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Oxidized Low-Density Lipoprotein Inhibits Hepatitis C Virus Cell Entry in Human Hepatoma Cells

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Cell entry of hepatitis C virus, pseudoparticles (HCVpp) and cell culture grown virus (HCVcc), requires the interaction of viral glycoproteins with CD81 and other as yet unknown cellular factors. One of these is likely to be the scavenger receptor class B type I (SR-BI). To further understand the role of SR-BI, we examined the effect of SR-BI ligands on HCVpp and HCVcc infectivity. Oxidized low-density lipoprotein (oxLDL), but not native LDL, potently inhibited HCVpp and HCVcc cell entry. Pseudoparticles bearing unrelated viral glycoproteins or bovine viral diarrhea virus were not affected. A dose-dependent inhibition was observed for HCVpp bearing diverse viral glycoproteins with an approximate IC_{50} of 1.5 μg/mL apolipoprotein content, which is within the range of oxLDL reported to be present in human plasma. The ability of lipoprotein components to bind to target cells associated with their antiviral activity, suggesting a mechanism of action which targets a cell surface receptor critical for HCV infection of the host cell. However, binding of soluble E2 to SR-BI or CD81 was not affected by oxLDL, suggesting that oxLDL does not act as a simple receptor blocker. At the same time, oxLDL incubation altered the biophysical properties of HCVpp, suggesting a ternary interaction of oxLDL with both virus and target cells. <u>In conclusion</u>, the SR-BI ligand oxLDL is a potent cell entry inhibitor for a broad range of HCV strains *in vitro*. These findings suggest that SR-BI is an essential component of the cellular HCV receptor complex. (HEPATOLOGY 2006;43:932-942.)

Over 170 million individuals worldwide are infected with hepatitis C virus (HCV), a leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Infectious HCV purified from plasma has been observed to complex low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), and high-density lipoprotein (HDL).[1-4] This association may account for the low buoyant density of HCV RNA containing particles in human plasma and may be relevant to the infectivity of HCV *in vivo*.[1,5,6] Interestingly, recent reports of cell culture–grown HCV (HCVcc) have found a similar low buoyant density of infectious particles.[7-9]

HCV pseudoparticle (HCVpp)[10,11] and HCVcc entry into permissive cells is mediated through an interaction of the viral encoded glycoproteins E1 and E2 with specific cell surface–expressed receptors that include CD81[12,13] and possibly scavenger receptor class B type I (SR-BI).[14,15] CD81 is critical for HCVpp and HCVcc infection, but CD81 expression alone is insufficient to render a cell permissive for HCVpp entry.[7-11,15,16] A role for SR-BI in HCVpp entry[11,17] is supported by the observation that HDL enhances HCVpp infectivity in an SR-BI–dependent manner.[18,19] However, whether this molecule plays an essential or facilitating role awaits clarification.

SR-BI is a member of the scavenger receptor family that is most highly expressed in the liver and steroidogenic tissues (see Boullier et al.[20] for review). Binding of HDL to SR-BI results in either the selective uptake of cholesteryl esters from HDL into the plasma membrane[21] or endocytosis of receptor-ligand complexes.[22,23] Like most scavenger receptors, SR-BI recognizes a wide range of ligands that include native HDL, LDL, and VLDL, as well

as various chemically modified HDL and LDL species.[24-27] At least two distinct ligand-binding sites have been reported to exist on the SR-BI molecule.[23,28] To further understand the role of SR-BI in HCV–cell entry, we investigated the effects of SR-BI ligands on viral infectivity. We found that oxidized LDL (oxLDL), a high-affinity ligand of SR-BI,[26] potently inhibits HCVpp and HCVcc infection, supporting a critical role for SR-BI in HCV entry and offering a potential target for therapeutic intervention.

Materials and Methods

Cells and Reagents. 293T, Hep3B, Huh-7, Huh-7.5, PLC/PRF5 and HepG2/CD81 cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS). Chinese hamster ovary (CHO) cells were maintained in DMEM/F-12 with 10% FBS. HepG2/CD81 cells were generated as previously reported[16] and grown on collagen-coated plastic. Lipoprotein-deficient serum was purchased from Sigma (St. Louis, MO).

Lipoproteins. LDL (1.019-1.063 g/mL) and HDL (1.063-1.21 g/mL) were isolated from normolipidemic human plasma via ultracentrifugation[29] and dialyzed against phosphate-buffered saline containing 0.3 mmol/L EDTA. Lipoproteins were oxidized via incubation of 100 μg/mL native lipoproteins with 10 μmol/L $CuSO_4$ for 18 hours at 37°C. The degree of oxidation was determined by measuring the amount of thiobarbituric acid–reactive substances. Butylated hydroxytoluene (20 μmol/L) and EDTA (0.1 mmol/L) were added to prevent further oxidation. $Na^{125}I$ (2,000 Ci/mmol) for labeling oxLDL[30] was obtained from ICN (Costa Mesa, CA). Apolipoproteins were isolated from lipoproteins by extracting lipids with ice-cold methanol/chloroform (1:1, v/v) as previously described.[31] The residual protein was washed with water/acetone and solubilized in octylglucoside (octylglucoside/protein = 30:1, w/w), and the detergent was removed by way of dialysis. The apolipoprotein B of native LDL was not prepared, because it cannot be resolubilized. The lipids from native and oxidized lipoproteins were isolated as described previously,[32] and their phospholipid concentration was determined via phosphorus assay.[33]

Lipoprotein Cell-Binding Assay. To determine the binding affinity of oxLDL to Hep3B, cells were incubated with various concentrations of ^{125}I-oxLDL for 5 hours at 37°C in the presence or absence of a 30-fold excess of unlabelled oxLDL. Cells were washed extensively and lysed with 0.2 N NaOH. Aliquots were taken to measure protein content and cell-associated γ-radiation. Specific binding was calculated as total binding minus nonspecific

binding in the presence of an excess of unlabeled oxLDL. In the competition assays, ^{125}I-oxLDL was added with the various unlabeled competitors for 5 hours at 37°C. Binding was then analyzed as described above.

