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Binding of Liver Derived, Low Density Hepatitis C Virus to Human Hepatoma Cells

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Abstract

HCV recovered from low density fractions of infected blood is associated with lipid and host apolipoproteins in lipo-viro-particles (LVP). It has been proposed that these particles are capable of binding and entering hepatocytes by viral glycoprotein independent mechanisms utilizing uptake pathways of normal host lipoproteins after binding to cell surface glycosaminoglycans (GAG), the low density lipoprotein receptor (LDL-r) or scavenger receptor B1 (SR-B1). In this study binding to human hepatoma cells of HCV low density RNA containing particles, semi-purified from macerates of infected human liver, is compared with that of normal host low density lipoprotein (LDL). Binding of both LDL and HCV low density RNA containing particles paralleled LDL-r but not SR-B1 expression on the recipient cells. Binding of both particle types was sensitive to suramin at 0°C but less so at 37°C suggesting that they both bind initially to GAG but, at 37°C, are internalized or transferred to a suramin resistant receptor. Suramin resistant uptake of both particles was blocked in the presence of excess LDL or oxidized LDL. However, whilst LDL uptake was blocked by anti-apoB-100, HCV low density RNA uptake was enhanced by anti-apoB100 and further enhanced by a cocktail of anti-apo-B100 and anti-apoE. Pre-incubation of HCV low density RNA containing particles with antibodies to the E2 glycoprotein had little or no effect on uptake. These data indicate that whilst liver derived HCV RNA containing particles are taken up by HepG2 cells by a virus glycoprotein independent mechanism, the mechanism differs from that of LDL uptake.

Keywords

lipo-viro-particles; apolipoprotein; LDL receptor; SR-B1

INTRODUCTION

Persistent infection with hepatitis C virus (HCV) is associated with perturbation of host lipid metabolism and with steatosis which may contribute to progression of fibrosis [Lonardo et al., 2004]. The mechanisms leading to fatty liver are poorly understood although the core protein of HCV has been shown to interact with lipid droplets [Hope and McLauchlan, 2000], to inhibit microsomal triglyceride transfer protein (MTP) and to interfere with secretion of host very low density lipoprotein (VLDL) [Perlemuter et al., 2002]. Liver MTP activity levels correlate with steatosis in HCV infected patients and with HCV RNA load in

HCV genotype 3 infected patients [Mirandola et al., 2006]. A proportion of virus recovered from the blood of infected patients is associated directly with host lipoprotein particles containing apolipoprotein B100 (apoB100) and apolipoprotein E (apoE), the predominant proteins of VLDL [Thomssen et al., 1992, 1993; Nielsen et al., 2006]. These viral RNA/host lipoprotein complexes have been termed lipo-viro-particles (LVP) [Andre et al., 2002]. Recent reports indicate that complexing of the nascent virus and host lipoprotein to form LVP may occur in the endoplasmic reticulum of the hepatoma cells and is necessary for release of virus [Gastaminza et al., 2006; Huang et al., 2007]. Formation of LVP may favor virus infectivity or persistence in the host and may interfere with the normal secretion and/or uptake of host cell lipoproteins and contribute to the pathology of persistent virus infection.

In the blood, LVP are most prevalent early in infection [Watson et al., 1996] and correlate with infectivity for chimpanzees [Hijikata et al., 1993]. In cell culture studies, binding and entry of blood derived particles containing viral RNA correlates with expression of low density lipoprotein receptor (LDL-r) on cells and can be completely inhibited by pre-incubating the cells with anti-LDL-r antibody, normal LDL or VLDL [Agnello et al., 1999] or by treating the virus with anti-apoB and anti-apoE antibodies [Andre et al., 2002]. LDL binding to the LDL-r is preceded by binding to cell surface glycosaminoglycans (GAG) and binding of blood derived LVP is also GAG dependent [Germi et al., 2002]. Serum derived virus/apo-B100 complexes have also been shown to bind to cell surface SR-B1 and enter cells via a mechanism which involves the acidification of endosomes. Uptake of viral RNA could be inhibited by antibodies to apo-B100 but not by anti-viral glycoprotein antibodies capable of neutralizing HCV pseudovirus particles [Maillard et al., 2006]. These studies indicate that interaction of host lipoprotein with cell surface receptors provides a route of binding and entry for lipoprotein associated virus which may be independent of viral glycoproteins and which may render the virus resistant to antiviral antibody mediated neutralization.

A patient S6 was described recently who suffered from common variable immunodeficiency complicated by iatrogenic HCV infection with a rapid course culminating in liver failure and transplantation. The transplantation was unsuccessful and the transplanted liver, S6b, was removed after 6 weeks at which time virus titers in the blood and in liver S6b were unusually high, $10^{7.5}$ international units (IU)/millilitre (ml) and 10^{10} IU/gram (g) respectively, in the absence of detectable antiviral antibody [Fenwick et al., 2006]. Following fractionation on iodixanol density gradients, 73% of the viral RNA in macerates of liver S6b had a density below 1.08 g/ml consistent with the density of LVP in serum [Nielsen et al., 2004, 2006]. The binding of S6b liver derived HCV low density RNA to human hepatoma cells in culture was compared in the present study with that of fluorescently labeled purified host lipoprotein particles to test the hypothesis that cellular uptake of HCV low density RNA is mediated by host lipoprotein receptor/ligand interactions.

