

The Low Density Lipoprotein Receptor plays a role in the infection of primary human hepatocytes by hepatitis C virus

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Short title : LDLR and HCV infection of normal hepatocyte

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Word count : 2994 (Introduction, Materials/Methods, Results, Discussion)

Abstract

Background/Aims: The direct implication of low density lipoprotein receptor (LDLR) in hepatitis C virus (HCV) infection of human hepatocyte has not been demonstrated. Normal primary human hepatocytes infected by serum HCV were used to document this point.

Methods: Expression and activity of LDLR were assessed by RT-PCR and LDL entry, in the absence or presence of squalestatin or 25-hydroxycholesterol that up- or down-regulate LDLR expression, respectively. Infection was performed in the absence or presence of LDL, HDL, recombinant soluble LDLR peptides encompassing full-length (r-shLDLR4-292) or truncated (r-shLDLR4-166) LDL-binding domain, monoclonal antibodies against r-shLDLR4-292, squalestatin or 25-hydroxycholesterol. Intracellular amounts of replicative and genomic HCV RNA strands used as end point of infection were assessed by RT-PCR.

Results: r-shLDLR4-292, antibodies against r-shLDLR4-292 and LDL inhibited viral RNA accumulation, irrespective of genotype, viral load or liver donor. Inhibition was greatest when r-shLDLR4-292 was present at the time of inoculation and gradually decreased as the delay between inoculation and r-shLDLR4-292 treatment increased. In hepatocytes pre-treated with squalestatin or 25-hydroxycholesterol before infection, viral RNA accumulation increased or decreased in parallel with LDLR mRNA expression and LDL entry.

Conclusion: LDLR is involved at an early stage in infection of normal human hepatocytes by serum-derived HCV virions.

Word count of abstract : 202, including *headings*

Keywords : virus receptor, virus entry, genome replication

1. Introduction

Hepatitis C virus (HCV) infection represents a serious health problem worldwide (1). HCV is a positive RNA strand enveloped virus classified as a *Hepacivirus* within the *Flaviviridae* family (2, 3). Hepatocytes represent the major site of replication of HCV, although this virus has been shown to infect other cell types (3-5). The mechanism by which HCV binds to and enters hepatocytes is not fully understood. Several *in vitro* models have been used to identify and characterize the HCV receptor, including cell lines infected with either plasma or serum from infected patients or virus-like particles (HCV-LP) expressed in insect systems (6), VSV/HCV pseudotype virus (7, 8), or pseudotyped retroviral particles (HCVpp) (9-11). More recently productive Huh-7-based cellular systems (HCVcc) have been described (12-15). Several receptor candidates have been identified, including CD81 (16), scavenger receptor-BI (17), DC-SIGN and/or L-SIGN (18), asialoglycoprotein receptor (19) and low-density lipoprotein receptor (LDLR) (20-22).

LDLR is a membrane glycoprotein that controls the primary pathway through which cholesterol enters the cells. The N-terminal ligand binding domain spans over residues 1-292 arranged in 7 repeats. Each repeat contains 6 S-S linked cysteines (23, 24). The physiological ligand LDL contains a single copy of apolipoprotein B-100 and carries 65-70% of plasma cholesterol. The receptor also binds apolipoprotein E-containing lipoproteins such as very low-density lipoproteins (β -VLDL). LDLR has been suspected to play a role in HCV infection on the basis of the well documented interaction between HCV and lipoproteins (25-27). Ultracentrifugation of infected plasma on density gradients revealed two compartments in which HCV is abundant. The first one (1.25 g/ml) contains poorly infectious particles linked to immunoglobulins. The second (1.06 g/ml) contains infectious lipovirions that are rich in triglycerides, and apolipoprotein B and E. Further observations supporting a role for LDLR in HCV infection came from analysis of the effects of lovastatin (22), anti-LDL antibodies (8, 20, 21, 28), LDLR genetic deficiency (21, 25, 28) and LDLR ectopic expression (21).

However, all these studies have been carried out using cell lines or virus particles that do not mimic the natural infection. In contrast, primary cultures of highly differentiated human hepatocytes (29-32, 33, 34) are likely to represent the most physiologically relevant model to investigate serum-derived HCV infection. Indeed,

previous studies have shown that human hepatocytes are sensitive to infection by HCV or hepatitis delta virus and permissive to their genome replication (35-37).