RNA Interference. SMARTpool silencing RNAs were purchased from Dharmacon (Lafayette, CO) and transfected into Hep3B cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Silencing efficiency was assessed via flow cytometry, and infection experiments were performed 48 and 72 hours after transfection. An irrelevant small interfering RNA (siRNA) with low homology to any human sequence was used as a negative control.

sE2 Cell-Binding Assay. CHOs were transduced with CD81, CD9, and SR-BI using the pTRIP lentiviral expression system. Transgene expression was confirmed via fluorescence-activated cell sorting using antibodies 1.3.3.22 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-CD9 (Ancell, Bayport, MN), and anti–Cla-1 (BD, Franklin Lakes, NJ) against CD81, CD9, and SR-BI, respectively. Genotype 1a soluble E2 (sE2) containing residues 384 to 661 was generated and its binding to cells was detected with rat anti-E2 monoclonal antibodies 9/75 and 7/59 as previously described.[12,34]

HCVpp Generation and Infection. HCVpp were generated as previously described.[10] Briefly using Lipofectamine 293T cells were cotransfected with the envelope-deficient HIV genome pNL4-3.Luc.R^-.E^- and a plasmid expressing any of the following viral glycoproteins: HCV strains H (genotype 1a), H77 (1a), OH8 (1b), or Con1 (1b); murine leukemia virus (MLV); vesicular stomatitis virus G protein (VSV-G); or an empty vector ("no envelope"). Medium was replaced with DMEM/3% FBS after 6 hours. Supernatants were collected after 48 hours and clarified via centrifugation. For infection experiments, supernatants were diluted in DMEM/3% FBS with 4 μg/mL polybrene with or without the desired lipoprotein species. The mixture was incubated at 37°C for 1 hour before being applied to the target cells for 4 to 6 hours. Pseudoparticles were removed, and the cells were incubated for a further 72 hours. Cells were lysed with 40 μL Cell Culture Lysis Reagent (Promega, Madison, WI), and infection was measured by quantifying the expression of the luciferase reporter using 50 μL luciferase substrate (Promega) and a Centro LB960 luminometer (Berthold Technologies, Bad Wildbad, Germany).

Sedimentation Velocity Centrifugation Over Sucrose Gradients. HCVpp containing supernatants were loaded onto linear sucrose gradients (5%-20% w/v, in TRIS-buffered saline) formed on a Gradient Master (Biocomp Inc., Fredericton New Brunswick, Canada) according to the manufacturer's instructions. Samples were
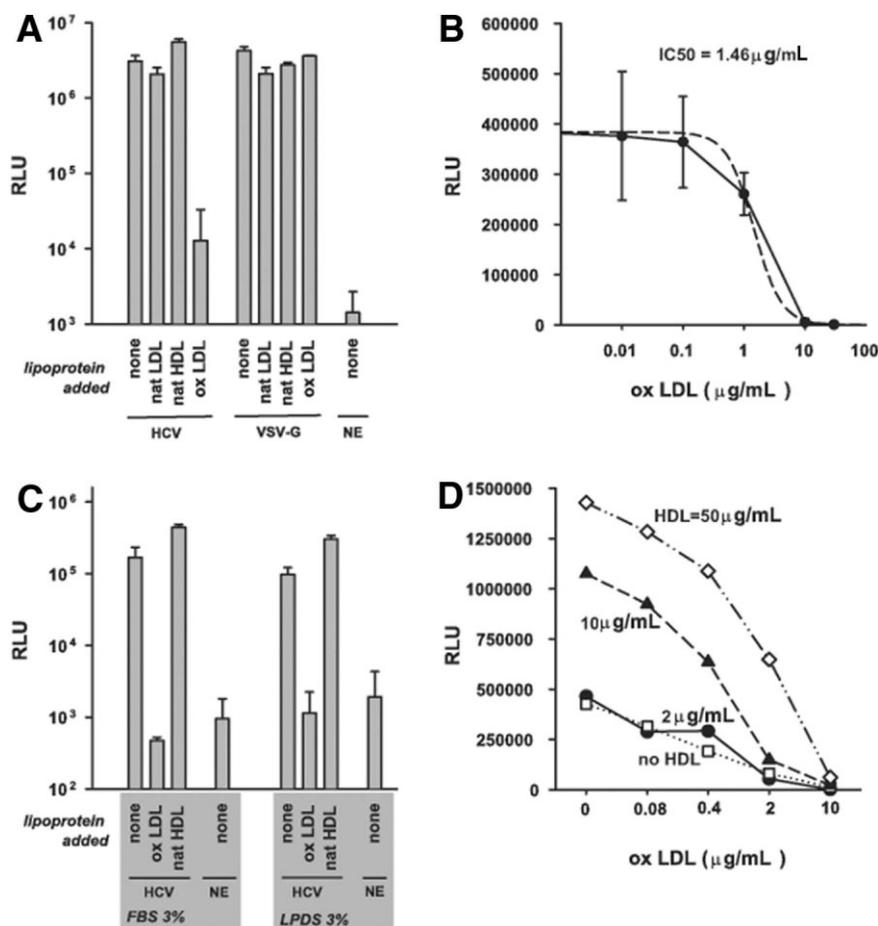
Fig. 1. HDL enhancement and oxLDL inhibition of HCVpp infectivity. (A) Effect of native LDL, native HDL, and oxLDL (10 μg/mL) on HCVpp and VSVGpp infection of Hep3B cells. (B) Dose response curve for oxLDL inhibition of HCVpp infectivity. (C) Effect of oxLDL and native HDL (10 μg/mL) on HCVpp generated and used for infection in DMEM with either 3% FBS or 3% lipoprotein-deficient serum. (D) Effect of oxLDL on HCVpp infectivity in the presence of HDL. All values are expressed as the mean $\pm$ SD of 3 independent experiments. RLU, relative light units; natLDL, native low-density lipoprotein; natHDL, native high-density lipoprotein; oxLDL, oxidized low-density lipoprotein; HCV, hepatitis C virus; VSV-G, vesicular stomatitis virus G protein; NE, no envelope; FBS, fetal bovine serum; LPDS, lipoprotein-deficient serum.

centrifuged in an SW41 rotor at 35,000 rpm for 20 minutes at 4°C. Fractions were collected from the top of the gradient.

p24 ELISA. Pseudoparticles were inactivated with 1% Empigen and subsequent incubation at 56°C for 30 minutes. Commercially available capture anti-p24, recombinant HIV-1 p24 standard (Aalto Bio Reagents, Dublin, Ireland), AP-conjugated anti-p24 (Cliniqa, Fallbrook, CA), and AMPAK AP-substrate system (DakoCytomation, Carpinteria, CA) were used according the Aalto Bio Reagents protocol (www.aaltobioreagents.ie/datasheet/protocol%201.pdf).

