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Characterization of the inhibition of hepatitis C virus entry by *in vitro* generated and patient derived oxidized low density lipoprotein

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List of abbreviations

HCV – hepatitis C virus; LDL – low density lipoprotein; oxLDL – oxidized low density lipoprotein; sE2 – soluble glycoprotein E2; SR-BI - scavenger receptor class B type I;

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Abstract

Oxidized low density lipoprotein (oxLDL) has been reported as an inhibitor of hepatitis C virus (HCV) cell entry making it the only known component of human lipid metabolism with an anti-viral effect on HCV. However, several questions remain open including its effect on full length cell culture grown HCV (HCVcc) of different genotypes or on other steps of the viral replication cycle, its mechanism of action and whether endogenous oxLDL shares the anti-HCV properties of *in vitro* generated oxLDL. We combined molecular virology tools with oxLDL serum measurements in different patient cohorts to address these questions.

We found that oxLDL inhibits HCVcc at least as potently as HCV pseudoparticles. There was moderate variation between genotypes with genotype 4 appearing most oxLDL sensitive. Intracellular RNA replication and assembly and release of new particles were unaffected. HCV particles entering target cells lost oxLDL sensitivity with time kinetics parallel to anti-SR-BI but significantly earlier than anti-CD81 suggesting that oxLDL acts by perturbing the interaction between HCV and SR-BI. Finally, in chronically HCV infected individuals endogenous serum oxLDL levels did not correlate with viral load, but in HCV-negative sera high endogenous oxLDL had a negative effect on HCV infectivity *in vitro*.

In conclusion oxLDL is a potent pan-genotype HCV entry inhibitor that maintains its activity in the context of human serum and targets an early step of HCV entry.

Introduction

Chronic hepatitis C virus (HCV) infection affects an estimated 160 million people worldwide (1). It is a frequent cause of end-stage liver diseases and hepatocellular carcinoma and a common indication for liver transplantation. Treatment remains problematic due to side effects and high cost. A particular problem is graft re-infection that occurs immediately following liver transplantation for HCV-associated end stage liver disease and often leads to transplant hepatitis and graft loss (2).

The HCV replication cycle is intricately linked to host lipid metabolism (3). The production of infectious viral particles is dependent on the cellular very low density lipoprotein (VLDL) assembly machinery and infectious particles in the blood are reminiscent of VLDL in being of low density, triglyceride and cholesterol rich and tightly associated with apolipoproteins B and E (3). Three lipid transport molecules on the hepatocyte surface have been implicated in viral entry: Low density lipoprotein (LDL-) receptor mediates cellular uptake of HCV RNA, but may be non-essential for productive infection (4). Scavenger receptor class B type I (SR-BI) is essential for HCV infection *in vitro* (5) and discussed in more detail below. Finally, Niemann-Pick C1-like 1 (NPC1L1), has been shown to support HCV entry, but its exact role is as yet unclear (6). Cell surface factors essential for HCV entry but not directly involved in lipid trafficking include the tetraspanin CD81 (7), and the tight junction components Claudin-1 (8) and Occludin (9).

SR-BI is a 509 amino acid protein with two transmembrane domains and a single large extracellular loop that is highly expressed in the liver and in tissues producing steroid hormones (10). It is the key high density lipoprotein- (HDL-) receptor and mediates a flux of free cholesterol from the lipoprotein into the cellular membrane (10, 11). Besides HDL, SR-BI interacts with diverse other ligands including VLDL, native LDL, oxidized LDL (oxLDL),

lipopolysaccharides, Triglyceride-rich lipoprotein (TRL) remnants and serum amyloid alpha (SAA) through multiple binding sites (10, 12-15).