The present study was undertaken to determine whether LDLR plays a role in the infection of these cells by HCV. For this purpose, specific tools were developed including recombinant soluble LDLR peptides and monoclonal antibodies. Hepatocytes were infected with HCV-positive sera in the absence or presence of LDLR peptides and anti-LDLR antibodies. Cellular levels of LDLR were modulated with squalestatin or 25-hydroxycholesterol. Based on the analysis of viral genome expression, the results suggest that LDLR plays a role in an early step of infection of human hepatocytes by serum-derived HCV virions.

2. Materials and methods

2.1. Cells

Hepatocytes were isolated from liver lobectomy specimens resected for medical reasons unrelated to our research program, as approved by the National Ethics Committee. Primary cultures of human hepatocytes were prepared from twenty two different patients free of HCV, HBV and HIV markers (Table 1), and cultured as described (31, 34). CHO-K1 and HepG2 cells were cultured in MEM supplemented with 5% fetal calf serum (FCS), 2mM glutamine, 100U/ml penicillin and 100µg/ml streptomycin, and DMEM supplemented with 10% FCS, 100U/ml penicillin and 100µg/ml streptomycin, respectively.

2.2. HCV-positive serum samples

HCV-positive serum samples (free of HBV and HIV markers) used to infect hepatocytes (Table 2) were prepared from blood collected for medical and biological diagnosis on HCV chronic carriers before treatment with interferon-ribavirine, and characterised and stored as described (35).

2.3. In vitro infection of hepatocytes and treatments

The procedure previously described was used here (35, 36). Hepatocytes were pre-treated or not with either: 0.62 to 620 nM of r-shLDLR4-166, r-shLDLR4-292 or u-shLDL4-166, or 2 to 8 µg/ml of mAb12.6, mAb28 and mAb29.8, for thirty minutes before and during infection. In some experiments, hepatocytes were treated with 62 nM r-shLDLR4-292 either at the time of infection (T0) or 0.5, 2 or 8 hours later, or for 24 hours with either 10 to 80 µM squalestatin (SQ, Sigma St Louis MO) or 25-hydroxycholesterol (25-OHC, Sigma).

2.4. Detection of HCV RNA and quantification by real-time PCR

Total cellular RNA was purified and intracellular HCV RNA strands were analyzed by strand-specific rTth/RT-PCR and quantitative RT-PCR as described (35, 36, 38, 39), and normalized to GAPDH RNA. In some experiments, cells were treated with 5 mM suramin for 30 minutes, before harvest and quantification of HCV RNA.

2.5. Expression and purification of soluble LDLR

Purification of u-shLDLR4-166. Purification of soluble LDL receptor from human urine (u-shLDLR4-166) was performed by immunoaffinity, diethyl amino-ethyl anion exchanger, size exclusion chromatography and reverse phase-high performance liquid chromatography.

Expression and purification of r-shLDLR4-166, r-shLDLR4-292 and r-shLDLR44-331. cDNA encoding repeats 1 to 4 of LDLR ligand-binding domain was cloned in pEAK12d vector and transfected in HEK293-EBNA cells. cDNAs encoding the 7 repeats of LDLR ligand-binding domain ending at Cys₂₉₂ or Cys₃₃₁ were cloned in pDHFR vector and transfected into CHO-DUKX cells. Secreted peptides were purified by anion exchange chromatography, copper chelating sepharose chromatography, followed by butyl-TSK HIC chromatography. Identity of peptides was ascertained by SDS-polyacrylamide gel electrophoresis, N-terminal sequencing, amino-acid analysis, ELISA and western blotting.

2.6. Monoclonal antibodies against r-shLDLR4-292

Peptide r-shLDLR4-292 in Complete Freund's Adjuvant was injected into 7-week-old female mice. After boosting successsively with 10 and 50 µg/mouse of immunogen, sera were tested by ELISA. Best responders were selected and hybridoma generated. Monoclonal antibodies (mAbs) generated by clones 12.6, 28 and 29.8 were purified by chromatography on Protein G column and identified as IgG1.

2.7. Expression of LDLR mRNA

One microgram of total RNA was reverse transcribed, using random hexamer primers and MMLV reverse transcriptase (Invitrogen, Carlsbad, CA). cDNA was analysed by real-time PCR, using forward 5'-ACTGGTGTGAGAGGACCACC-3', and reverse 5'-CAAAGGAAGACGAGGAGCAC-3' LDLR primers.