HCVcc Infection and NS5A Immunostaining. Infectious HCV J6/JFH particles were generated as previously described.[7] Briefly, RNA was transcribed *in vitro* from a chimeric full-length J6/JFH (both genotype 2a) genome and electroporated into Huh-7.5 cells. After 72 hours, supernatants were collected, filtered, and applied to naïve Huh-7.5 target cells in the presence or absence of human lipoproteins. Successful infection was detected 48 hours after infection via methanol fixation and staining for NS5A antigen using a mouse monoclonal anti-NS5A primary and HRP-conjugated anti–mouse immunoglob-

ulin G secondary antibody. Fifty percent tissue culture infectious dose values were determined via limiting dilution assay.[7,35]

Statistical Analysis. All *P* values were determined with the unpaired *t* test using Prism4 (GraphPad Software, San Diego, CA). Error bars indicate the SD of duplicate experiments.

Results

OxLDL Inhibits HCVpp Infectivity. We evaluated the effect of SR-BI ligands on HCVpp (clone H77) infection of Hep3B cells. Incubation of HCVpp with oxLDL (at 10 μg/mL apolipoprotein content), but not native LDL, reduced infectivity (as measured by expression of the luciferase reporter) by more than 100-fold, whereas VSV-Gpp and MLVpp were only modestly affected (less than threefold in several independent experiments) (Figs. 1A, 2B). The copper-containing buffer in which oxLDL was generated alone or mixed with native LDL had no effect on HCVpp infectivity (not shown). The inhibition was dose-dependent, with a half-maximal effect at 1.5 μg/mL (Fig. 1B). To rule out interference by bovine li-

Fig. 2. Target cell binding and inhibition of HCVpp infectivity. (A) HCVpp and (B) MLVpp infection of Hep3B in the presence of lipoproteins or their protein or lipid moieties. Whole lipoproteins were used at 10 μg/mL protein content; apolipoprotein and lipid preparations were used at 100 μg/mL protein or phospholipid, respectively. Values are expressed as the mean $\pm$ SD of 3 experiments. (C) Binding of ^{125}I-labeled oxLDL to Hep3B cells. Values are expressed as the mean $\pm$ SD of 4 independent experiments. (D) Binding of lipoprotein components to target cells. Components of native and oxidized lipoproteins were tested for their ability to compete with ^{125}I-oxLDL (5 μg/mL) binding to Hep3B cells. Competitors were used in 30-fold excess. Values are expressed as the mean $\pm$ SD of 2 independent experiments. RLU, relative light units; n.d., not determined; oxLDL, oxidized low-density lipoprotein; oxApoB, oxidized apolipoprotein B; natHDL, native high-density lipoprotein; apo, apolipoproteins; lipids; lipoprotein derived lipid fractions; oxHDL, oxidized high-density lipoprotein; natLDL, native low-density lipoprotein.
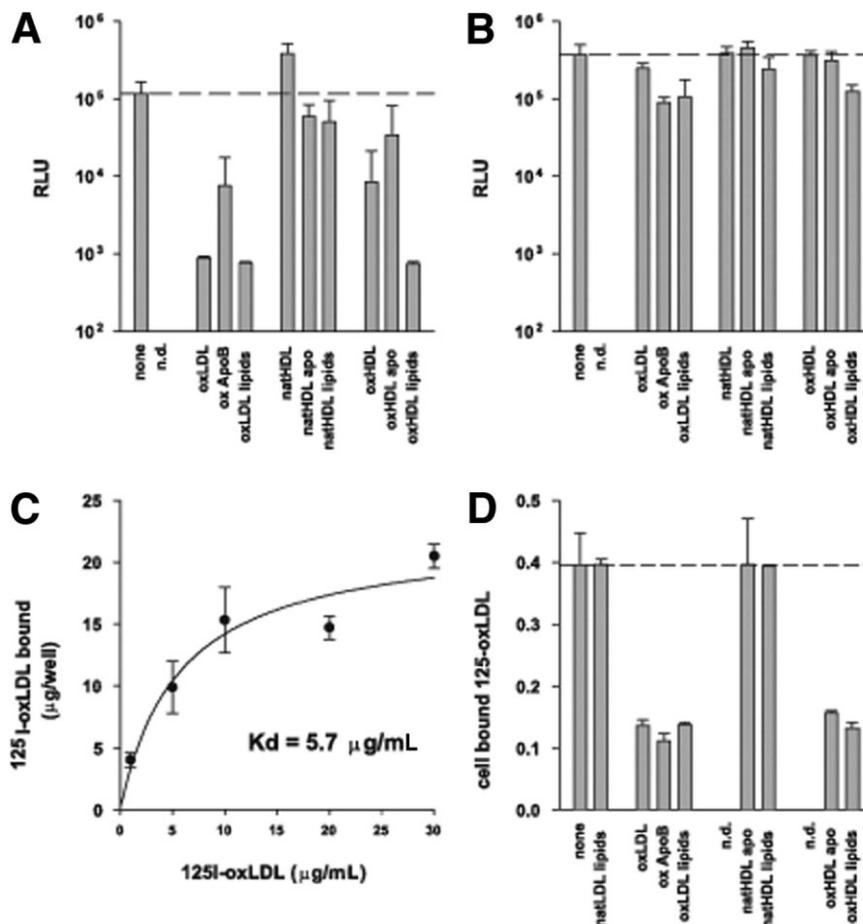
poproteins within the tissue culture media, pseudoparticles were generated and used to infect target cells in media containing 3% lipoprotein-deficient serum. oxLDL reduced HCVpp infectivity in the presence and absence of bovine lipoproteins (Fig. 1C). HDL enhanced HCVpp infection (two- to fourfold) and had no effect on VSV-Gpp as previously reported (Fig. 1A,C).[18] In the presence of various concentrations of HDL, oxLDL was always able to inhibit more than 95% of HCVpp infectivity with the IC50 remaining at 0.5-1.5 μg/mL. However, more than 99% inhibition was only seen in the absence of HDL (Fig. 1D). Maximal HDL enhancement of infectivity (*i.e.*, the ratio of the signal in the presence of 50 μg/mL HDL over the signal in the absence of HDL) was three- to fourfold when oxLDL was 0.4 μg/mL or less but increased to 12-fold and 79-fold when 2 μg/mL and 10 μg/mL oxLDL, respectively, were present. oxLDL inhibited HCVpp (clone H77) infection of different target cells, whereas native LDL had no effect (Table 1). Preincubation of HCVpp with HDL enhanced infection of Hep3B, Huh-7, and PLC/PRF5 cells but had minimal effect on HepG2/CD81 cells. All target cells expressed both SR-BI and CD81 on the cell surface (data not

shown). HCVpp bearing diverse glycoproteins of genotype 1a (H, H77) and 1b (Con1, OH8) strains were all inhibited by oxLDL.