First proposed as an HCV-receptor because of its interaction with the viral glycoprotein E2 (16), SR-BI was subsequently shown to be essential for HCV entry in numerous studies (5, 9, 17, 18). Animal data indicate that SR-BI is also important for HCV infection *in vivo* (19) and an inhibitor of the HCV-SR-BI interaction is the only HCV entry inhibitor currently in clinical trials (20). Recent work has suggested that SR-BI fulfils at least two distinct functions during viral entry, one related to initial attachment and another during post-binding (5, 21, 22).

Among SR-BI ligands HDL has a modest enhancing effect on HCV entry (23), while oxLDL has been found to be a strong inhibitor (24). oxLDL is a modified form of LDL that is generated early on in the pathogenesis of atherosclerosis, when native LDL deposited in early atherosclerotic lesions ("fatty streaks") undergoes oxidative modifications (25). The exact nature and driving forces behind these modifications are incompletely understood (26), but they allow LDL to be taken up in great quantities by macrophages through an LDL-receptor-independent route creating foam cells, a hallmark of atherosclerotic lesions (27).

Previous work on oxLDL has almost exclusively relied on HCV pseudoparticles (HCVpp) and there may be important differences in the cell entry properties of HCVpp and full-length HCV particles grown in cell culture (HCVcc) (6). Moreover the mechanism of HCV entry inhibition by oxLDL is unclear. An SR-BI dependent mechanism of action has been suspected, but there is no experimental evidence for this. We undertook the present study with a three main objectives: to characterize the effect of oxLDL on the full HCV replication cycle, to test if oxLDL inhibition is indeed linked to SR-BI and to probe the anti-HCV properties of endogenous oxLDL present in patient sera.

Materials and Methods

LDL isolation and in vitro peroxidation

Isolation of the low density lipoprotein (LDL) fraction of human plasma was performed by sequential gradient ultracentrifugation (28). Briefly, the LDL fraction was dialysed at 4°C against 1x PBS and protein concentration was determined by the Bradford method. The native LDL (100 µg/ml) was incubated with 10 µmol/ml Copper (II)-sulfate for 24 hours at 4°C. LDL peroxidation was stopped by adding 20 µmol/l butylhydroxytoluol and 5mmol/l EDTA. Oxidation state was determined by the detection of conjugated diene formation by measuring UV absorbance at 234 nm (29).

Virus production and infection assay

Firefly reporter (CSFlucW2) lentiviral pseudoparticles were generated as described (30). Production of HCVcc was performed by electroporation of Huh-7.5 cells with 5µg RNA of a full length HCV genome as previously described (31). Supernatant was harvested 48 hours post transfection. Infections were performed in the presence or absence of oxLDL mostly at a concentration corresponding to the IC₉₀ of that specific batch of oxLDL (range 10 to 50 µg/ml). Activity varies from batch to batch most likely due to the oxidation state of the particular batch. Infection was quantified after 72 hours. For the TCID₅₀ assay infected cells were stained with a NS5A antibody (9E10, a kind gift from C. Rice). Luciferase assay was performed as described (32). Intra- and extracellular HCV core was measured by a commercially available ELISA (Architect HCV Ag Assay, Abbott Laboratories, USA).

oxLDL ELISA

Serum oxLDL levels were quantified using two commercial ELISA systems offered by Mercodia (Uppsala, Sweden) and Immundiagnostik (Bensheim, Germany) as indicated in the manufacturers' manuals.

Results

oxLDL inhibits infection of hepatitis C virus across genotypes

oxLDL but not native LDL inhibits HCVcc of genotype 2a (Fig. 1A). Using a single batch of oxLDL we found similar IC_{50} of 0.74 ± 0.35 and 0.40 ± 0.22 $\mu\text{g/ml}$ for HCVcc and HCVpp, respectively (Fig. 1B). As expected, there was some batch-to-batch variation in the IC_{50} most likely reflecting the degree of oxidation of a given batch. Chimeras of the structural region of genotypes 1a to 7a and the JFH1 non-structural region (33) showed a clear inhibitory effect of oxLDL treatment on all genotypes (Fig. 1C). There was limited inter-genotype variation with genotype 6 appearing least and genotype 4 most sensitive when using the same multiplicity of infection (MOI) (Fig. 1D). Thus oxLDL is a potent inhibitor of HCV infection *in vitro* with pan-genotype activity.