2.8. Preparation of lipoproteins

Lipoproteins were isolated from normolipidemic donor plasmas by ultracentrifugation and ionidated as described (40). More than 97% of radioactivity was associated with protein.

2.9. Treatments of hepatocytes with BODIPY FL LDL

Hepatocytes, pre-treated or not with 10-80 μM SQ or 25-OHC, were exposed to 3-6 $\mu\text{g/ml}$ BODIPY FL LDL (Molecular Probe, L-3483) for 2-48 hours in the absence or presence of a 25-fold excess of unlabeled LDL.

Fluorescence-activated cell sorting. Cell monolayers were washed with PBS and cells were detached with 5 mM EDTA and resuspended in PBS. Cell fluorescence was evaluated by FACScalibur (Becton Dickinson) and analysed with CellQuest software.

Microscopic analysis. Cell monolayers were washed with PBS and fixed for 20 min with 30 mM PIPES, 12.5 mM HEPES, 5 mM EDTA, 5 mM MgCl_2 , 3% formaldehyde, 0.05% glutaraldehyde, pH 6.9. Fixed cells were exposed to 1/1000 diluted Hoescht 33258, mounted in DAKO Fluorescent medium and viewed with LEICA or Zeiss Axioplan microscope.

2.10. Solid phase assay for ^{125}I -lipoprotein binding to r-shLDLR4-331/292/166 and u-shLDLR4-166

96 well polystyrene Stripwell™ plates (Corning) were coated with mAb 29.8 (500ng) in buffer A (50 mM Tris-HCl buffer, pH 8.0, 2 mM CaCl_2). After washing and blocking with buffer A-5% BSA, 50 ng r-shLDLR4-331, r-shLDLR4-292, r-shLDLR4-166 or u-shLDLR4-166 were added and incubated at 4°C overnight. Next, 500 ng/well of ^{125}I -lipoprotein (LDL, VLDL, HDL) were added in absence or presence of 50-fold excess of unlabeled lipoprotein in buffer A-5% BSA. After washing proteins were dissociated and counted (Cobra Auto-Gamma counter Packard). In competition experiments, mAbs 12.6, 28 or 29.8 was added before incubation with ^{125}I -lipoprotein.

2.11. ^{125}I -lipoprotein binding to and entry into CHO, HepG2 cells and hepatocytes

CHO or HepG2 cells in lipoprotein-deficient medium received 10 $\mu\text{g/ml}$ ^{125}I -lipoprotein in absence or presence of 40-fold excess of unlabeled lipoprotein, and r-shLDLR peptides or mAbs. Lipoprotein binding and degradation were determined as described (41). Hepatocytes, pre-treated or not with 10-80 μM SQ or 25-OHC, received 2.5 nM ^{125}I -radiolabelled LDL (3380 cpm/ng) in absence or presence of 25-fold excess of unlabeled lipoprotein. Lipoprotein binding and entry were determined as described (40).

3. Results

In this work, we have used primary cultures of highly differentiated normal human hepatocytes infected with serum-derived HCV to evaluate the role of LDLR in virus entry. This cellular model closely mimics the physiological situation. Indeed, we have shown that these cultures retain several liver phenotypic markers including secretion of plasma proteins (31) and blood coagulation factors (30), expression and inducibility of detoxification enzymes (33, 34, 42, 43), expression of C/EBP transcription factors (32), activation of cytokine signal transmission (29, 35), and are sensitive to infection by HCV or hepatitis delta virus and permissive to their genome replication (35-37). Intracellular accumulation of replicative and genomic HCV RNA strands, assessed by rTth-RT-PCR and quantitative RT-PCR (35, 36, 38, 39), respectively, were used as experimental end-points. Detection of the replicative strand was taken as a proof of viral genome replication. However, our conclusions are based *exclusively* on the quantitative analysis of the genomic strand. Washing of cells with suramin (44) before quantification of HCV RNA to eliminate residual cell surface bound HCV prior to analysis revealed that externally bound HCV does not significantly affect intracellular HCV RNA amount (*Fig. Suppl-1*). On average, the number of HCV genome copies detected per hepatocyte is 0.18 but varies widely from culture/serum combination to another (range : 0.1 to 36).