Protein and Lipid Components of oxLDL Inhibit HCVpp Infectivity and Bind to Hep3B Cells. To understand the mechanism of oxLDL inhibition of HCVpp infectivity, we separated the protein (*i.e.*, apolipoproteins) and lipid fractions of oxLDL, native HDL, and oxidized HDL (oxHDL) and evaluated their effect on HCVpp infectivity and their ability to bind Hep3B target cells. Both the protein and lipid fractions of oxLDL and oxHDL inhibited HCVpp infectivity (Fig. 2A). There was only a minor nonspecific inhibitory effect on MLVpp infectivity (Fig. 2B). In contrast, HDL-enhanced HCVpp infectivity was only observed with intact HDL particles. Because oxLDL is reported to bind SR-BI with high affinity (Kd $\approx$4 μg/mL),[26] it is feasible to hypothesize that it inhibits HCVpp infection in an SR-BI dependent manner. Consistent with this hypothesis, we found that oxLDL bound Hep3B cells with a similar Kd of 5.7 μg/mL (Fig. 2C). Because both apolipoprotein and lipid moieties of oxLDL inhibited HCVpp infectivity, we were interested to determine whether the same fractions would

Table 1. Effect of HDL, oxLDL, and natLDL (10 μg/mL) on HCVpp Infection of Different Target Cells and HCVpp Bearing Diverse HCV E1E2 gps

E1E2 Clone (Genotype)	Target Cell	Percent Change from Baseline		
		HDL	oxLDL	natLDL
H77 (1a)*	Hep3B*	+239.5 ± 61.3	−99.6 ± 0.4	+13.4 ± 13.6
H77 (1a)	Huh-7	+151.1 ± 38	−99.3 ± 0.1	−30 ± 2.4
H77 (1a)	PLC/PRF5	+227.4 ± 48.5	−98.7 ± 0.1	−27.1 ± 46.7
H77 (1a)	HepG2/CD81	+67 ± 70.2	−92.7 ± 0.6	+40.9 ± 114.9
H77 (1a)*	Hep3B*	+326.5 ± 106.6	−98.3 ± 0.1	ND
H (1a)	Hep3B	+206.7 ± 76.7	−98.8 ± 0.9	ND
OH8 (1b)	Hep3B	+67.1 ± 15.6	−99.1 ± 0	ND
Con1 (1b)	Hep3B	−7.7 ± 27.4	−99.6 ± 0	ND

NOTE. Values are given as change versus values obtained in the absence of human lipoproteins (mean of n = 3 replicates ± SD).

Compared with Hep3B, the signal of strain H77 HCVpp was four-fold, six-fold, and 40-fold reduced on Huh7, PLC/PRF5, and HepG2/CD81 cells, respectively. Compared with HCVpp of strain H, the infectivity on Hep3B cells was three-fold, five-fold, and two-fold lower for strain H77, OH8, and Con1, respectively.

Abbreviations: HDL, high-density lipoprotein; oxLDL, oxidized low-density lipoprotein; natLDL, native low-density lipoprotein; HCVpp, hepatitis C virus pseudoparticles; ND, not determined.

*Data dealing with target cells (upper half) and E1E2 clones (lower half) were obtained using different preparations of HCVpp.

compete with iodinated oxLDL binding to Hep3B cells. Both apolipoproteins and lipid fractions of oxLDL and oxHDL, but not lipids from native lipoproteins, competed for cell binding (Fig. 2D). Thus binding to Hep3B cells associates with the inhibition of HCVpp infectivity.

Pretreatment of Hep3B Cells With oxLDL Does Not Inhibit HCVpp Infection. To investigate whether oxLDL inhibits HCVpp infection by blocking a cellular binding site, we examined the effect of preincubating Hep3B target cells with oxLDL for 1 hour prior to virus infection. Cells were washed and infected with HCVpp or MLVpp (Table 2). Alternatively, pseudoparticles and oxLDL were mixed, incubated at 37°C for 1 hour, and then added to the target cells. Both at 37°C or 4°C significant HCV-specific inhibition was observed only when pseudoparticles and oxLDL were present on the target cell simultaneously. Preincubation of target cells with oxLDL had a modest effect on the luciferase signal that was indistinguishable between HCVpp and MLVpp. Conversely, HCVpp that had been allowed to bind to (but not enter) target cells on ice for 1 hour prior to the addition of oxLDL were still sensitive to oxLDL inhibition.

sE2 Interaction With SR-BI and CD81 Is Not Affected by oxLDL. To determine if oxLDL directly inhibits HCV-glycoprotein interactions with SR-BI and/or CD81, we monitored sE2 binding to CHO cells expressing human SR-BI, CD81, or CD9, a tetraspanin closely related to CD81 but unable to interact with sE2.[12] Anti-E2 monoclonal antibodies (mAbs) could distinguish between SR-BI–bound and CD81-bound sE2 (Fig. 3A-B), such that monoclonal antibody 9/75 (specific for E2 amino acids 524-532) recognized sE2–SR-BI complexes but not sE2 bound to CD81. Conversely, monoclonal antibody 7/59 specific for the hypervariable region of E2

was able to recognize sE2–CD81 but not sE2–SRBI complexes. Neither anti-E2 nor an antibody against a histidine tag at the C-terminus of sE2 detected significant sE2 binding to CHO/CD9 (Fig. 3C and not shown). In neither case was the measured amount of sE2–receptor complexes modulated by the presence or absence of oxLDL (up to 50 μg/mL) or native LDL (Fig. 3D-F). Furthermore, cell surface binding of anti SR-BI was only minimally affected by the presence or absence of oxLDL (data not shown). Our data suggest that oxLDL does not simply compete with HCV for a cellular binding site but favors a model in which entry steps subsequent to virus–receptor binding are being perturbed and HCV–lipoprotein receptor ternary complexes are required for inhibition to occur.