MATERIALS AND METHODS

Cell Culture

HepG2 cells (ATCC, Manassas, VA) and Vero cells (Clinical Virology Unit, Royal Victoria Infirmary, Newcastle upon Tyne, UK) were cultured in six well plastic plates (Corning, Schiphol-Rijk, Netherlands) in 5% carbon dioxide at 37 degrees celsius (°C), in growth medium consisting of Eagle's minimum essential medium (containing 1% non-essential amino acids for HepG2 only), 1% 200 millimolar (mM) glutamine, 100 U/ml penicillin, 100 U/ml streptomycin solution (Invitrogen, Paisley, UK) and 10% foetal calf serum (PAA laboratories Ltd, Somerset, UK). Prior to inoculation, cells monolayers were incubated for 3 days in normal growth medium containing 2.5 micrograms (µg)/ml 25-hydroxy-cholesterol

(Sigma, Dorset, UK) or in 10 µg/ml insulin (Sigma) containing growth medium with 8.5% lipoprotein deficient foetal calf serum, (LPDS, prepared by the method of Bierman and Albers [1977]).

Antibodies

Monoclonal and polyclonal antibodies are detailed in Table I. Where appropriate antibody solutions were dialyzed three times against PBS at 4°C, to remove preservative.

Preparation of Low Density HCV RNA From S6b Liver

Patient S6 has previously been described [Fenwick et al., 2000; Nielsen et al., 2004]. 1g fragments of liver, S6b, collected 6 weeks post-transplant, were macerated in the presence of protease inhibitors as described by Nielsen et al. [2004] 2.3 ml liver macerate was mixed with 4.2 ml 60% iodixanol (Axis-Shield, Dorset, UK), 3.5 ml 0.25 M sucrose (BDH, Dorset, UK), 200 microlitres (µl) 1 M Tris hydrochloride (BDH) pH 7.4 and 100 µl 0.5 M ethylenediamine-tetra-acetic acid (EDTA, BDH). A self-forming iodixanol gradient was achieved by centrifugation in a Beckman Ultracentrifuge (Beckman Coulter UK Ltd, Buckinghamshire, UK) for 24 hr at 4°C and 165,480g. Five hundred-microliter layers were collected from the top of the gradient, working downwards. The density of each fraction was measured with a refractometer (Atago, Tokyo, Japan) and those less than 1.066 g/ml were pooled, and concentrated in Vivasciences 6 ml concentrators, with a molecular weight cut-off of 100 kDa, (Fischer, Leicestershire, UK). After concentration, the titer was $1-8 \times 10^6$ IU HCV RNA/µl.

Preparation of Lipoprotein

LDL was prepared from normal human blood by the method of Mackness and Durrington [1992] and VLDL labeled with DiI (Sigma) by the method of Arnold et al. [1992] and adjusted to 0.4 µg/µl. All lipoprotein and DiI-labeled lipoprotein were stored with 10% sucrose (BDH) at -80°C to prevent degradation.

Binding Studies

Cells monolayers were inoculated with 1 ml of 7.5 µg/ml DiI-LDL in growth medium containing 8.5% LPDS or 800 µl of the same medium containing between 3×10^5 and 3×10^7 IU of HCV RNA from S6b liver macerate as appropriate or 8.6×10^6 IU HCV RNA from the serum of patient S6. Inoculum and cells were incubated for 3 hr at 37°C or at 0°C, as specified, in 5% carbon dioxide. DiI-labeled cultures were kept in the dark as described in Stephan and Yurachek [1993]. The cells were washed three times with PBS containing 0.5% bovine serum albumin (BSA) (BDH) or, washed once and incubated for 30 min at 4°C under either 2 ml of 10 mg/ml dextran sulfate (BDH) in PBS with 1 mM EDTA or 2 ml of 10 mg/ml suramin (Sigma) in PBS, before a final wash.

Fluorescence Activated Cell Sorting (FACS) Analysis of Cells Stained With DiI-Labeled Lipoprotein

DiI-LDL labeled cells were detached by incubating at 37°C for 5 min in 1.07 mM EDTA and 0.025% trypsin (Gibco, Paisley, UK), collected in growth medium and filtered through an 11 µm nylon filter (Millipore, Watford, UK). Ten thousand cells per sample were analyzed on FACScan, (Becton Dickinson, Devon, UK), using excitation and emission wavelengths of 488 nanometres (nm) and 550-600 nm respectively. Parallel samples of cells were stained with 1 mg/ml propidium iodide (Sigma) to assess viability and cultures were discarded if viability was <85%.

Real Time RT-PCR

Cells were scraped into 1 ml of 40 mM sodium citrate (BDH) pH 7.0 containing 4 M guanidinium thiocyanate (Sigma), 0.5% sodium-N-lauryl sarcosine (Sigma) and 100 mM 2-mercaptoethanol (BDH). They were homogenized using a QIAshredder (Qiagen, Sussex, UK), overlaid onto 4 ml of 5.7 M cesium chloride (Sigma) and centrifuged at 146,000g at 18°C for 18 hr in a Ti50 rotor in a Beckman L7 ultracentrifuge (Beckman Coulter UK Ltd, Buckinghamshire, UK). The pellet was resuspended in 350 µl of lysis buffer from an RNeasy mini kit (Qiagen) and RNA was further purified using the mini kit. RT-PCR assay was carried out using primers and probe annealing between positions 120 and 290 in the 5' non-coding region of the HCV 1a genome as described previously [Nielsen et al., 2006]. The assay was calibrated against World Health Organisation international standard for HCV 96/790 (National Institute of Biological Standards and Controls, Hertfordshire, UK).