3.1. LDLR expression and activity in primary human hepatocytes

Sterol regulatory element-binding proteins (45) up- or down-regulate LDLR expression depending on cholesterol/sterols intracellular levels. Hepatocytes treated with squalestatin (SQ, inhibitor of squalene-synthase) or 25-hydroxycholesterol (25OHC) exhibited increased or decreased LDLR mRNA levels (*Fig. 1A*), respectively. Saturable binding of LDL corresponding to a single binding site was observed in agreement with others (46) (*Fig. Suppl-2*). Following treatment of hepatocytes (n=9) with SQ or 25OHC, LDL entry (and binding, not shown) exhibited on average 4-fold increase or 2-fold decrease, respectively (*Fig. Suppl-3*). In the presence of a 200-fold molar excess of unlabeled LDL neither entry nor binding of ¹²⁵I-LDL was observed ; similar excess of HDL had no effect. These results were confirmed by microscopy examination of cells exposed to BODIPY-FL LDL. The number and size of fluorescent vesicles increased or decreased in SQ- or 25OHC-treated cells (*Fig. Suppl-4*). Fluorescence activated cell sorting (FACS) analysis

confirmed these results (Fig. 1B). Thus, in cultured hepatocytes, LDLR is functional and regulated as expected by the cholesterologenic status of cells.

3.2. Effect of r-shLDLR peptides and monoclonal and polyclonal antibodies against r-shLDLR4-292

Specific tools were developed to evaluate LDLR implication in HCV infection. These included recombinant soluble LDLR peptides r-shLDLR4-331, r-shLDLR4-292, and r-shLDLR4-166 or urinary u-shLDLR4-166, and monoclonal antibodies (mAb) generated against r-shLDLR4-292: mAb12.6, mAb28 and mAb29.8 (*Fig. Suppl-5*). Both r-shLDLR4-292 and r-shLDLR4-331 equally bound LDL, while r-shLDLR4-166 and u-shLDLR4-166 did not. When compared to LDL, VLDL bound moderately to r-shLDLR331, while HDL did not. HDL entry was not inhibited, while inhibition of VLDL entry was intermediate (*Fig. Suppl-6A-C*). Both r-shLDLR4-292 and r-shLDLR4-331 equally inhibited LDL entry in CHO-K1 cells while r-shLDLR4-166 and u-hLDLR4-166 did not (Fig. 2A).

Competition effect of r-shLDLR4-292, r-shLDLR4-166 and u-shLDLR4-166 was tested during hepatocyte infection. r-shLDLR4-292 markedly inhibited the accumulation of both replicative (*Fig. Suppl-7A*) and genomic (Fig. 2B) RNA strands, while in contrast, r-hsLDLR4-166 or u-hsLDLR4-166 had no effect. Control experiments revealed that GAPDH mRNA was not affected by shLDLR peptides (not shown). Next, cells were treated with r-shLDLR4-292 at the time of infection (T0) or later. When treatment started at the time of infection, replicative (*Fig. Suppl-7B*) and genomic (Fig. 2C) strand levels decreased markedly, while inhibition diminished progressively when treatment was applied later. Similar observations were made, irrespective of the viral genotype. Thus, only peptide r-shLDLR4-292 which binds LDL is a significant inhibitor of an early step of infection, while r-shLDLR4-166 and u-shLDLR4-166 which do not bind LDL, are not inhibitor.

mAb28 exhibited the strongest inhibitory effect on LDL binding to r-shLDLR4-331, followed by mAb12.6 and mAb29.8 (*Fig. Suppl-8*). Consistently, mAb28 strongly inhibited LDL entry in HepG2 cells while inhibition mediated by mAb12.6 and mAb29.8 was weaker (Fig. 3A).

Competition effect of mAb12.6, mAb28 and mAb29.8, and an anti-LDLR polyclonal antibody known to inhibit LDL entry into human fibroblasts (kindly provided by John Chapman, Paris) was tested during hepatocyte infection. mAb28 markedly

inhibited accumulation of both replicative (*Fig. Suppl-9*) and genomic (Fig. 3B) strand (>90%), while mAb12.6 and mAb29.8 produced approximately 66% inhibition at the greater concentration. Interestingly, the inhibitory effect of mAbs on infection paralleled their inhibitory effect on LDL binding to LDLR and cellular entry. The polyclonal anti-LDLR antibody was less potent than mAb28 but produced marked inhibition as well. Mouse IgG-1 and anti-CD29 (integrin- β 1) monoclonal antibody (Immunotech, clone K20), used as controls, had no significant effect (Fig 3C). Thus, antibodies that inhibit LDL binding to LDLR also inhibit HCV infection. Whether the antibodies compete with HCV-lipovirions binding or inhibit receptor internalisation is unknown.

