebola virus-binding capacity to leukemic cells, participates in antigen uptake and internalization, and therefore it functions as a pathogen receptor [17]. However, the potential role of LSECtin in HCV binding to LSECs was not fully understood.

In this study, we present evidence that LSECtin could interact with DC-SIGNR and CD81, the two previously identified HCV receptors. We further showed that LSECtin could bind to the HCV E2 glycoprotein in a calcium-dependent manner. These results might contribute to the understandings of the complicated mechanism of HCV infection.

Materials and methods

Cell lines and antibodies

CHO cells stably expressing the Fc-LSECtin fusion protein were cultured in DMEM and 10% fetal bovine serum (HyClone) supplemented with 400 µg/ml Geneticin (G418; Gibco) and 100 units/ml penicillin/streptomycin. LSECtin and DC-SIGNR were subcloned into the A site of the pIRES expression vector. LSECtin, DC-SIGNR and empty vectors (for mock cells) were individually transfected into NIH3T3 cells, and 800 µg/ml G418 was added after 48 h. Clones that stably expressed LSECtin or DC-SIGNR were screened, and the expression was confirmed by western blotting and fluorescence-activated cell sorter (FACS) analysis, using anti-LSECtin mouse monoclonal antibody we previously raised [18] and anti-DC-SIGNR antibody (IgG2b; R&D Systems). The anti-LSECtin monoclonal antibody could specifically recognize LSECtin and its specificity was further shown in Supplementary Fig. 1.

Coimmunoprecipitation and western blotting

Full-length DC-SIGNR and CD81 were cloned into the pcDNA3.1 vector (Invitrogen). Coimmunoprecipitation was carried out as described previously [19].

Expression and purification of HCV E2 protein

The HCV E2 (384–664 aa) fused to human IgG1-Fc was generated. CHO clones with stable Fc-E2 were obtained. The Fc-E2 proteins were purified by affinity chromatography with the rProtein AFF column (Amersham Biosciences). The amount of E2 antigen was quantified by ELISA using anti-Fc monoclonal antibody (Sigma). HCV E2 was also cloned into the pSecTag2/Hygro vector for producing Myc-tagged E2.

Soluble E2 binding and inhibition

NIH3T3 cells (2×10^5) with stable LSECtin or DC-SIGNR expression were seeded in six-well plates. The cells were then harvested with the cell dissociation solution (Sigma) and washed three times with an FACS buffer. After adding 20 µg/ml mannan or 5 mM EGTA, the cells were incubated with the mock antigen or soluble E2 glycoprotein in the FACS buffer for 1 h at 4°C. After washing three times, the adherence of E2 was quantified by FACS using anti-Fc or anti-Myc antibody. For the soluble LSECtin protein inhibition experiment, HEK293T cells were transfected with pFlag-CMV-LSECtin. The cells were preincubated with the soluble LSECtin protein before addition of E2. Fc protein was used as negative control and data was normalized by the total input protein.

Pseudotype particles production and cell infection

Plasmid containing HCV E1E2 was constructed with 5′-GCT AAG CTT GGA TGG CCG ACC TCA TGG GGT AC-3′ (Hind III) and 5′-CGA GAA TTC CGC CTC CGC TTG GGA TAT-3′ (EcoR I) using HCV genome-containing pBRTM-HCV1-3011 vector (infectious molecular clone H77 (GenBank accession No. AF009606) as the template. The PCR product was digested and cloned into pcDNA3.1 vector. To generate the pseudotype viral particles, the envelope-deficient pNL4-3.Luc.R-E-plasmid and the HCV E1E2 plasmid were cotransfected into HEK293T cells. The culture supernatant was harvested, sterile filtered, divided into aliquots, and stored at −80°C. The bound pseudotype particles were measured using ELISA. Stable LSECtin or DC-SIGNR NIH3T3 cells (2×10^5) were seeded 24 h before infection in 12-well plates. After washing three times with serum-free DMEM, 200 µl of the pseudotype retroviral particles in medium were added to the cells followed by incubation for 3–5 h at 37°C under gentle agitation every 15 min. Subsequently, 300 µl of DMEM was added, and the luciferase activity in cell lysates was determined at 48 h after infection (assay kit was from Promega).