Labeling of Cells With Antibody and FACS Analysis

HepG2 cells treated with either 25-hydroxy cholesterol or insulin were stained with antibody against scavenger receptor B1 (SR-B1) or the LDL receptor. Briefly, treated cells were washed with PBS, then washed with and incubated for 20 min at 37°C under 1.07 mM EDTA in PBS. The cells were resuspended in growth medium. An equal volume of FACS rinse buffer (1% BSA and 0.1% sodium azide in PBS) was added and the suspension was filtered using an 11 µm filter (Millipore). The filtered cells were collected by centrifugation at 405g for 5 min at 4°C in a Chillspin centrifuge (Jouan, Waltham, MA) and fixed for 30 min at room temperature with an equal volume of 4% paraformaldehyde (Sigma). The fixed cells were collected by centrifugation, washed twice in PBS, and permeabilized by incubating for 20 min at room temperature in an equal volume of 0.1% saponin (Sigma) in PBS, recovered by centrifugation as before and resuspended in FACS diluent (FACS rinse buffer with 5% swine serum: Sigma) to give 2×10^5 cells/25 µl. Equal volumes of SR-B1 rabbit polyclonal antibody, anti-LDL-r rabbit polyclonal antibody, or normal rabbit immunoglobulin G (IgG) control antibody, diluted in FACS diluent with 0.1% saponin (Sigma), were added to fixed cell suspensions and mixed for 30 sec on an Easiashaker (Medgenix Diagnostics, Brussels, Belgium) before incubation at 4°C for 1 hr. Cells were rinsed once with 200 µl of FACS diluent and twice with FACS rinse buffer. Fifty microliters of 1/50 swine anti-rabbit IgG fluorescein isothiocyanate (DAKO, Cambridgeshire, UK) was added to each well and incubated for 1 hr at 4°C. The cells were rinsed as before, resuspended in 300 µl FACS rinse buffer and analyzed on the FACScan as above.

Confocal Microscopy

DiI-labeled cells were incubated overnight at 37°C, in growth medium and wrapped in foil for confocal microscopy the following day. Cells were analyzed on a Leica TCS SP2 UV CLSM microscope (Leica Microsystems, Heidelberg, Germany). Leica Confocal Software Lite was used to analyze the images.

RESULTS

Binding of HCV Low Density RNA to HepG2 Cells

HepG2 cells were cultured in LPDS medium with insulin, to raise, or medium containing normal foetal calf serum and 25-hydroxy-cholesterol to lower LDL-r expression. Cells treated with lipoprotein deficient serum plus insulin (LPDS/insulin) or foetal calf serum plus 25-hydroxyl-cholesterol (FCS/hydroxy-cholesterol) were incubated for 3 hr at 37°C with low density S6b liver macerate containing 3×10^6 IU HCV RNA. After binding, cells were washed three times with PBS plus 0.5% BSA, extracted for RNA and cell associated HCV RNA was estimated by quantitative RT-PCR. Culture of cells in FCS/hydroxy-cholesterol

significantly reduced binding of HCV low density RNA threefold ($P<0.01$) as compared to cells cultured in LPDS/insulin. In parallel experiments a similar reduction in DiI labeled LDL (DiI-LDL) binding, measured by flow cytometry, was observed (2.2-fold, $P=0.01$) (Table II).

Rhainds et al. [2003] have reported that binding of LDL to HepG2 cells occurs via both the LDL receptor and SR-B1. To assess whether these receptors were regulated differentially by insulin and 25-hydroxyl-cholesterol, treated cells were stained with 10 $\mu\text{g/ml}$ of anti-LDL receptor or 15 $\mu\text{g/ml}$ of anti-SR-B1 and analyzed by flow cytometry. The difference in expression of LDL-r was 2.74-fold ($P<0.01$) whilst that for SR-B1, was 1.15-fold (Fig. 1). Thus low density HCV RNA containing particles and DiI-LDL binding more closely paralleled LDL-r rather than SR-B1 expression.

Estimation of the Proportion of HCV Low Density RNA Containing Particles Bound to Cell Surface Glycosaminoglycans

In order to estimate labeled lipoprotein internalized following binding, material bound to the external surface of cells via both GAG and LDL-r, is removed with dextran sulfate [Al Rayyes and Floren, 1998]. Dextran sulfate is a potent inhibitor of PCR and so was unsuitable for the removal of surface bound HCV low density RNA containing particles in these experiments. Confocal microscopic analysis demonstrated that the polyanion suramin was also efficient at stripping cell surface DiI-LDL (data not shown) and, unlike dextran sulfate, suramin is compatible with subsequent RT-PCR [Garson et al., 1999] and could thus be used to wash GAG and LDL-r bound low density HCV RNA containing particles from the cell surface. To investigate how efficient suramin is at removing cell surface bound low density HCV RNA, the effects of a suramin wash on low density HCV RNA and DiI-LDL binding to insulin/LPDS treated HepG2 cells was determined both at 37°C and at 0°C, at which temperature membrane fluidity is decreased, blocking internalization of host lipoprotein [Jarvis, 2000]. The HCV RNA recovered was adjusted for recovery of total cell RNA to compensate for any loss of cells during the washing steps and the results are presented in Table III. At 0°C only traces of DiI-LDL was found associated with the cells after a suramin wash and HCV low density RNA bound was only 17% of that taken up at 37°C. At 37°C over 50% of HCV low density RNA were found to be suramin resistant and thus may be internalized. However, it cannot be excluded that the suramin resistant HCV RNA remains on the cell surface and has transferred to a putative suramin resistant receptor.