Since LDL is also a high-affinity ligand for SR-BI, it was pertinent to evaluate the effect of r-shLDLR peptides and monoclonal antibodies on SR-BI-mediated entry of HDL into the cells. Neither r-shLDLR292, r-shLDLR166, mAbs (28, 12.6 or 29.8), SQ or 25OHC had significant effect (*Fig. Suppl-10*), arguing in favor of the specificity of their effects.

3.3. Effect of LDL and HDL

Hepatocytes were infected with HCV-positive serum in the absence or presence of 1.5-150 nM LDL; 15-1500 nM HDL2 or HDL3 were used as control since these lipoproteins use a distinct receptor, SR-BI. LDL produced a strong inhibitory effect at 150 nM on both replicative (*Fig. Suppl-11*) and genomic (Fig. 4) strand while HDL3-mediated inhibition was only observed at 1500 nM, and HDL2 had no effect.

3.4. Effect of SQ and 25OHC

Since SQ and 25OHC modulate LDLR expression, we tested whether pretreatment of hepatocytes with these compounds modulate HCV infection. Interestingly, accumulation of both replicative (*Fig. Suppl-12*) and genomic (Fig. 5) strands was increased in cells pretreated with SQ, but declined in cells pretreated with 25OHC. Collectively, these results support the notion that LDLR plays a role in the *in vitro* infection of human hepatocytes by serum-derived HCV virions.

4. Discussion

Our results show that LDLR plays a critical role at an early step of infection. This conclusion is based on the following observations: i) SQ and 25OHC, two compounds that modulate LDLR expression and activity, modulate in parallel HCV infection; ii) LDL is one order of magnitude more efficient than HDL in inhibiting HCV infection; iii) a soluble hLDLR peptide encompassing the entire LDL-binding domain, markedly inhibits HCV infection while shorter peptides, which do not bind LDL, do not; iv) anti-LDLR monoclonal and polyclonal antibodies markedly inhibit HCV infection; v) similar observations were made with hepatocyte cultures prepared from different donors inoculated with different sera, irrespective of HCV genotype. A likely hypothesis is that lipovirions formed by HCV virions bound to the low-density lipoproteins, are recruited at the cell membrane *via* interaction with LDLR (20, 21, 25). Although glycoprotein E2 binds neither LDL nor LDLR (8, 28, 47), the possibility that virus entry results from an interaction between glycoprotein E1 and LDLR (8) cannot be excluded.

The current results corroborate previous observations in which human sera were used as inoculum to infect various cell lines (20-22, 25, 28). However, our results differ from other data generated with HCV-LP or HCVpp. Indeed several reports have questioned the role of LDLR in the cellular entry of HCV. Triyatni et al. (48) observed no inhibition of HCV-LP entry into MOLT4 cells by anti-LDLR antibodies. Bartosch et al. (9, 10) and Hsu et al. (11) observed no inhibition of HCVpp infection of Huh-7 cells by LDL, or by anti-LDLR antibodies, respectively. A likely explanation for these differences is that HCV-LP and HCVpp do not fully mimic the natural virions, especially in terms of association with lipoproteins (25).

LDLR is expressed in many cell types and not just in hepatocytes. Moreover, expressing this receptor ectopically does not restore cell responsiveness to HCV (9-11). This indicates that LDLR is probably not the sole player to mediate HCV entry. Indeed, other receptors, including CD81, SR-BI, the asialoglycoprotein receptor, and DC/L-SIGN have also been implicated in HCV cell entry (16, 17-19). In particular, CD81 and SR-BI are known to interact with HVR1 region of E2, and it has been emphasized that all cells permissive to HCV infection express LDLR, CD81 and SR-BI (9). However, none of these receptors, expressed alone or in combination, define permissiveness to infection (11). The current observation that high concentrations of HDL3 inhibit HCV genome replication in human hepatocytes (Fig. 4) is consistent

with a role for SR-BI in HCV entry into these cells. Taken together, these observations suggest that a network of proteins needs to be present at the cell surface to mediate HCV entry.