Virus-binding assay

The HCV virus-positive and negative donors' sera (all HIV negative) were obtained from the General Hospital of PLA, and informed consent was obtained from each subject. The cells were blocked with 10% heat-inactivated goat serum for 20 min at 37°C. After washing, the sera were diluted in an adhesion buffer and were allowed to incubate with the cells for 1 h at 37°C; the unbound viruses were removed. The HCV RNA copies bound to the cells were measured by quantitative PCR using the HCV kit (ShenZhen PG BIO-TECH). For the inhibition assays, mannan (20 µg/ml) or EGTA (5 mM) was preincubated for 20 min at room temperature or the HCV virions were preincubated with the soluble LSECtin protein for 20 min at 4°C.

Results

LSECtin interacts with DC-SIGNR and CD81

We have previously demonstrated that similar to its close paralogue DC-SIGNR, LSECtin could form homo-tetramer [13]. DC-SIGNR and CD81 are known HCV receptors expressed on LSECs. This knowledge prompted us to investigate whether LSECtin could interact with DC-SIGNR or CD81. First we detected the physical interactions of LSECtin with DC-SIGNR using co-immunoprecipitation assay (Co-IP) in the HEK293T cells co-transfected with LSECtin and DC-SIGNR expressing plasmids. As shown in Fig. 1a, ectopic expressed LSECtin could be coimmuno-precipitated with Myc-tagged DC-SIGNR in HEK293T cells. The reciprocal Co-IP assays also indicated that DC-SIGNR could be coimmunoprecipitated with LSECtin (Fig. 1b). Then the soluble LSECtin adhesion assay was performed to confirm the interaction. The soluble LSECtin protein consisting of only the central ectodomain (aa 55–162) was incubated with the HEK293T cells transfected with DC-SIGNR or with vector-mock cells. Their associations were assessed by FACS assays with anti-LSECtin antibody. About a half of the DC-SIGNR-HEK293T cells were bound to the soluble Fc-LSECtin but not control-Fc protein, whereas only 6% of the mock cells were adhered to LSECtin (Fig. 1c). Since soluble LSECtin contains only the ectodomain, this result implicated that the ectodomain of LSECtin was sufficient for its interaction with DC-SIGNR.