Barth et al. [2003] have suggested that highly sulfated heparin sulfate may be the principle GAG involved in the initial docking of HCV. Accordingly, binding of HCV low density RNA and DiI-LDL to untreated cells and to cells pre-treated with heparinase I was assessed before and after a suramin wash (Table IV). In all cases suramin removed a somewhat greater proportion of the cell associated material than heparinase 1 and the effects of the two treatments were only partially additive, suggesting that heparan sulfate is not the only GAG involved.

To study increases in virus binding with inoculum concentration, HepG2 cells cultured in LPDS/insulin were incubated with serial dilutions of low density S6b liver macerate containing 3×10^7 IU HCV RNA and incubated for 3 hr at 37°C. Cells were washed either with PBS/BSA alone or with suramin before HCV RNA extraction. Results are shown in Figure 2. Bound HCV RNA was linearly related to input RNA with no sign of saturation. 2.5% to 3.2% of input HCV RNA bound to cells, equivalent to approximately one RNA molecule bound per cell. Suramin resistant uptake of HCV RNA by insulin/LPDS treated HepG2 cells was also linearly related approximately to the input concentration (Fig. 2) and consisted of 1.5-2% of the inoculum. A similar proportion (1.5%) of low density RNA from serum of the same patient was taken up by LPDS/insulin treated cells. However, low density

RNA uptake was not liver specific as 2.2% of liver derived low density HCV RNA was taken up by Vero monkey kidney cells (data not shown).

Inhibition of Suramin Resistant HCV Low Density RNA and DiI-LDL Binding by Excess LDL and Oxidized LDL

Duplicate wells of LPDS/insulin treated HepG2 cells were incubated with purified LDL or oxidized LDL at concentrations of 75-1,000 $\mu\text{g/ml}$ for 15 min at 37°C before addition of DiI-LDL or low density liver macerate containing 1.8×10^7 IU HCV RNA. After incubation for 3 hr at 37°C, cells were washed with suramin and analyzed by FACS or RT-PCR. LDL (Fig. 3A) and oxidized LDL (Fig. 3B) blocked binding of both DiI-LDL and HCV low density RNA in a dose dependent manner achieving in excess of 60% inhibition for both. Inhibition of binding of HCV RNA was somewhat greater with oxidized LDL.

The Effect of Anti-apoB100 and Anti-apoE on DiI-LDL and HCV Low Density RNA Containing Particle Binding

Binding of LDL to LDL-r is mediated by apoB100 and can be inhibited by anti-apoB100 [Milne et al., 1989]. To evaluate the role of apoB100 in HCV low density RNA containing particle uptake by LPDS/insulin treated HepG2 cells, 20 μg of rabbit polyclonal anti-apoB was incubated with 1 ml of 7.5 $\mu\text{g/ml}$ DiI-LDL or 1 ml of low density liver macerate containing 1.5×10^6 IU HCV RNA for 30 min at 37°C. Parallel preparations were treated with 20 μg normal rabbit immunoglobulin as a control. Suramin resistant binding of the antibody treated particles to LPDS/insulin treated HepG2 cells was assessed as previously. Whilst anti-apoB100 antibody blocked binding of DiI-LDL by 99% ($P < 0.01$; Table V), it enhanced binding of HCV low density RNA by 59% ($P < 0.05$). As serum derived LVP contain apoE as well as apoB100 [Nielsen et al., 2006], the effect of anti-apoE on binding of liver derived HCV low density RNA was also assessed. Fifty micrograms of Dako polyclonal rabbit anti-apoE antibody marginally increased binding of low density RNA although this was not statistically significant. Similar results (not shown) were obtained with rabbit polyclonal anti-apoE from a different source (Biogenesis, Dorset, UK) and with monoclonal antibody ID7, which has been shown previously to bind to the receptor binding site of apoE [Weisgraber et al., 1983]. Combinations of anti-apoB100 with anti-apoE also showed an enhancing effect with binding over double that by HCV low density RNA treated with non-immune serum ($P < 0.01$).

Antibody to the Virus E2 Glycoprotein Fails to Inhibit Binding

Nielsen et al. [2006] have demonstrated that a polyclonal antibody against the HCV E2 glycoprotein, B65581G, and a monoclonal antibody with broad cross-reactivity for the hypervariable region 1 of E2, 3C7-C3, are capable of binding to the E2 glycoprotein present in S6b liver and of immunoprecipitating HCV RNA from the low density fractions of serum. We investigated whether these antibodies can block binding of liver derived HCV low density RNA containing particles to LPDS/insulin treated HepG2 cells. One hundred micrograms of polyclonal B65581G antibody or monoclonal 3C7-C3 were added to 1.5×10^6 IU low density HCV RNA from liver in 1 ml growth medium with 8.5% LPDS, and incubated for 30 min before inoculation onto LPDS plus insulin treated HepG2 cells. Normal goat IgG purified from goat serum (DAKO, Cambridge, UK) and anti-respiratory syncytial virus (2G122) monoclonal antibody were tested in parallel as controls. None of the antibodies tested produced significant effects upon HCV low density RNA binding although marginal decrease in binding was observed with the polyclonal anti-E2 antibody (Table V).