oxLDL inhibits high and low density fractions of HCV

Next we assessed the effect of oxLDL on different density fractions of HCVcc isolated by ultracentrifugation over a density gradient. In all fractions containing detectable infectivity (more than 2 TCID₅₀/ml, i.e. fractions 1-7 corresponding to 1.03 – 1.18 g/ml) we observed inhibition by oxLDL (Fig. 2 and Suppl. Fig. 1). In the lowest density fraction (fraction 1 corresponding to 1.03-1.05 g/ml) the degree of oxLDL inhibition appeared slightly lower, however, this fraction also contained low baseline infectivity compared to the following fractions, which may have impaired our ability to quantify the degree of inhibition (Suppl. Fig. 1).

oxLDL has no effect on HCV RNA replication or on assembly and release of new viral particles

In Huh-7.5 cells transfected with Fluc-Jc1 virus RNA application of oxLDL or the highly potent antibody C167 directed against SR-BI had no effect on RNA replication (Fig. 3A). Moreover, intracellular core and core released from transfected cells was not different (Fig. 3B). In addition, we performed a TCID₅₀ assay using the supernatant from oxLDL treated or untreated producer cells and found that similar infectious titers were released (Fig. 3C). Thus the inhibitory effect of oxLDL on HCVcc appears solely due to an inhibition of viral entry.

oxLDL treatment does not affect protein expression level of HCV entry receptors

Previously, it was reported that oxLDL inhibition of HCV entry is not mediated through competition for the E2 binding site on SR-BI (24). Another conceivable mechanism for the observed entry inhibition would be down regulation of an essential cellular HCV entry factor. We performed western blot and FACS analyses to determine the expression levels of five known HCV entry factors (SR-BI, CD81, CLDN1, OCLN and NPC1L1) in untreated and oxLDL treated Huh-7.5 cells and found similar expression levels in all cases (Fig. 4A+B). These findings argue against altered expression of a cellular entry factor being responsible for oxLDL inhibition of HCV entry.

oxLDL inhibition parallels anti-SR-BI inhibition

In a time course experiment with synchronized infection of target cells by multiple virions and timed addition of inhibitors oxLDL lost its effect in parallel to anti-SR-BI (34) and

significantly earlier than anti-CD81 (Fig. 5). This suggests that oxLDL mediates entry inhibition in a SR-BI dependent manner.

We next tested the effect of oxLDL on two HCV variants that have been reported to less dependent on SR-BI compared to wildtype HCV. The IC_{50} for oxLDL inhibition of the Δ HVR1 variant where the entire hypervariable region 1 (HVR1) has been deleted from the E2 glycoprotein (35) was about 18-fold higher compared to unmodified HCV (Fig. 6A), but this observation did not reach statistical significance. The IC_{50} for oxLDL against the G451R point mutant (36) was similar to wildtype (Fig. 6B).

oxLDL inhibition is maintained in the presence of altered SR-BI levels

Next we were interested if modulation of cellular SR-BI levels would affect the sensitivity of HCV to oxLDL. When cellular SR-BI expression levels were increased by overexpression or decreased by RNA interference no major change in oxLDL sensitivity was seen (Suppl. Fig. 2).

oxLDL inhibition is maintained in the presence of SR-BI variants

Since SR-BI is a multi-ligand receptor, we hypothesized that it might be possible by deleting the oxLDL binding site from SR-BI to generate a variant that functions as an HCV receptor but is insensitive to oxLDL inhibition. The location of the oxLDL binding site in SR-BI is unknown, but for the closely related CD36 it has been reported (37). Alanine scanning of the homologous region in SR-BI (aa 154 to 183) was performed by replacing blocks of five amino acids with alanine residues and expressing wild-type SR-BI or variants in Huh-7.5. Overexpression of wild-type SR-BI resulted in a 2- to 3-fold enhancement of HCV infection as

previously reported (18, 31) while SR-BI variants containing alanine-blocks in the putative oxLDL binding site region did not - suggesting that they are non-functional as HCV receptors (Suppl. Fig. 3). Moreover, oxLDL inhibition was maintained in all cases. Thus we were not able to generate an SR-BI variant that is functional as an HCV receptor yet oxLDL insensitive.