Silencing of SR-BI Does Not Abrogate the oxLDL Effect. To determine whether modulation of SR-BI expression affects oxLDL inhibition of HCVpp infectivity, Hep3B cells were transfected with siRNA against SR-BI or CD81. Specific downregulation of the target molecules was confirmed via flow cytometry sorting for cell surface–expressed SR-BI and CD81 (Fig. 4A-B). While the SR-BI–specific signal detected was only 1.5-fold above the isotype-control signal even in cells treated with irrelevant siRNA, its decrease to almost background in SR-BI–silenced cells was reproducibly seen in independent experiments (n = 6; data not shown). Also, a loss of HDL enhancement of HCVpp infectivity was observed in SR-BI but not in CD81-silenced cells. Silencing CD81 clearly inhibited HCVpp infection both in the presence and absence of HDL, whereas the effect of silencing SR-BI on infectivity reached statistical significance only when HDL was present. VSVGpp infectivity was not significantly affected (Fig. 4D). The inhibitory effect of ox-

Table 2. Effect of Sequential Versus Simultaneous Application of oxLDL and Pseudoparticles to Target Cells

Experimental Protocol			Inhibition by oxLDL (10 μg/mL)	
Step 1	Step 2	Step 3	HCVpp	MLVpp
Incubate cells with oxLDL for 1 h at 37°	Wash twice	Add pp to cells for 1 h at 37°C	22.1%	20.4%
Incubate cells with oxLDL for 1 h at 4°	Wash twice	Add pp to cells for 1 h at 4°C	26.5%	31.9%
Incubate pp with oxLDL for 1 h at 37°C	—	Add pp + oxLDL to cells for 1 h at 37°C	98.3%	19.3%
Incubate pp with oxLDL for 1 h at 37°C	Chill to 4°C	Add pp + oxLDL to cells for 1 h at 4°C	97.2%*	42.3%
Incubate pp with oxLDL for 1 h at 37°C	Chill to 4°C	Add pp + oxLDL to cells for 1 h at 4°C	98.0%*	ND
Incubate cells with HCVpp for 1 h at 4°	Wash twice	Add oxLDL to cells for 1 h at 4°C	87.1%	ND

NOTE. Hep3B cells were exposed to pseudoparticles and oxLDL in the order shown. Values are given as percent of the signal obtained when performing the same protocols without oxLDL (means of n = 2).

Abbreviations: oxLDL, oxidized low-density lipoprotein; HCVpp, hepatitis C virus pseudoparticles; MLVpp, murine leukemia virus pseudoparticles; pp, pseudoparticles.

*Values do not match because data in top 4 and bottom 2 rows, respectively, were obtained on 2 separate occasions.

LDL on HCVpp infectivity was maintained under all conditions.

Interaction of HCVpp With an Oxidized Lipid Species. Because HCV is thought to associate with β-lipoproteins, we were interested to determine whether oxLDL associates with HCVpp independent of binding the target cell. HCVpp were incubated for 1 hour at 37°C with either a mixture of ^{125}I-oxLDL and unlabeled oxLDL or an equal total amount of unlabeled oxLDL. Subsequently, the mixture, pseudoparticles alone, or oxLDL alone were subjected to sedimentation velocity centrifugation over a sucrose gradient (5%-20%), and fractions were assayed for p24 antigen to detect pseudoparticles (Fig. 5A), infectivity (Fig. 5B), and gamma-radiation to locate oxLDL (Fig. 5C). After centrifugation, more than 90% of the labeled oxLDL was detected in the first 2 fractions closest to the top of the gradient, independent of the presence or absence of HCVpp. The vast majority of p24 antigen and infectivity was located within fractions 4 through 8, with the gradient containing pseudoparticles
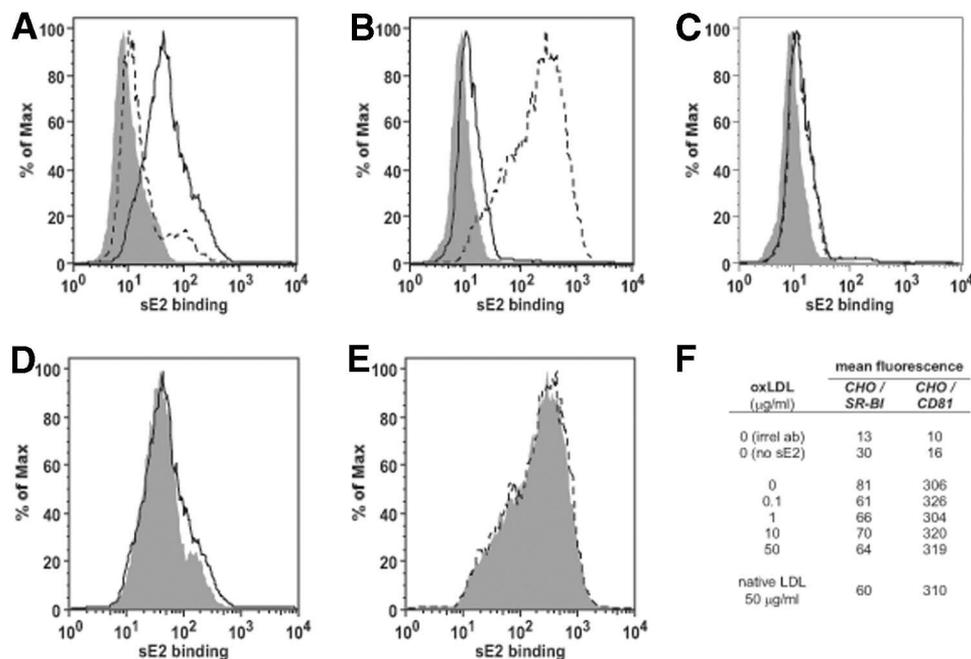
Fig. 3. Binding of sE2 in the presence of oxLDL. CHO cells expressing human (A) SR-BI, (B) CD81, or (C) CD9 were incubated with sE2 for 1 hour, and bound sE2 was visualized with anti-E2 antibodies 7/59 (dashed line) and 9/75 (solid line) or an irrelevant isotype control (shaded area). (D) sE2 binding to CHO/SR-BI in the presence (solid line) or absence (shaded area) of oxLDL (50 μg/mL) detected with anti-E2 9/75. (E) sE2 binding to CHO/CD81 in the presence (dashed line) or absence (shaded area) of oxLDL (50 μg/mL) detected with anti-E2 7/59. (F) Mean fluorescence intensity in the presence of increasing concentrations of oxLDL. Antibodies 9/75 and 7/59 were used for detection of sE2 in the case of CHO/SR-BI and CHO/CD81, respectively. Max, maximum; sE2, soluble E2; oxLDL, oxidized low-density lipoprotein; CHO, Chinese hamster ovary; SR-BI, scavenger receptor class B type I; LDL, low-density lipoprotein; irrel ab, isotype control.