LDLR and/or LDLR-related proteins have been reported to promote cellular entry of other viruses including GB virus type C, (20), human rhinovirus (49), avian leukosis and sarcoma viruses (50). In addition, interferon was shown to induce the release of the 292 N-terminal domain of LDLR from human WISH cells, a process apparently protecting cells from infection by vesicular stomatitis virus (51). It seems therefore that LDLR represents a potent, although not exclusive, factor for virus recruitment at the cell membrane. The involvement of LDLR hepatocyte infection by HCV suggests therapeutic strategies based on the use of anti-LDLR antibodies or peptides like r-shLDLR4-292.

In conclusion, the current results, obtained with highly differentiated normal human hepatocytes inoculated with serum-derived HCV virions, support the notion that LDLR plays a role in an early step of virus entry. Other membrane proteins are likely to be responsible for the unique capacity of HCV to bind and enter hepatocytes. Testing the role of these proteins including CD81 and SR-BI is currently under investigation using this primary hepatocyte culture system.

Acknowledgements: We thank Prof J Chapman and Dr T Huby for kindly providing us with the polyclonal antibody to LDLR and for many helpful discussions, Laurent Menoud for the recombinant LDLR protein purification, and Camille Malval for her help in LDL binding experiments. This work was supported in part by grant 01031 from the “Agence Nationale de Recherches sur le SIDA et les hépatites virales” (ANRS) and grant 5869 C from “Association pour la Recherche sur le Cancer” (ARC) to C. F-W. Both V.C and S.M were supported by an ANRS fellowship.

List of abbreviations

LDL:low density lipoprotein;LDLR:low density lipoprotein receptor;VLDL:very low density lipoprotein;HDL:high density lipoprotein;r-shLDLR:recombinant soluble human LDLR;u-shLDLR:urinary soluble human LDLR;SQ: squalestatin;25-OHC:25-hydroxycholesterol.

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Figure legends

Figure 1. LDLR expression and activity in primary human hepatocytes

A. Hepatocytes (FT179) were plated and maintained for 5 days in long-term serum-free culture medium. At day 4, cells were treated with 1 or 10 μ M squalenstatin (SQ), or 5 or 20 μ M 25-hydroxycholesterol (25-OHC) for 24 hours. Cells were then harvested and total RNA was prepared and analysed for LDLR mRNA by quantitative RT-PCR (LightCycler) using specific primers. Data from triplicate experiments are presented. These experiments are representative of other hepatocyte cultures : FT197, FT199 and FT257.

B. At day 1 after plating, hepatocytes (FT223) were treated with 10 μ M SQ or 25OHC for 48 hours. At day 3, this treatment was continued for another 48 hours, in the presence of 3 μ g/ml BODIPY-FL LDL. Cells were then analysed by FASC. From the data presented, SQ increased fluorescence by 25% while 25OHC decreased fluorescence by 27%. On average (n=4) SQ increases fluorescence level by 62.5%, while 25OHC or competing unlabeled LDL decreases fluorescence by 25 and 62.5%, respectively. These experiments are representative of other hepatocyte cultures : FT221, FT224, and FT228.

Figure 2. Effect of r-shLDLR molecules on HCV RNA replication

A. Effect of LDLR soluble forms on LDL entry in CHO cells. The indicated amounts of r-shLDLR4-331, r-shLDLR4-292, r-shLDLR4-166 and u-shLDLR4-166 were incubated for 1 hour at 37°C with 20 nM ¹²⁵I-LDL in the absence or presence of an excess of unlabeled LDL (40-fold). CHO cells were then exposed to this mixture for 5 hours at 37°C. After evaluation of ¹²⁵I-LDL binding by radioactivity counting, the data were normalised first with respect to the total protein concentration and second with respect to the binding obtained in control cells (no peptide) taken arbitrarily at 100.