Next we were interested to see if the sensitivity of HCV to oxLDL would be changed in the presence of SR-BI variants with altered E2 binding properties. Thus we overexpressed murine SR-BI (mSR-BI) that has been reported to not bind sE2 yet support HCV infection and two point mutants where individual amino acids from mSR-BI were introduced into human SR-BI. From the set of point mutants generated by Catanese and colleagues (34) we chose the ones with the reportedly highest (S101A) and lowest (E210A) sE2 binding. When overexpressed in Huh-7.5 cells mSR-BI and the point mutants but not CD36 showed enhanced HCV entry with maintained oxLDL inhibition (Fig. 7A+B). Similar results were observed when the molecules were expressed in a Huh-7.5 subclone where endogenous SR-BI expression was down regulated by RNA interference (Fig. 7C+D). When overexpressed in CHO cells human SR-BI, the points mutants and mSR-BI appeared to bind HCVcc (Suppl. Fig. 4A). However, as previously reported by others, neither mSR-BI nor CD36 bound sE2 (Suppl. Fig. 4B).

Endogenous oxLDL has no effect on virus load but correlates with in vitro infectivity

Low levels of oxLDL are present in human serum although its exact chemical composition is incompletely defined (38). Using the Mercodia ELISA assay we measured serum oxLDL levels in three different patient cohorts: healthy volunteers (n = 29), chronically hepatitis B virus (HBV) infected individuals (n = 36) and chronically HCV infected individuals (n = 67; Suppl.

Table 1). In HCV infected individuals with a high viral load (>800,000 IU/ml) oxLDL levels were slightly lower, but this was not statistically significant (Fig. 8A). However, when sera from the healthy cohort were heat inactivated and added at 1:10 final dilution to an *in vitro* infection of Huh-7.5 cells with HCVcc (Fluc-Jc1) infectivity was significantly reduced in the third of individuals with the highest serum oxLDL compared to the lowest and middle third (Fig. 8B). oxLDL measurements using a second commercial oxLDL ELISA offered by Immundiagnostik did not correlate with the measurements of the Mercodia assay (Suppl Fig. 5) and hence failed to show the same correlation. However, using the Mercodia system, the observation that high endogenous oxLDL levels contained in human serum reduce HCV infectivity *in vitro* was reproduced in the HBV infected cohort (Fig. 8C). In a reverse experiment we found that *in vitro* generated oxLDL retains its inhibitory effect in the presence of human serum (data not shown). Thus both *in vitro* generated and endogenous oxLDL seem to have an anti-HCV effect and are active in the context of human serum.

Discussion

We found that oxLDL is an effective inhibitor of HCV cell entry across a broad range of genotypes. Moreover, we provide evidence suggesting that it acts through an SR-BI dependent mechanism and that both *in vitro* generated and endogenous oxLDL have anti-HCV activity.

Our data show that oxLDL is an entry inhibitor that is equally or more active against HCVcc compared to HCVpp and does not affect RNA replication or viral particle assembly and release. Moreover, oxLDL inhibits all HCV genotypes although the effect may be more pronounced for some genotypes such as 4. This would be of some interest, since genotype 4 is difficult to treat with currently available regimens.

When the inhibitory effect of oxLDL on HCV was discovered, it was thought likely that oxLDL perturbs the interaction between HCV and SR-BI on grounds that oxLDL is a known ligand of SR-BI (24). However, there was no experimental evidence supporting this assumption and, in fact, binding of sE2 to SR-BI was found to occur normally in the presence of oxLDL. We can now show that the timing when infecting virions become inhibitor insensitive is parallel for oxLDL and anti-SR-BI and occurs significantly earlier compared to anti-CD81.