Fig. 4. Silencing of SR-BI and CD81 in Hep3B cells. (A) Forty-eight hours after transfection of siRNA specific for SR-BI (dashed line) or an irrelevant control siRNA (solid line), gene knockdown was confirmed via cell surface staining of SR-BI. Isotype control staining is indicated by the shaded 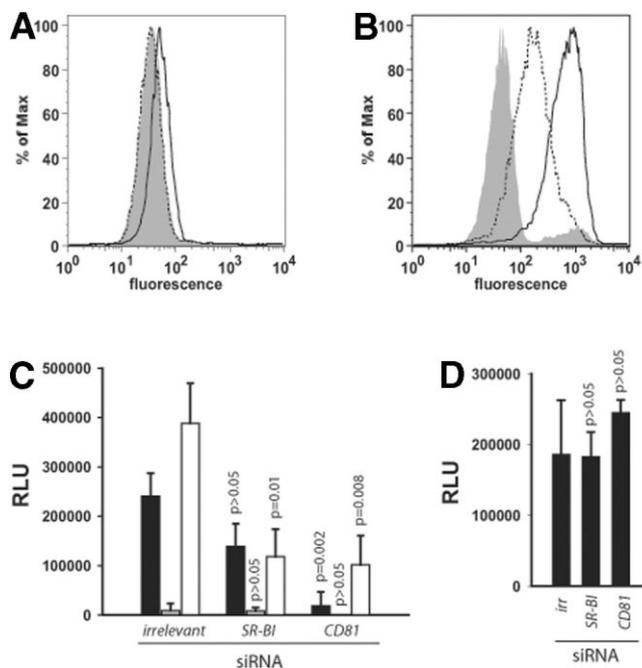area. (B) A parallel experiment using siRNA specific for CD81 (dashed line) or irrelevant control siRNA (solid line) demonstrated reduced CD81 expression. (C) HCVpp infection of cells treated with the respective siRNAs in the absence of human lipoprotein (black bars) or presence of 10 μg/mL oxLDL (gray bars) or HDL (white bars). (D) VSVGpp infection of siRNA-treated cells. *P* values indicate differences from the analogous experiments in cells transfected with irrelevant siRNA. Max, maximum; RLU, relative light units; SR-BI, scavenger receptor class B type I; irr, irrelevant; siRNA, small interfering RNA.

mixed with oxLDL showing a slight shift of the p24 peak toward a lower sedimentation velocity (Fig. 5B). This shift was reproducibly observed in 4 independent experiments and was not seen when MLVpp was studied (n = 2; data not shown). Thus, although the labeled apolipoprotein moiety of oxLDL and HCVpp do not appear to form a stable interaction, there appears to be a change in the biophysical properties of the HCVpp after exposure to oxLDL. The infectivity of HCVpp that had been incubated with oxLDL was markedly reduced, suggesting an inactivation of viral infectivity that persists after separation of virus and oxLDL (Fig. 5C). To test this conclusion, we incubated HCVpp with oxLDL at 5 μg/mL for 1 hour at 37°C, diluted the virus-lipoprotein mixture in plain media to a point where the oxLDL concentration was well below the IC50 and tested the infectivity of the resulting mixture. In this case, the inhibitory effect was reduced upon dilution, suggesting that the virus was not irreversibly inactivated after contact with oxLDL (Fig. 5D).

oxLDL Potently Inhibits Cell Culture–Grown HCV Particles. Finally, we assessed the effect of oxLDL on HCVcc (J6/JFH) infection of Huh-7.5 cells.[7] After 72 hours, a significant percentage of HCVcc-infected but not mock treated cells stained positive for NS5A (Fig. 6A-B). Incubation of J6/JFH with native LDL (10 μg/mL) for 1 hour at 37°C prior to infection had no discernable impact on the percentage of NS5A-positive cells in this semiquantitative assay (Fig. 6C), whereas oxLDL (10 μg/mL) led to a marked reduction (Fig. 6D). The inhibitory effect of oxLDL was quantified by limiting dilution assays in the presence of increasing amounts of oxLDL. A dose-dependent inhibition was observed at concentrations of 1 μg/mL and above (Fig. 6E), suggesting a similar potency of oxLDL for the inhibition of HCVcc and HCVpp (Fig. 1B). In a plaque assay, oxLDL had no effect on the titer of bovine viral diarrhea virus (strain NADL) in MDBK cells (1.6 × 10^{6} PFU/mL with and without oxLDL; data not shown).

Discussion

The SR-BI ligand oxLDL demonstrates a potent and specific inhibition of HCVpp and HCVcc infectivity (Figs. 1, 6). An association of HCV with lipoproteins was first reported over 10 years ago[1,36]; herein, we report an inhibitory effect of a lipoprotein species on HCV. The inhibitory effect is conserved across diverse HCV genotypes (genotypes 1a, 1b, 2a) and for all target cells tested (Table 1). The inhibition of the lipoprotein is most likely exerted by an oxidized lipid species contained in oxLDL (Fig. 2). Furthermore, we provide evidence to suggest that inhibition does not occur through oxLDL simply acting as a receptor antagonist (Fig. 4; Table 2) but involves a ternary interaction of virus, receptor, and oxLDL (Figs. 2, 5; Table 2).