LSECtin protein contains an N-terminal transmembrane domain (TM), a potential encocytic motif (aa 6–9), a central ectodomain (aa 55–162) including a coiled-coil region (aa 96–136), and a C-terminal carbohydrate
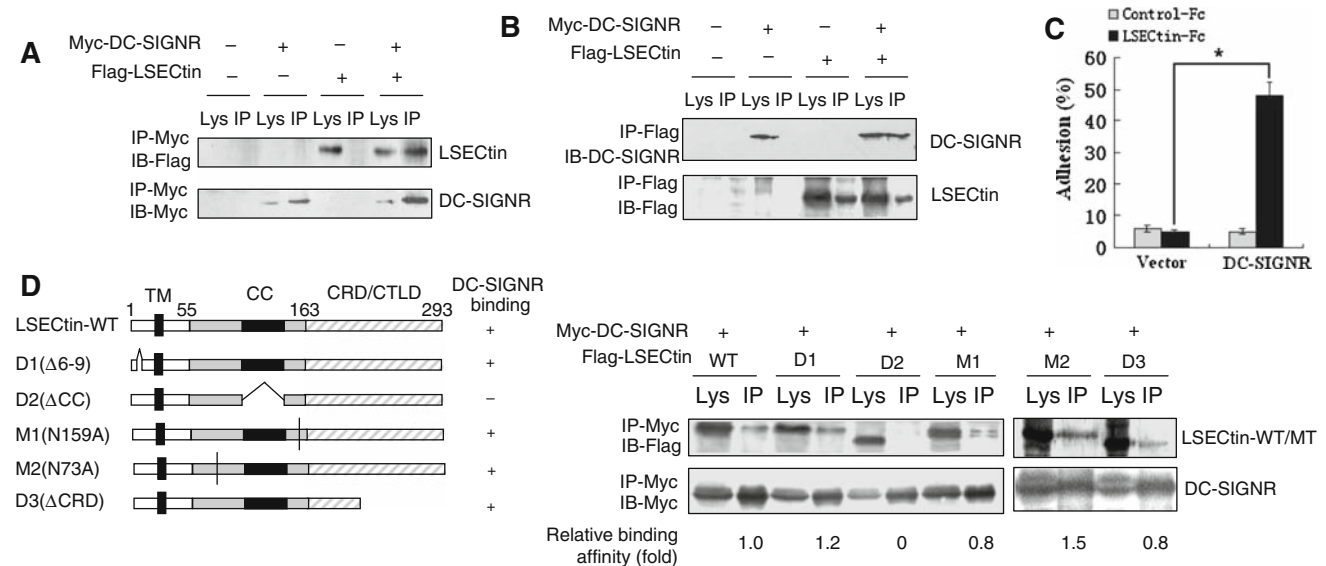

Fig. 1 Interactions between LSECtin and DC-SIGNR. **a** HEK293T cells were transfected with Flag-LSECtin and Myc-DC-SIGNR. After 30 h, cell lysates were prepared and immunoprecipitated (IP) with anti-Myc monoclonal antibody and examined by immunoblotting (IB) with anti-Myc and Flag antibodies. **b** The reciprocal coimmunoprecipitation was performed using an anti-Flag antibody. **c** At 24 h posttransfection, HEK293T cells expressing DC-SIGNR or control cells were incubated with the soluble Fc-tagged LSECtin ectodomain or control-Fc protein. After washing times, the cells with adhered LSECtin were detected by FACS using an anti-Fc monoclonal antibody. Asterisk indicates that the difference is significant. **d** Co-IP assays for Myc-DC-SIGNR and Flag-tagged LSECtin wild-type and the indicated mutants. The 5% inputs and IP products were run on SDS-PAGE gel and detected by western blotting. The interaction of the distinct LSECtin mutants with DC-SIGNR was quantified and normalized to express the results as "relative to the level of expression of the different LSECtin mutants". The binding affinity of wild-type LSECtin to DC-SIGNR was indicated as onefold. The data are representative of three independent experiments in (**a**), (**b**), and (**c**). Mean values ± SD (standard deviation) of the three experiments are shown in (**c**)

recognition domain (CRD)/C-type lectin-like domain (CTLD) (aa 163–293). To further investigate whether the ectodomain of LSECtin was required for and whether other regions were involved in the interaction with DC-SIGNR, a series of LSECtin truncates or point mutants were constructed and used in the Co-IP assays. The expressions of these mutants in cells were adjusted to a comparable level and the lysates in same amount were used as input for immunoprecipitation. The 5% inputs and IP products were run on SDS-PAGE gel and detected by western blotting. As shown in Fig. 1d, deletion of the coiled-coil region within the ectodomain (indicated by D2) completely abolished the interaction of LSECtin with DC-SIGNR. In contrast, deletion of the potential endocytic motif (indicated by D1) or mutation of each N-linked glycosylation sites N73, N159 to Ala (M1, M2) had no significant effect on the interaction, and the deficiency of CRD/CTLD domain (D3) also had modest effect on the interaction which might be caused by conformation changes. Compared with the binding affinity of wild-type LSECtin to DC-SIGNR (indicated as onefold), the affinities of various LSECtin mutants to DC-SIGNR were D1, 1.2; D2, 0; M1, 0.8; M2, 1.5; D3, 0.8, respectively. These results clearly showed that the central ectodomain is critical for the LSECtin-DC-SIGNR interaction.