DISCUSSION

Approximately 2% of low density HCV RNA derived from the low density fractions of infected liver was taken up by both human liver cells, HepG2, and primate kidney cells, Vero, a percentage which approximated closely the proportion of serum derived HCV RNA taken up in similar assays. The saturability of binding is a key issue in differentiating “non-specific” adherence of the LVP as, for example due to charge differences, from “specific” binding to a definable receptor. It was not possible to saturate HepG2 cells with HCV RNA at levels over 200 times higher than required by Germi et al. [2002] to saturate Vero cells with plasma derived virus. However, the total virus bound with the highest concentration of HCV RNA we could muster corresponds to only one HCV RNA copy per cell and such low levels of binding could not be expected to saturate a cell surface receptor. The saturability of HepG2 cells with liver derived low density HCV RNA containing particles therefore remains untested. The ready saturation of receptors with virus from plasma may reflect the presence of other factors in the serum absent in the partially purified low density fractions of liver macerate utilized here.

Binding and entry of cell culture derived HCV involves interaction of the virus with SR-B1 and subsequently with CD81 and then claudin-1 [Cocquerel et al., 2006; Evans et al., 2007] whereas in vivo derived lipo-viro-particles (LVP) derived from serum of infected patients appear to bind and enter cells via a CD81 independent interaction of host apo-lipoprotein in the virus complex with cell surface LDL-r or SR-B1 [Agnello et al., 1999; Monazahian et al., 2000; Wunschman et al., 2000; Andre et al., 2002; Maillard et al., 2006; Molina et al., 2007]. As HepG2 cells do not express CD81, the uptake of liver derived low density RNA containing particles studied here must be CD81, and thus probably claudin-1, independent. Uptake was elevated in cells cultured in lipoprotein deficient serum and insulin suggesting that LDL-r, which, unlike SRB-1, was also elevated, is the major receptor for liver derived low density HCV particles and that, if SRB-1 is involved, it must be in association with other, insulin sensitive factors.

Germi et al. [2002] were able to show that binding of serum derived HCV to Vero cells could be inhibited 30% by heparinase I, which degrades highly sulfated domains in heparan sulfate, implicating cell surface glycosaminoglycans in the initial stages of virus attachment. In the present study, after incubation of the liver derived low density HCV on the cells at 37°C for 3 hr, heparinase 1 pretreatment of cells inhibited an equivalent amount of uptake. However, further reduction of liver derived virus bound to heparinase treated cells by post-attachment washing with suramin suggesting that other species of GAG may also be involved. Although a similar pattern of results was exhibited by DiI-LDL, the reduction in uptake of liver low density HCV RNA by both heparinase and suramin was greater. This suggests that heparin sulfate may play a larger role in the attachment of the HCV particles. As DiI-LDL bound to cells at 0°C was almost completely removed by suramin, this result also suggests that, at 37°C, a greater proportion of LDL than HCV is internalized.

The ability of excess LDL to block binding of HCV has previously been taken as evidence of uptake via the LDL-r [Agnello et al., 1999; Germi et al., 2002]. However, Rhainds et al. [2003] have shown that LDL-r interactions account for only a minority of the uptake of LDL by HepG2 cells and that SR-B1 mediated interactions also play a significant role and Maillard et al. [2006] reported that VLDL and to a lesser extent LDL block uptake of serum derived virus via SR-B1. In this study it was observed that, whilst excess LDL was able to block both DiI-labeled LDL and low density HCV RNA binding, oxidized LDL was too, and produced a somewhat greater inhibition of HCV binding than LDL. As ApoB-100 is fragmented in oxidized LDL and LDL-r does not bind oxidized LDL [Viita et al., 1999] these results suggest that mechanisms other than apo-B/LDL-r interactions mediate the

binding of low density HCV RNA in this system. This conclusion is reinforced by our observation that liver derived low density HCV RNA binding was enhanced, not blocked, by pre-incubation with anti-apolipoprotein. These results contrast markedly with those of Maillard et al. [2006] who found that binding of blood derived virus to SR-B1 was blocked by anti-beta-lipoprotein antibodies and also to those of Agnello et al. [1999] and Andre et al. [2002] who have reported that, whilst anti-apoB100 and anti-apoE are insufficient alone to block serum derived virus binding to the LDL-r, together they reduce binding by over 65%.

Both LDL and Ox-LDL bind to SR-B1 but to conclude that the >60% reduction of binding achieved by incubation with these host lipoproteins is due to blockage of SR-B1 mediated binding is inconsistent with our prior observation that the elevated binding to LPDS/insulin treated cells did not correlate with a commensurate increase in SR-B1 expression. This suggests that LDL and Ox-LDL inhibit binding of liver derived low density RNA containing particles by a mechanism which is independent of their ability to bind either LDL-r or SR-B1.

Finally, whereas Germei et al. [2002] were able to block binding of serum derived virus with a monoclonal antibody to the E2 glycoprotein, we were unable to block binding of liver derived virus with either a polyclonal antiserum to E2 or a monoclonal antibody to the hypervariable region, both of which have been shown capable of binding to HCV from this liver by immunoprecipitation [Nielsen et al., 2006]. Although we cannot exclude the possibility that some epitopes on the E2 glycoprotein or other viral surface proteins are involved in binding, the findings, are consistent with liver derived HCV binding to HepG2 cells via host cell receptor ligand interactions in a viral glycoprotein independent manner.