Other than SR-BI, the only known oxLDL receptor expressed on Hep3B cells is CD36 (P. Balfe, personal communication), but CD36 does not interact with sE2.[14] The LDL receptor has been proposed as a possible HCV receptor,[37,38] but it recognizes only native LDL, not ox-LDL.[39,40] SR-BI was initially reported as a candidate receptor for HCV because of its ability to bind to sE2.[14] Subsequent reports have shown that antibodies and siRNA targeting SR-BI inhibit HCVpp entry[15,17,41] (Fig. 4). However, this inhibition is generally modest (30%-80%) and varies between different HCV genotypes.[11,17] The anti–SR-BI monoclonal antibody used in this study (BD anti-ClaI) and several noncommercially available antisera (a kind gift from D. Silver) failed to inhibit HCVpp infectivity (data not shown). HDL enhancement of HCVpp infection is clearly dependent on expression of a functional SR-BI molecule. However, HCVpp infection,

Fig. 5. Preincubation and subsequent separation of HCVpp and oxLDL. HCVpp, ^{125}I-oxLDL, or both were incubated for 1 hour at 37°C, loaded on a 5%-20% sucrose/TRIS-buffered saline gradient, and separated via sedimentation velocity centrifugation. Fractions were measured for (A) p24 antigen, (B) infectivity on Hep3B cells, and (C) gamma emission. (D) In a separate experiment, HCVpp and oxLDL (5 μg/mL) were incubated for 1 hour at 37°C and then diluted in media alone (gray bars) or in media containing oxLDL (5 μg/mL) (white bars). Infectivity values are shown relative to HCVpp that were incubated without oxLDL and then diluted in media (black bars). Values are expressed as the mean $\pm$ SD of 3 independent experiments. HCVpp, HCV pseudoparticles; oxLDL, oxidized low-density lipoprotein; RLU, relative light units;.
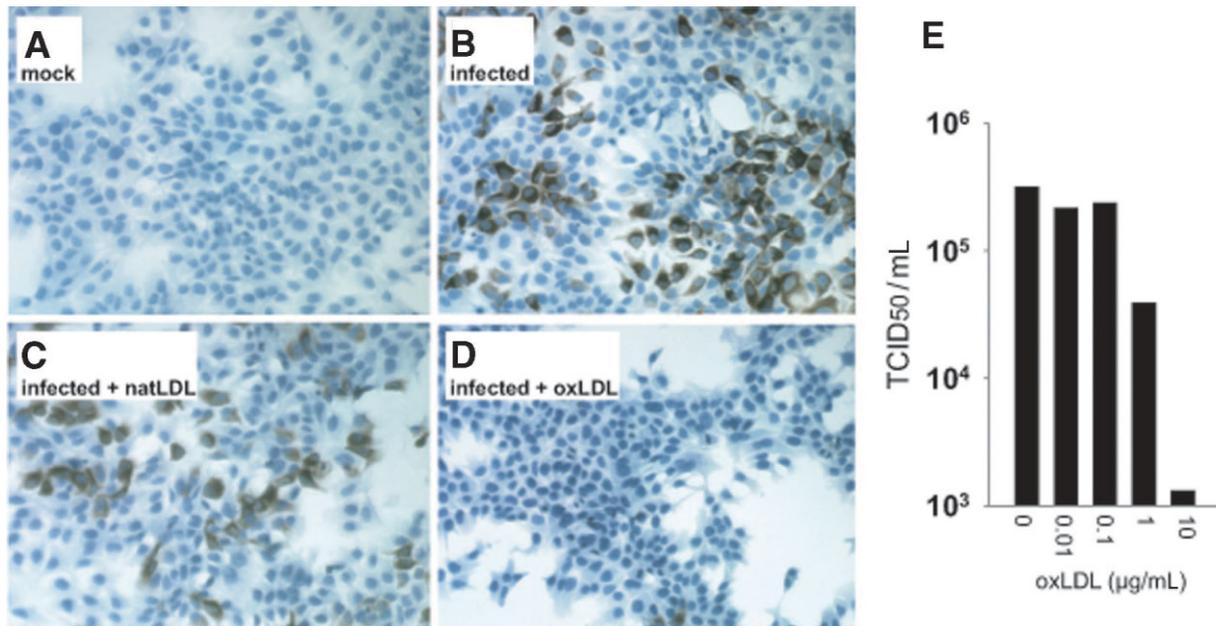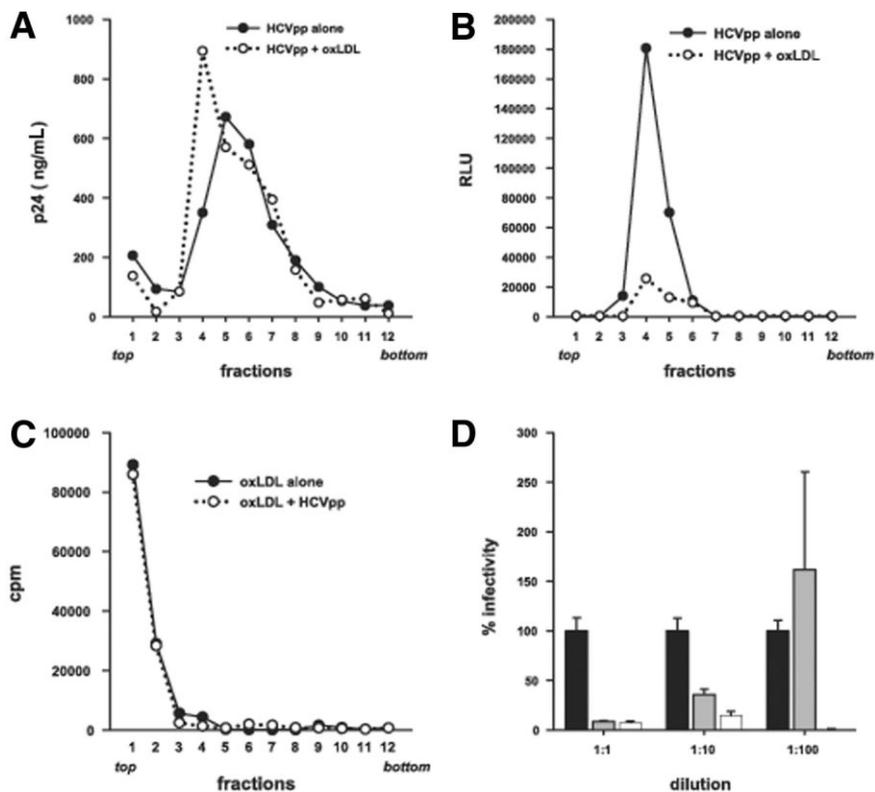
Fig. 6. Inhibition of HCVcc infection by oxLDL. (A) Naïve Huh-7.5 cells mock infected with supernatant from Huh-7.5 cells harboring a subgenomic genotype 2a replicon and stained for NS5A antigen. (B,C,D) Naïve Huh-7.5 infected with supernatant from Huh-7.5 harboring a full-length J6/JFH genome. Infection was performed either (B) in the absence of human lipoproteins or in the presence of 10 μg/mL (C) native LDL or (D) oxLDL. A representative visual field from each plate is shown. Note the 2 NS5A-positive cells near the lower margin in panel D. (E) Fifty percent tissue culture infectious dose per milliliter of HCVcc as determined by limiting-dilution assay in the presence of increasing concentrations of oxLDL. natHDL, native high-density lipoprotein; oxLDL, oxidized low-density lipoprotein; TCID$_{50}$, 50% tissue culture infectious dose.

even in the presence of HDL, is CD81-dependent such that CD81-negative HepG2 cells remain uninfectable in the presence of HDL[18,19] (Fig. 4 and unpublished data). The potent inhibition of HCV cell entry via the SR-BI ligand oxLDL supports an essential role of SR-BI in the cell entry of HCVpp and HCVcc.