To extend our conclusion, we further tested the interaction of LSECtin with another HCV receptor CD81. Co-IP assays and FACS analysis both showed that LSECtin could also interact with CD81 (Fig. 2a–c). Due to the fact that HEK293T expresses high level of endogenous CD81, we also detected the interaction between Flag-LSECtin with endogenous CD81 (Fig. 2b). Adhesion assay result implicated that ectodomain of LSECtin was sufficient for its interaction with CD81, although the affinity with CD81 seems to be weaker than that with DC-SIGNR (compare Fig. 2c with Fig. 1c).

LSECtin binds to soluble HCV E2 protein

Since LSECtin could interact with DC-SIGNR and CD81, which are the well-defined HCV receptors, we asked whether LSECtin could interact with HCV by binding HCV E2 glycoprotein similar to the manner of DC-SIGNR and CD81. NIH3T3 cells stably expressing LSECtin or DC-SIGNR were generated and the expressions of these proteins on the cell surface were roughly comparable (Supplementary Fig. 1A). Then the cells were incubated with purified soluble Fc-tagged HCV E2 fusion protein and washed and binding was assessed by flow cytometry. The soluble Fc-tagged E2 but not control-Fc protein significantly bound the NIH3T3-LSECtin or NIH3T3-DC-SIGNR expressing cells but not the mock cells (Fig. 3a).
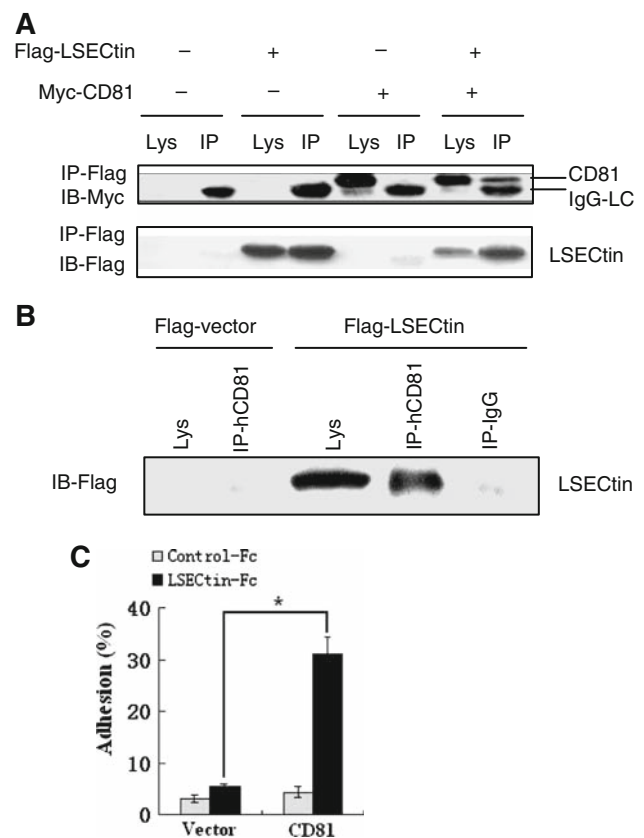

Fig. 2 Interactions between LSECtin and CD81. (**a**) Exogenous LSECtin interaction with exogenous CD81. Co-IP assays were performed as described in Fig. 1. IgG LC, IgG light chain. (**b**) Exogenous LSECtin interaction with endogenous CD81 in HEK293T cells. Co-IP assays were performed with anti-CD81 or isotope IgG. (**c**) Adhesion of Fc-LSECtin or control-Fc protein with CD81-expressing cells