Together these data indicate that liver derived low density HCV is taken up by HepG2 cells by an insulin sensitive host cell receptor/ligand mechanism which has not previously been described. Uptake is apparently unaffected by antibodies to the E2 glycoprotein of the virus and may play a role in persistence of the virus in the presence of anti-viral antibodies in vivo.

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REFERENCES

- Agnello V, Abel G, Elfahal M, Knight G, Zhang Q-X. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. *Proc Natl Acad Sci.* 1999; 96:12766–12771. [PubMed: 10535997]
- Al Rayyes O, Floren CH. Troglitazone upregulates LDL receptor activity in HepG2 cells. *Diabetes.* 1998; 47:1193–1198. [PubMed: 9703316]
- Andre P, Komurian-Pradel F, Deforges S, Perret M, Berland J, Sodoyer M, Pol S, Brechot C, Paranhos-Baccala G, Lotteau V. Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. *J Virol.* 2002; 14:6919–6928. [PubMed: 12072493]
- Arnold, K.; Innerarity, T.; Pitas, R.; Mahley, R. Lipoprotein-receptor interactions. In: Converse, C.; Skinner, E., editors. *Lipoprotein analysis: A practical approach.* Oxford: Oxford University Press; 1992. p. 165-166.
- Barth H, Schafer C, Adah MI, Zhang F, Linhardt RJ, Toyoda H, Kinoshita-Toyoda A, Toida T, Van Kuppevelt TH, Depla E, Von Weizsacker F, Blum HE, Baumert TF. Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. *J Biol Chem.* 2003; 278:41003–41012. [PubMed: 12867431]
- Bierman EL, Albers J. Regulation of low density lipoprotein receptor activity by cultured human arterial smooth muscle cells. *Biochim Biophys Acta.* 1977; 488:152–160. [PubMed: 196655]

- Cocquerel L, Voisset C, Dubuisson J. Hepatitis C virus entry: Potential receptors and their biological functions. *J Gen Virol*. 2006; 87:1075–1084. [PubMed: 16603507]
- Evans MJ, von Hahn T, Tscherne DM, Syder AJ, Panis M, Wolk B, Hatzioannou T, McKeating JA, Bieniasz PD, Rice CM. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature*. 2007; 446:801–805. [PubMed: 17325668]
- Fenwick F, Agarwal K, Bevitt D, Burt A, Hubscher S, Pumeechockchai W, Bassendine M, Toms G. Immunohistochemical detection of HCV antigen in paraffin embedded sections of human liver from immunosuppressed patients but not from immunocompetent patients. Australia: Gold Coast; 2000. p. 231
- Fenwick F, Bassendine MF, Agarwal K, Bevitt D, Pumeechockchai W, Burt AD, Toms GL. Immunohistochemical assessment of hepatitis C virus antigen in cholestatic hepatitis after liver transplantation. *J Clin Pathol*. 2006; 59:174–178. [PubMed: 16443734]
- Garson J, Lubach D, Passa J, Whitby K, Grant P. Suramin blocks hepatitis C binding to human hepatoma cells in vitro. *J Med Virol*. 1999; 57:238–242. [PubMed: 10022794]
- Gastaminza P, Kapadia S, Wood M, Chisari FV. Intracellular hepatitis C virus particles are infectious and secreted by the lipoprotein export machinery of the cell. In: Gowans, E.; Beard, MR.; McCaughan, G., editors. 13th International Meeting on Hepatitis C Virus and Related Viruses; Queensland, Australia: Cairns; 2006. p. 7727–31 August 2006. Abstract 317
- Germi R, Crance J-M, Garin D, Guimet J, Lortat-Jacob H, Ruigrok R, Zarski J-P, Drouet E. Cellular glycosaminoglycans and low density lipoprotein receptor are involved in hepatitis C virus adsorption. *J Med Virol*. 2002; 68:206–215. [PubMed: 12210409]
- Hijikata M, Shimizu Y, Kato H, Iwamoto A, Shih J, Alter H, Purcell R, Yoshikura H. Equilibrium centrifugation studies of hepatitis C virus: Evidence for circulating immune complexes. *J Virol*. 1993; 67:1953–1958. [PubMed: 8383220]
- Hope R, McLauchlan J. Sequence motifs required for lipid droplet association and protein stability are unique to the hepatitis C virus core protein. *J Gen Virol*. 2000; 81:1913–1925. [PubMed: 10900028]
- Huang H, Sun F, Owen D, Weiping L, Chen Y, Gale M, Ye J. Hepatitis C virus production by human hepatocytes dependent on assembly and secretion of very low-density lipoproteins. *Proc Natl Acad Sci USA*. 2007; 104:5848–5853. [PubMed: 17376867]
- Jarvis, SM. Assay of membrane transport in cells and membrane vesicles. In: Baldwin, SA., editor. *Membrane transport*. Oxford: Oxford university press; 2000.
- Lonardo A, Adinolfi LE, Loria P, Carulli N, Ruggiero G, Day CP. Steatosis and hepatitis C virus: Mechanisms and significance for hepatic and extrahepatic disease. *Gastroenterology*. 2004; 126:586–597. [PubMed: 14762795]
- Mackness, M.; Durrington, P. Lipoprotein separation and analysis for clinical studies. In: Converse, C.; Skinner, E., editors. *Lipoprotein analysis—A practical approach*. Oxford: Oxford University Press; 1992. p. 1–42.
- Maillard P, Huby T, Andreo U, Moreau M, Chapman J, Budkowska A. The interaction of natural hepatitis C virus with human scavenger receptor SR-BI/Cla1 is mediated by ApoB-containing lipoproteins. *FASEB J*. 2006; 20:735–737. [PubMed: 16476701]
- Milne R, Theolis R Jr, Maurice R, Pease RJ, Weech PK, Rassart E, Fruchart JC, Scott J, Marcel YL. The use of monoclonal antibodies to localize the low density lipoprotein receptor-binding domain of apolipoprotein B. *J Biol Chem*. 1989; 264:19754–19760. [PubMed: 2479639]
- Mirandola S, Realdon S, Iqbal J, Gerotto M, Dal Pero F, Bortoletto G, Marcolongo M, Vario A, Datz C, Hussain MM, Alberti A. Liver microsomal triglyceride transfer protein is involved in hepatitis C liver steatosis. *Gastroenterology*. 2006; 130:1661–1669. [PubMed: 16697730]
- Molina S, Castet V, Fournier-Wirth C, Pichard-Garcia L, Avner R, Harats D, Roitelman J, Barbaras R, Graber P, Ghersa P, Smolarsky M, Funaro A, Malavasi F, Larrey D, Coste J, Fabre JM, Sa-Cunha A, Maurel P. The low-density lipoprotein receptor plays a role in the infection of primary human hepatocytes by hepatitis C virus. *J Hepatol*. 2007; 46:411–419. [PubMed: 17156886]
- Monazahian M, Kippenberger S, Muller A, Seitz H, Bohme I, Grethe S, Thomssen R. Binding of human lipoproteins (low, very low, high density lipoproteins) to recombinant envelope proteins of hepatitis C virus. *Med Microbiol Immunol*. 2000; 188:177–184. [PubMed: 10917154]