The IC_{50} of oxLDL against two HCV mutants with reduced – but not absent – SR-BI dependency, Δ HVR1 (35) and G451R (36) did not differ significantly from wildtype. However, in the case of Δ HVR1 numerically the IC_{50} was markedly (18-fold) higher compared to wildtype. We believe, there may in fact be a true difference in oxLDL sensitivity between Δ HVR1 and wildtype, but given the variability of the assay our experiments were not sufficiently powered to prove or refute this.

While deletion of the entire HVR1 is a drastic alteration to the viral glycoprotein, Δ HVR1 is in our hands a variant with only slightly (about 5-fold) decreased titers (35). Interestingly,

although Δ HVR1 and G451R share a decreased sensitivity to anti-SR-BI sera and increased sensitivity to soluble CD81. Of note, their major difference – G451R but not Δ HVR1 directly bind SR-BI – has been shown only with sE2 but not complete virions (35, 36).

Given that SR-BI is thought to act at more than one step of the HCV entry process (21, 22), further work will clearly be needed to define the exact effects of the different viral variants and inhibitors of the virus-SR-BI interactions. In addition, it is interesting to speculate how oxidative modification of apolipoproteins such as apoE and apoC1 now known to be associated with HCV would affect HCV infectivity.

In samples from chronically HCV infected individuals we did not observe a significant correlation between serum oxLDL and viral load. Given that *in vivo* HCV viral load varies between and within individuals over a wide range and is likely influenced by a multitude of variables this is not very surprising. Differently from our data, a recent study reported a positive correlation between oxLDL and HCV viral load (39), however, it appears that this hinged strongly on a small number of outliers with very high oxLDL levels and viral load while the majority of the population was similar to our cohort showing a weak negative correlation that likely falls short of statistical significance.

Human sera with a high oxLDL content as measured by the Mercodia ELISA system was associated with a mild inhibitory effect on HCV infectivity *in vitro*. This observation was made in two independent cohorts, one healthy and one chronically infected with HBV. Although correlation does not prove causality, we see this as suggestive that endogenous oxLDL has an inhibitory effect on HCV entry similar to *in vitro* generated oxLDL. Most studies investigating the biological effects of oxLDL including ours use copper oxidized LDL as it is thought to share many properties of endogenous oxLDL (25). However, oxidation likely introduces a multitude of chemical alterations to all parts of the LDL particle and to what

extent these are truly alike in copper oxidized and endogenous oxLDL is unknown. Our observation that the available oxLDL ELISA systems offered by Mercodia and Immundiagnostik do not correlate is in line with what has been reported by others (40) and underscores the complex nature of oxLDL.

The introduction of NS3/4A protease inhibitors into HCV therapy has improved response rate for genotype 1 infection but side effects, contraindications, drug-drug interactions, selection of resistant variants and high cost remain significant shortcomings. Moreover, HCV entry inhibitors would be useful to prevent the presently universal graft re-infection after liver transplantation. ITX 5061, an SR-BI inhibitor with an unclear mechanism of action, is in phase 1 and currently the only HCV entry inhibitor in clinical trials (www.clinicaltrials.gov). The published *in vitro* data indicates up to 90% inhibition which is likely less than what can be achieved with oxLDL (20). Given its chemical complexity and potential involvement in the pathogenesis of atherosclerosis oxLDL is an unlikely therapeutic lead compound. However, a smaller chemically defined compound that mimics its anti-HCV mechanism of action would be an attractive lead. Encouragingly, we have previously shown, that oxidized phospholipids are similarly effective compared to whole oxLDL particles (24).

In conclusion, oxLDL is a potent SR-BI dependent pan-genotype HCV entry inhibitor that maintains its activity in the context of human serum. Further studies should address whether its mechanism of action can be mimicked by smaller chemically defined compounds and whether these show promise as leads for anti-HCV therapy.