To further confirm the binding of E2 with LSECtin, HEK293T cells were transiently transfected with pFlag-CMV-LSECtin and incubated with E2. The soluble E2 glycoprotein was found to bind efficiently with the HEK293T-LSECtin cells in a dose-dependent manner but not with the empty vector (Fig. 3b and Supplementary Fig. 1B). Pre-incubation with the soluble LSECtin protein resulted in a dramatic decrease in the affinity of HCV E2 to the HEK293T-LSECtin cells (Fig. 3c). The binding of Myc-tagged E2 to the HEK293T-LSECtin cells was also inhibited by the soluble His-tagged LSECtin protein (Fig. 3d). Previous studies have shown that EGTA, chelators of calcium ion, and mannan could block the binding of the virus to DC-SIGN and DC-SIGNR [9–12]. LSECtin can bind to carbohydrates such as mannose, GlcNAc, and fucose [13]; this prompted us to investigate whether the interaction between the E2 glycoprotein and LSECtin was influenced by these agents. The binding of E2 with NIH3T3-LSECtin stable transfectants was efficiently inhibited by EGTA and less
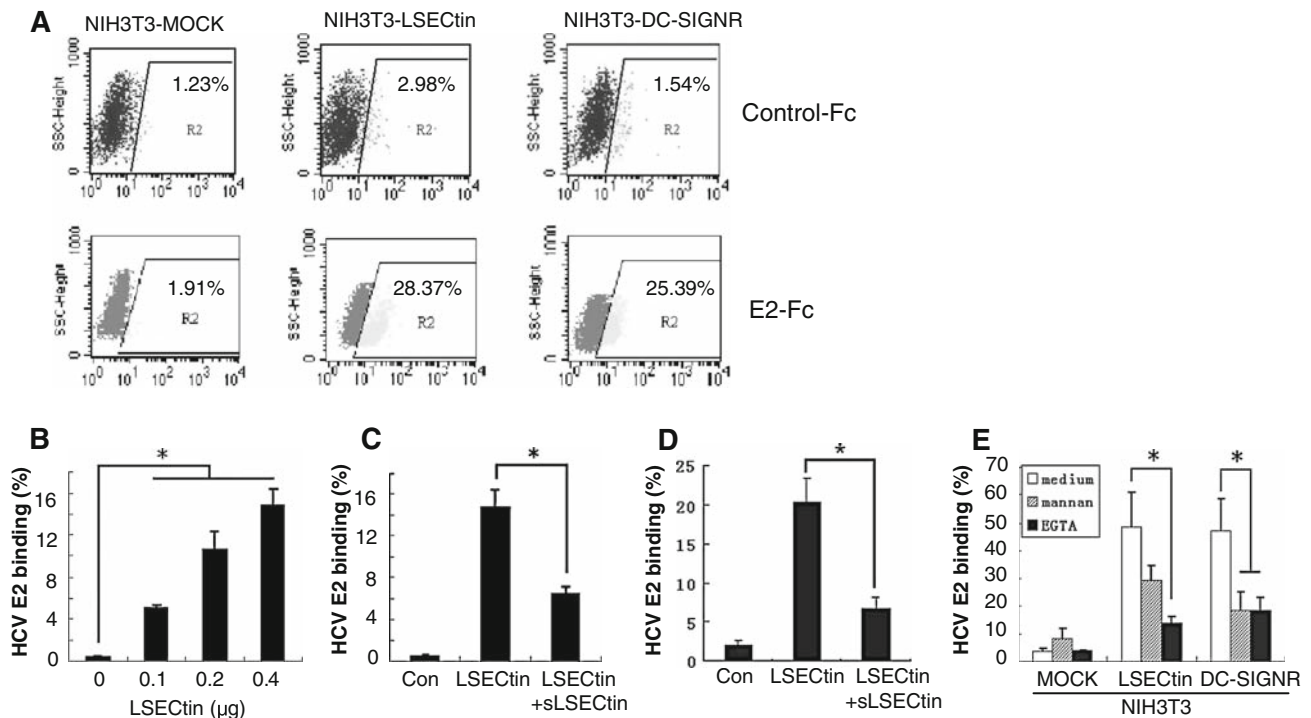
Fig. 3 Soluble HCV E2 glycoprotein binds to LSECtin. (**a**) LSECtin-expressing, DC-SIGNR-expressing, and control NIH3T3 cells were incubated with the soluble HCV E2 glycoprotein. After washing 3 times with PBS, their binding with E2 on the cell surface were quantified by FACS using FITC-coupled anti-Fc antibody. The percentage of cells bound to E2 is indicated. One representative result from the three independent analyses is shown. (**b**) HEK293T cells were transiently transfected with 0, 0.1, 0.2, or 0.4 μg pFlag-CMV-LSECtin plasmid. After 48 h, the ratio of HCV E2 binding to these cells was measured using FACS. (**c**, **d**) For the inhibition assay,