- Nielsen SU, Bassendine MF, Burt AD, Bevitt DJ, Toms GL. Characterization of the genome and structural proteins of hepatitis C virus resolved from infected human liver. *J Gen Virol.* 2004; 85:1497–1507. [PubMed: 15166434]
- Nielsen SU, Bassendine MF, Burt AD, Martin C, Pumeechockchai W, Toms GL. Association between hepatitis C virus and verylow-density lipoprotein (VLDL)/LDL analyzed in iodixanol density gradients. *J Virol.* 2006; 80:2418–2428. [PubMed: 16474148]
- Perlemuter G, Sabile A, Letteron P, Vona G, Topilco A, Chretien Y, Koike K, Pessayre D, Chapman J, Barba G, Brechot C. Hepatitis C virus core protein inhibits microsomal triglyceride transfer protein activity and very low density lipoprotein secretion: A model of viral-related steatosis. *FASEB J.* 2002; 16:185–194. [PubMed: 11818366]
- Rhainds D, Brodeur M, Lapointe J, Charpentier D, Falstraalt L, Brissette L. The role of human and mouse hepatic scavenger receptor class B type I (SR-BI) in the selective uptake of low-density lipoprotein-cholesteryl esters. *Biochemistry.* 2003; 42:7527–7538. [PubMed: 12809509]
- Stephan ZF, Yurachek EC. Rapid fluorometric assay of LDL receptor activity by DiI-labeled LDL. *J Lipid Res.* 1993; 34:325–330. [PubMed: 8381454]
- Thomssen R, Bonk S, Propfe C, Heermann K, Kochel H, Uy A. Association of hepatitis C virus in human sera with beta-lipoprotein. *Med Microbiol Immunol (Berl).* 1992; 181:293–300. [PubMed: 1335546]
- Thomssen R, Bonk S, Thiele A. Density heterogeneity of hepatitis C virus in human sera due to binding of beta-lipoproteins and immunoglobulins. *Med Microbiol Immunol.* 1993; 182:329–334. [PubMed: 8121333]
- Viita H, Narvanen O, Yla-Herttuala S. Different apolipoprotein B breakdown patterns in models of oxidized low density lipoprotein. *Life Sci.* 1999; 65:783–793. [PubMed: 10466744]
- Watson J, Bevitt D, Spickett G, Toms G, Bassendine M. Hepatitis C virus density heterogeneity and viral titre in acute and chronic infection: A comparison of immunodeficient and immunocompetent patients. *J Hepatol.* 1996; 25:599–607. [PubMed: 8938533]
- Weisgraber KH, Innerarity TL, Harder KJ, Mahley RW, Milne RW, Marcel YL, Sparrow JT. The receptor-binding domain of human apolipoprotein E. Monoclonal antibody inhibition of binding. *J Biol Chem.* 1983; 258:12348–12354. [PubMed: 6313653]
- Wunschman S, Medh J, Klinzmann D, Schmidt W, Stapleton J. Characterization of hepatitis C virus (HCV) and HCV E2 interactions with CD81 and low-density lipoprotein receptor. *J Virol.* 2000; 74:10055–10062. [PubMed: 11024134]

Fig. 1.

The effect of LPDS/insulin and FCS/hydroxyl-cholesterol on HepG2 cells. Staining of cells with rabbit anti-LDL-r antiserum ■, rabbit anti-SR-B1 antiserum ◐ and normal rabbit serum ◻ assessed by flow cytometry. Means and standard deviations were calculated from the results of six replicates in each case. LPDS, lipoprotein deficient serum; FCS, foetal calf serum; LDL-r, low density lipoprotein receptor; SR-B1, scavenger receptor B1.