The hypothesis that oxLDL inhibits HCV infection by interfering with virus–receptor interactions is supported by an association between the ability of lipoprotein components to compete with labeled oxLDL for target cell binding (Fig. 2D) and their inhibition of HCVpp infectivity (Fig. 2A). In relative terms, oxLDL was more effective in the absence of HDL, whereas, HDL enhanced HCVpp infectivity most efficiently in the presence of higher concentrations of oxLDL (Fig. 1D; data not shown). The oxLDL IC50 remained unchanged in the presence of HDL, suggesting that HDL and oxLDL may act as noncompetitive antagonists. Preincubation of cells with oxLDL had no inhibitory effect on their ability to support HCVpp infection (Table 2), nor did oxLDL inhibit binding of sE2 to CHO cells expressing SR-BI (Fig. 3). Although sE2 may imperfectly model the behavior of HCVpp, these data argue against oxLDL acting as a competitive receptor antagonist. Rather, it may suggest that subsequent steps of the entry process that cannot be modeled using sE2 are inhibited by oxLDL. This is in keeping with the observation that HCVpp prebound to the target cell can still be inhibited by oxLDL (Table 2).

If oxLDL acts by interfering with the interaction between the virus and SR-BI, one could predict that its inhibitory effect will be preserved or will be even more pronounced when fewer receptor molecules are present. Accordingly, when silencing SR-BI or CD81, we found that oxLDL retained its inhibitory effect regardless of the presence or absence of HDL and the siRNA employed (Fig. 4). HDL enhancement of HCVpp infectivity seemed more pronounced in CD81-silenced cells than in cells treated with irrelevant siRNA and, as reported by others,[18,19] was abrogated when SR-BI was silenced. However, because gene silencing is rarely complete and few remaining molecules of a virus receptor may conceivably still suffice for virus entry, the incomplete inhibition of HCVpp infectivity by siRNA against SR-BI cannot rule out an involvement of this receptor.

Several lines of evidence suggest that an oxidized lipid contained in oxLDL is the active component inhibiting HCV cell entry: First, HCVpp are inhibited by both oxLDL and oxHDL, which contain common lipid species but completely distinct apolipoprotein components (Fig. 2A). Second, the lipid fractions of oxLDL and oxHDL inhibit HCVpp infectivity more effectively than the apolipoprotein preparations, even though the latter were used at a 10-fold higher apolipoprotein concentration compared with intact oxLDL. Third, during the oxidation process, lipids form adducts with apolipoproteins such that apolipoprotein preparations derived from oxidized lipoproteins may still contain lipids, whereas the lipid fraction can be assumed to be apolipoprotein-free.[42] In keeping with this, the recognition of oxLDL by SR-BI is thought to be mediated by an oxidized phospholipid species.[26]

Although we failed to detect a physical interaction between HCVpp and radiolabeled oxLDL (Fig. 5A,C), we observed a change in the biophysical properties of HCVpp after incubation with oxLDL and subsequent separation via velocity sedimentation centrifugation (Fig. 5A). Only the apolipoprotein moiety of oxLDL is labeled, which may be due to an interaction of HCVpp and a lipid component of oxLDL. However, attempts to detect such an association using lipophilic fluorescent DiO as a noncovalently bound tracer failed to show any interaction (data not shown). The infectivity of HCVpp that had been incubated with and then separated from oxLDL by gradient analysis was markedly reduced (Fig. 5B). This suggests a direct inactivation of viral infectivity by oxLDL that persists even after the virus and oxLDL had been separated. However, when HCVpp was incubated with oxLDL and then diluted in media without oxLDL to a point where the oxLDL concentration is biologically ineffective, viral infectivity was unaltered (Fig. 5D), arguing against an irreversible inactivation of the virus. We cannot definitively explain this difference; however, it is feasible that oxLDL-treated virus is more sensitive to inactivation in the environment that it is subjected to during sucrose gradient analysis.

We propose that oxLDL inhibition of HCVpp and HCVcc infectivity involves a ternary interaction between virus, SR-BI, and oxLDL, requiring the simultaneous presence of all 3 agents. An interaction of oxLDL with both virus and cell would maintain the validity of the findings that show oxLDL alters the biophysical properties of HCVpp (suggesting an interaction with the virus) and the association between cell binding of lipoprotein components and inhibitory activity (suggesting an interaction with the target cell). However, better tools to experimentally dissect the different steps of the cell entry process may be required. We may then be able to determine a more complete pathogenesis and discover at what entry step oxLDL inhibits HCV infectivity. Moreover, whether the same entry routes are used by HCV *in vivo* remains to be defined.

In vivo oxLDL is generated via oxidative modification of native LDL in the subendothelial space in a setting of atherosclerosis (see Lusis[43] for a review), and its levels are increased in cardiovascular disease.[44,45] The heterogeneous nature of oxLDL *in vivo* makes comparative studies difficult; however, levels close to the IC50 observed in our assay have been reported in healthy volunteers.[45] An agent capable of blocking cell entry by diverse HCV strains would be of great clinical value in the setting of transplantation for HCV-induced end-stage liver disease. Presently, outcome after transplantation is unfavorable because of universal and rapidly progressing reinfection.[46] A means of blocking HCV cell entry during the perioperative period might prevent reinfection of the graft. The potency and specificity of oxLDL as well as its ability to inactivate a broad range of HCV genotypes provide a valuable tool. We can use this to study the molecular mechanisms of HCV cell entry as well as develop therapeutics targeting the early steps in the HCV life cycle.

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