HEK293T cells were transfected with 0.4 μg pFlag-CMV/LSECtin or empty vector. After 48 h, the E2-Fc (**c**) or E2-myc (**d**) glycoprotein was preincubated with the soluble Fc-LSECtin or His-LSECtin protein separately, following which binding assays were performed. (**e**) Inhibition assays with EGTA or mannan preincubation. HEK293T cells were transfected as in (**c**), and the cells were harvested, preincubated with 20 μg/ml mannan or 5 mM EGTA, and then HCV E2 binding assays were performed. The mean values ± SD of the three independent experiments are shown for all assays. Asterisks indicate significant differences

efficiently by mannan (Fig. 3e); the latter was a little different from that in the case of DC-SIGNR. Taken together, these results indicated that the soluble HCV E2 protein could bind to LSECtin in the cell surface.

LSECtin binds to HCV pseudotype particles

To evaluate whether the HCV glycoprotein binds to LSECtin in the context of a viral particle, we tested the binding of the HIV pseudotype particles bearing the HCV E1 and E2 chimeras to the NIH3T3-LSECtin cells. The affinity of the HCV pseudotypes with LSECtin was approximately fivefolds that of the control pseudotype particles alone (Fig. 4a, middle). As a positive control, the binding of HCV pseudotypes to DC-SIGNR-expressing cells was eightfolds than the control pseudotype particles (right). Interestingly, LSECtin adherence could be blocked by EGTA but not by mannan whereas DC-SIGNR adherence could be blocked by both (Fig. 4a), indicating their specificity in carbohydrate binding.