B. Three days after plating, hepatocytes (FT197) were left untreated (UT) or treated with 0.62, 6.2, 62, or 620 nM r-shLDLR292 (MW : 32.3 KD), or 11, 110 or 1100 nM r-shLDLR166 (MW : 18.2 KD) or u-hsLDLR166 at the time of infection. Cells were infected overnight with 25 μ l of HCV(+) human serum (S155). After extensive washes the next day, cells were cultured for 5 days, the medium being renewed every 48 hours. At day 5 post-infection, cells were scraped and total RNA was extracted. One μ g of the same RNA was analysed by real-time PCR for the genomic strand (*for*

negative strand see [Figure S-7A](#)). RNA quality control was evaluated by performing a quantification of GAPDH mRNA in each sample. Amounts of HCV RNA were normalized to GAPDH mRNA; note that in this experiment the average number of HCV genome per cell is 0.6 in untreated cells. Results are expressed as percent of intracellular viral RNA relative to the amount obtained in *in vitro* infected hepatocytes in the absence of treatment (UT). Data are mean of three independent assays. These results are representative of observations made with other hepatocyte cultures (FT) from different donors infected with different serum samples (S) including, FT161-S42, FT167-S42, FT168-S42, FT171-S42, FT176-S42/-S12/-S31, FT195-S155, FT199-S151, FT200-S182, FT200-S183, and FT269-S241.

C. Three days after plating, hepatocytes (FT176) were exposed to HCV(+) human serum (S42). Cells were left untreated (UT) or treated with 62 nM r-shLDLR292 added either at the time of inoculation with HCV (+) serum (T0), or 30 min, 2h, or 8h later. Following overnight exposure cells were washed three times and the culture was continued. Three days post-inoculation, cells were washed three times and total cellular RNA was extracted. One μ g of the same RNA samples was analyzed for the genomic strand by real time RT-PCR (*for negative strand* see [Figure S-7B](#)). Cellular RNA quality control was evaluated by performing a quantification of GAPDH mRNA in each sample. Amounts of HCV RNA were normalized to GAPDH mRNA; note that in this experiment the average number of HCV genome per cell is 36 in untreated cells. Results are expressed as percent of intracellular viral RNA relative to the amount obtained in *in vitro* infected hepatocytes in the absence of treatment (UT). Data are the mean of three independent assays. These results are representative of observations made with another hepatocyte culture (FT171) infected with serum samples S12, S31, and S42.

Figure 3. Effect of monoclonal and polyclonal antibodies against hLDLR on HCV RNA replication

A. Effect of monoclonal antibodies on LDL entry in HepG2 cells. Ten μ g of the indicated antibodies were incubated for 1 hour at 37°C with 4 nM 125 I-LDL in the absence or presence of an excess of unlabeled LDL (40-fold). HepG2 cells were then exposed to this mixture for 5 hours at 37°C. After evaluation of 125 I-LDL binding by radioactivity counting, the data were normalised first with respect to the total protein

concentration and second with respect to the binding obtained in control cells (none) taken arbitrarily at 100.

B and C. Three days after plating, primary hepatocytes FT168 (or FT269 for anti-CD29 antibodies control experiments) were exposed to 25 μ l of HCV(+) serum S42 (or S241 for CD29 experiments), in the absence (UT) or presence of 2 or 8 μ g/ml mAbs12.6, 28 or 29.8 against hLDLR or 20 to 80 μ g/ml anti-LDLR polyclonal antibody or 4 μ g/ml mouse IgG1 isotype (BD Biosciences), or 2 or 20 μ g/ml of anti-CD29 antibodies (Immunotech, clone K20). FACS analysis revealed that more than 70% cells express CD29 and that the degree of binding by antibodies is 62.3 and 90.8% at 2 and 20 μ g/ml, respectively. Following overnight exposure, cells were washed three times and the culture was continued. Five days post-inoculation, cells were washed three times and total cellular RNA was extracted. One μ g of RNA was analysed by real-time PCR for the genomic strand (*for negative strand see [Figure S-9](#)*). RNA quality control was evaluated by quantification of GAPDH mRNA in each sample. Amounts of HCV RNA were normalized to GAPDH mRNA; note that in this experiment the average number of HCV genome per cell is 4.2 and 1.5 in untreated cells from FT168 and FT269, respectively. Results are expressed as percent of intracellular viral RNA relative to the amount obtained in *in vitro* infected hepatocytes in the absence of treatment (UT). Data are mean of three independent assays. These results are representative of observations made with other hepatocyte cultures (FT) from different donors infected with different serum samples (S) including, FT167-S42, FT197-S151, FT200-S183, FT203-S183, FT259-S298 and FT269-S241.