Fig. 2.
Dose dependence of the binding of HCV RNA in the low density fractions of macerates of liver S6b to HepG2 cells cultured in lipoprotein deficient serum and insulin. ●● without suramin wash; ○—○ with suramin wash.

Fig. 3.

The inhibition of DiI-LDL and HCV RNA uptake by HepG2 cells in the presence of (A) purified LDL or (B) oxidized LDL (●—● mean fluorescence intensity (DiI-LDL binding) (○—○) HCV RNA (IU) per μg host RNA recovered. DiI-LDL, dioctadecylindocarbocyanine-low density lipoprotein; LDL, low density lipoprotein.

TABLE I

Monoclonal and Polyclonal Antibodies

Antibody	Species	Isotype	Source
Anti-F RSV 2G122	Mouse	IgG1	Novacastra Labs. (Newcastle upon Tyne, UK)
Anti-human apoB100	Rabbit	IgG polyclonal	DAKO (Cambridgeshire, UK)
Anti-human apoE	Rabbit	IgG polyclonal	DAKO (Cambridgeshire, UK)
Anti-human apoE	Rabbit	IgG polyclonal	Biogenesis (Dorset, UK)
Anti-LDL-r	Rabbit	IgG polyclonal	Progen Biotechnik (Heidelberg, Germany)
Anti-rabbit SR-B1	Rabbit	IgG polyclonal	Novus Biologicals (Littleton, CO)
Normal rabbit IgG	Rabbit	IgG polyclonal	DAKO (Cambridgeshire, UK)
ID7 monoclonal to human apoE	Mouse	IgG1	Ottawa Heart Institute, Canada
Anti-HCV glycoprotein E2 B65581G	Goat	IgG polyclonal	Biodesign (Saco, ME)
Anti-HCV glycoprotein E2 hypervariable region 1 clone 3C7-C3	Mouse	IgG1	Prof. Mario Mondelli, University of Pavia, Italy

RSV, respiratory syncytial virus; apoB100, apolipoprotein B100; apoE, apolipoprotein E; apoA1, apolipoprotein A1; LDL-r, low density lipoprotein receptor; SR-B1, scavenger receptor B1; IgG, immunoglobulin G.

TABLE II

DiI-LDL and HCV Low Density RNA Uptake by HepG2 Cells Treated With FCS/Hydroxy-Cholesterol or LPDS/Insulin

	DiI-LDL	HCV low density RNA
FCS/hydroxy-cholesterol	784±49 ^a	30±16 ^b
LPDS/insulin	1,740±68	93±22
Increment	2.2	3.1

DiI-LDL, dioctadecylindocarbocyanine-low density lipoprotein; FCS, foetal calf serum; LPDS, lipoprotein deficient serum.

^aMean fluorescence intensity.

^b10³ IU of HCV RNA.

TABLE III

The Effects of Suramin on LDL and HCV Low Density RNA Taken Up by LPDS/Insulin Treated HepG2 Cells

Temperature	Suramin wash	LDL ^a	HCV low density RNA ^b
37°C	-	n.d.	6,919±326
	+	2,335±246	4,482±189 ^b
0°C	-	n.d.	2,507±929
	+	15.4±1.2	744±134

LDL, low density lipoprotein.

^aMean fluorescence intensity.

^bIU HCV RNA per µg host RNA recovered.

TABLE IV

Inhibition of Host Lipoproteins or HCV Low Density RNA Binding to LPDS/Insulin Treated HepG2 Cells by Pre-Incubation of Cells With Heparinase and/or a Post-Binding Wash With Suramin

Treatment	Percentage reduction in binding ^a	
	DiI-LDL	HCV low density RNA
Heparinase	14.2	30.5
Suramin	19.4	44.3
Suramin + heparinase	39.8	58.3

DiI-LDL, dioctadecylindocarbocyanine.

^aValues were calculated from means taken from at least two cell cultures analyzed for DiI fluorescence by FACS or six cultures analyzed for HCV RNA in real-time RT-PCR. In no case did the standard deviations of the means exceed 23%.

TABLE V

Effect of Anti-Apolipoprotein and Anti-HCV E2 Antibodies on Binding of LDL and HCV Low Density RNA to LPDS/Insulin Treated HepG2 Cells

Antibody treatment	Dil-LDL		HCV low density RNA		P ^c
	% increment in binding ^a		% increment in binding ^b		
Anti apo-B100	-99		+59		<0.04
Anti apo-E	n.d.		+7		>0.05
Anti apo-B100 + anti apo-E (Dako)	n.d.		+116		<0.01
Anti-E2 glycoprotein, B65581G	n.d.		-13		>0.05
Anti-E2 Mab, 3C7-C3	n.d.		0		>0.05

Dil-LDL, dioctadecylindocarbocyanine; apo-B100, apolipoprotein B100; apo-E, apolipoprotein E.

^aThe change in percentage of Dil-LDL bound compared to that when cells were incubated with Dil-LDL and 20 µg of normal rabbit serum.

^bThe change in percentage of HCV RNA bound, and P values from student's *t* tests.

^cWhen compared to that when cells were incubated with HCV low density RNA and, as appropriate, normal rabbit immunoglobulin, normal goat immunoglobulin or mouse monoclonal anti-respiratory syncytial antibody 2G122 at equivalent concentration.