Previous studies showed that DC-SIGN and DC-SIGNR augment infection of various viruses such as HIV, HCV, ebola virus, CMV in *trans*-infection form, suggesting that they capture viruses and then transfer them to nearby other target cells rather than mediate virus entry into the cells where they are expressed [reviewed in ref. 9]. Similar to DC-SIGNR, LSECtin is expressed on liver sinusoidal endothelial cells, which are not the host cells in HCV infection. So we speculated whether LSECtin could capture viruses and had any effects on HCV infection in a manner similar to that of DC-SIGN and DC-SIGNR. NIH3T3 transfectants and Hep3B hepatocellular carcinoma cells (positive control) were infected with the HCV pseudotype particles and the luciferase activities were measured 48 h post-infection. Hep3B cells are highly permissive to HCV pseudovirus entry. By contrast, the infection of the NIH3T3-mock, NIH3T3-DC-SIGNR, and NIH3T3-LSECtin cells resulted in only low levels of luciferase activities, indicating that neither DC-SIGNR nor LSECtin facilitated HCV infection of NIH3T3 cells (Fig. 4b).
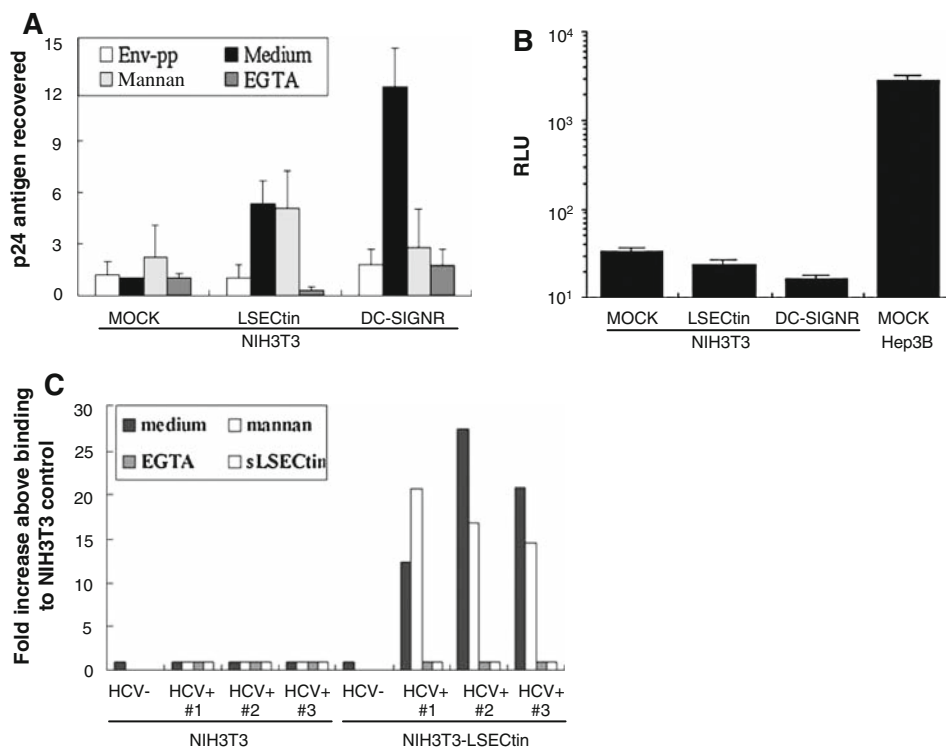
Fig. 4 HCV pseudotype particles and HCV virions in patients' sera bind to LSECtin. (**a**) The indicated cells were incubated for 3 h with the envelope-deficient HCV viral particle or HCV pseudotype particles containing 1 ng normalized p24 antigen. After washing and lysis in 1% Triton X-100, the amount of bound p24 antigen was measured using ELISA. The data are shown as the percentage of the recovered antigen. (**b**) NIH3T3 cells and Hep3B cells were infected with HCV pseudoviruses. Luciferase activities (relative light units, RLU) were measured in the cell lysates at 48 h postinfection. (**c**) NIH3T3 cells were incubated with the sera from 3 HCV-infected patients for 2 h, washed three times, and then lysed. The HCV RNA was extracted and measured using real-time qPCR assay. The y-axis represents the log-fold increases above NIH3T3 cell binding in the graph

LSECtin binds to HCV virions

To detect and confirm the binding of the HCV virions present in the HCV-positive sera to LSECtin, we performed the virus-binding assay with patient serum. NIH3T3-LSECtin cells were bound to the HCV virions in all the three high-titer HCV-positive sera (above 10^5 copies), and the binding levels of LSECtin ranged from 10 to 30-folds that of the control cells (Fig. 4c). The results suggested that LSECtin could interact with HCV virions in the HCV-positive sera. To investigate the binding specificity, we performed the inhibition experiments. The binding effects of NIH3T3-LSECtin cells to HCV virions could be blocked by either soluble LSECtin or EGTA, indicating the binding specificity and the Ca^{2+}-dependence (Fig. 4c). Consistently with the conclusion of the soluble HCV-E2 binding assay (Fig. 3), LSECtin binding to HCV virions could not be inhibited by mannan (Fig. 4c), suggesting the similarity but not identity of LSECtin with DC-SIGNR in the HCV adhesion.