Figure 4. Effect of LDL and HDL on HCV RNA replication

Three days after plating, hepatocytes (FT257) were left untreated (UT) or treated with 1.5, 15 or 150nM LDL or 15, 150 or 1500nM HDL for 30 min before inoculation with 25 μ l of HCV(+) serum S298. Following overnight exposure, cells were washed three times and the culture was continued. Five days post-inoculation, cells were washed three times and total cellular RNA was extracted. One μ g of the same RNA sample was analysed by real-time PCR for the genomic strand (*for negative strand see [Figure S-11](#)*). RNA quality control was evaluated by performing a quantification of GAPDH mRNA in each sample. Amounts of HCV RNA were normalized to GAPDH mRNA; note that in this experiment the average number of HCV genome per cell is 0.12 in untreated cells. Results are expressed as percent of intracellular viral RNA

relative to the amount obtained in *in vitro* infected hepatocytes in the absence of treatment (UT). Data are the mean of three independent assays. These results are representative of observations made with other hepatocyte cultures (FT) from different donors infected with different serum samples (S) including, FT197-S151 and FT259-S297.

Figure 5. Effect of SQ on HCV genome replication

Hepatocytes (FT197) were plated and cultured under standard conditions. At day 2, cells were then treated with 0.1 to 80 μ M squalestatin (SQ) or 0.1 to 40 μ M 25-hydroxycholesterol (25OHC) for 24 hours. Then, cells were infected overnight with 25 μ l of HCV(+) human serum (S155) at day 3. After extensive washes the next day, cells were cultured for 5 days, the medium being renewed every 48 hours. At day 5 post-infection, cells were scraped and total RNA was extracted.

A. (SQ treatment) and B (25OHC treatment). One μ g of the same RNA was analysed by real-time PCR for the genomic strand (*for negative strand see [Figure S-12](#)*). RNA quality control was evaluated by performing a quantification of GAPDH mRNA in each sample. Amounts of HCV RNA were normalized to GAPDH mRNA. Results are expressed as percent of intracellular viral RNA relative to the amount obtained in *in vitro* infected hepatocytes in the absence of treatment (UT); note that in this experiment the average number of HCV genome per cell is 0.6 in untreated cells. Data are the mean of three independent assays. These results are representative of observations made with other hepatocyte cultures (FT) from different donors infected with different serum samples (S) including, FT179-S155 and FT199-S151.

Table I. Clinical characteristics of liver donors

Patient ID	Age	Sex	Reason for surgery/Cause of death
FT161	35	M	organ donor ^a
FT167	57	M	metastasis of colic tumor
FT168	75	F	metastasis of colic tumor
FT171	48	M	metastasis of colic tumor
FT176	69	F	metastasis of colic tumor
FT195	17	M	organ donor ^b
FT197	79	M	metastasis of colic tumor
FT199	42	M	hepatic lesion due to Caroli's disease
FT200	71	M	metastasis of colic tumor
FT203	72	M	metastasis of colic tumor
FT223	74	M	hepatocellular carcinoma on normal liver
FT224	73	M	metastasis of colic tumor
FT228	76	M	metastasis of colic tumor
FT233	28	M	angiocarcinoma
FT236	60	M	metastasis of colic tumor
FT240	63	M	metastasis of colic tumor
FT245	73	M	metastasis of colic tumor
FT246	50	F	organ donor ^c
FT256	43	F	adenoma
FT257	30	F	hydatid cyst
FT259	76	M	hepatocellular carcinoma on normal liver
FT269	46	M	adenocarcinoma on normal liver

a. This patient became an organ donor after a head traumatism due to a traffic accident. His liver was not transplanted because a tumor of the pancreas was discovered in this patient

b. This patient became an organ donor after a head traumatism due to a traffic accident. His liver was not transplanted because the recipient patient died during operation, just before transplantation

c. This patient became an organ donor after a cerebral haemorrhage. Her liver was not transplanted because of a too high level of steatosis

Table 2. Characteristics of HCV positive serum samples

Serum ID	Genotype	Viral load (IU/ml)
S12	1b	1.5 10 ⁶
S31	2	13 10 ⁶
S42	1b	6 10 ⁶
S151	1a	1.7 10 ⁶
S155	1a	1.0 10 ⁶
S182	1b	6.7 10 ⁶
S183	3a	2.6 10 ⁶
S241	1b	4.4 10 ⁶
S297	3a	1.5 10 ⁶
S298	3a	1.5 10 ⁶