Discussion

Our present study suggests that LSECtin, a recently identified C-type lectin, could interact with DC-SIGNR and CD81 and was involved in HCV binding. The coimmunoprecipitation assays and the soluble LSECtin adherence assays showed the interaction of LSECtin with DC-SIGNR and CD81. Moreover, liver sinusoidal endothelial cells (LSECs) serve as an active barrier between circulating blood and hepatocytes. During HCV infection in the liver, LSECs capture the circulating HCV particles, transmit the captured particles to the endothelial cells, and present them to the entry-permissive hepatocytes. The adherence of CD81-expressing cells to soluble LSECtin also implicated a potential interaction between CD81 on hepatocytes and LSECtin on LSECs, which may be involved in the transmission of the HCV virions to hepatocytes. This possibility should be further investigated in the future.

Mapping analysis showed that the central ectodomain of LSECtin was both sufficient and necessary for interaction with DC-SIGNR and CD81. Deletion of the coiled-coil

region which might mediate the homo-tetramerization or hetero-oligomerization completely abolished the binding of LSECtin to DC-SIGNR (Fig. 1d). In contrast, neither the N-terminal endocytic motif nor C-terminal CRD domain was required for the interaction. Our further investigations of LSECtin binding to HCV E2 glycoprotein suggested that both the ectodomain and the CRD domain are important for HCV binding, due to the fact that preincubation with the soluble ectodomain of LSECtin protein or EGTA, the Ca^{2+} binding chelator, could significantly blocked the LSECtin-HCV binding (Figs. 3c, d, e, and 4a, c). These results also implicated that the binding of DC-SIGNR with the ectodomain of LSECtin might contribute to the capacity of LSECtin for HCV adherence.

Powlesland et al. demonstrated that LSECtin bind very distinct sets of glycan ligands compared with L-SIGN [15]. L-SIGN showed high affinity to high mannose oligosaccharides, but LSECtin bound with high selectivity to glycoproteins terminating in GlcNAcβ1-2Man. We noted that mannan, a mannose polymer obtained from yeast cell wall, could not significantly block the binding of the E2 glycoprotein and HCV pseudotype particles with LSECtin (Fig. 3e, 4a and c), while mannan could compete for binding to L-SIGN. These results suggested that the presence of various types of incompletely matured glycans may facilitate the interaction of enveloped viruses with multiple cell surface receptors. In addition, we observed similar affinities of LSECtin and DC-SIGNR to HCV-E2 in binding assays with soluble E2 proteins (Fig. 3e) but different affinities in the assays with pseudotype particles (Fig. 4a). This might be caused by different conformations of E2 proteins in different contexts or different length of ectodomains of LSECtin and DC-SIGNR. In this aspect, ectodomain length of type 2 C-lectins has been demonstrated critical for pathogen recognition [10, 11, 17], and LSECtin ectodomain is shorter than DC-SIGNR ectodomain [13, 16].

Gramberg et al. previously reported that they could not detect the binding of HCV pseudotype particles to LSECtin expressed cells [14], which is inconsistent with our results. This inconsistency may be from the different affinity of LSECtin to the variants of E2 protein from different HCV isolates. HCV can be classified into six major genotypes and further subdivided into at least 70 subtypes, which exhibit different phenotypic properties. It was reported that a soluble form of E2 protein had a significant genotype-specific difference in CD81 binding assay. In our present study, HCV E1E2 glycoprotein constructs were amplified using the infectious molecular clone H77 as the template. Another possibility is the different sensitivity in detecting the p24 antigen recovery with the different titer of HCV pseudotype particles because the affinity of LSECtin with HCV pseudotype particles was only half of DC-SIGNR.

In summary, the present study suggests that LSECtin interaction with DC-SIGNR might contribute to HCV binding to liver sinusoidal endothelial cells.

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