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Figure legends

Fig. 1: oxLDL inhibits HCV of all genotypes.

(A) Naïve Huh-7.5 cells were inoculated with Fluc-Jc1 with native LDL (nLDL) or oxLDL. Bars represent means $\pm$ standard deviation of $n=4$ measurements done within a representative of several independent experiments. **(B)** Huh-7.5 were inoculated with HCVcc or HCVpp with the indicated concentrations of oxLDL. Infectivity in the absence of oxLDL is set to 100%. Individual values represent mean of 3 experiments. **(C)** In a TCID₅₀ assay Huh-7.5 cells were inoculated with supernatant containing different HCV genotypes with or without oxLDL (20 $\mu\text{g/ml}$). A representative of three independent experiments is shown. **(D)** Naïve Huh-7.5 cells were infected with a similar MOI of HCV genotype 4a or 6a plus different oxLDL concentrations (0, 0.1, 1, 10, 50 $\mu\text{g/ml}$). A representative of three independent experiments is shown.

Fig. 2: oxLDL inhibits different density fractions of HCV.

oxLDL (20 $\mu\text{g/ml}$) inhibition of HCVcc contained in fractions of a density gradient ranging from very low (fraction 1; approx. 1.03 g/ml) to high (fraction 7; approx. 1.17 g/ml). Infectivity in the respective serum fractions without the addition of oxLDL is set to 100%. Means of three independent experiments are shown.

Fig. 3: oxLDL has no effect on non-entry steps of viral replication cycle.

(A) Luciferase activity in Huh-7.5 cells transfected with Fluc-Jc1 RNA with or without oxLDL (10 $\mu\text{g/ml}$) or anti-SR-BI C167 (1 $\mu\text{g/ml}$) ($n=4 \pm$ SD). **(B)** 48h hours post transfection supernatant was harvested and intra- and extracellular HCV core was quantified. A

representative of two independent experiments is shown. **(C)** Supernatant was further used to infect naïve Huh-7.5 cells in a TCID₅₀ assay. A representative of two independent experiments is shown.

Fig. 4: Protein expression levels of known HCV entry factors are not affected by oxLDL.

Huh-7.5 cells seeded 24h previously were treated with oxLDL (20 µg/ml) for four hours. **(A)** Cells were then lysed and expression of SR-BI, CLDN1, OCLN and NPC1L1 were determined by immunoblotting. **(B)** Expression of CD81 was measured by FACS. A representative of three independent experiments is shown.

Fig. 5: oxLDL inhibition parallels inhibition by anti-SR-BI

To study cell entry kinetics Huh-7.5 cells were inoculated on ice and inhibitors added at defined time points as described in the supplemental methods section.

Fig. 6: oxLDL inhibits HCV variants with reduced SR-BI-dependency.

Huh-7.5 cells were inoculated with supernatant containing Fluc-Jc1 and either **(A)** Fluc-Jc1/ΔHVR1 or **(B)** the Jc1/G451R variant plus different concentration of oxLDL.

Fig. 7: The inhibitory effect of oxLDL is maintained in SR-BI receptor variants.

(A) SR-BI protein levels in Huh-7.5 cells were transduced with different SR-BI receptor variants. **(B)** Fluc-Jc1 infection of Huh-7.5 expressing SR-BI variants with or without oxLDL (50 µg/ml). **(C)** SR-BI expression and **(D)** infectivity with or without oxLDL in Huh-7.5 cells harbouring a regulated shRNA against SR-BI (7.5/SR-BIkd). A (+) indicates that the shRNA is switched on.

Fig. 8: oxLDL serum level has an effect on *in vitro* infectivity but not on viral load.

(A) In serum samples of chronically HCV infected patients (n = 67) oxLDL level was determined using the Mercodia ELISA. Viral loads above 800,000 IU/ml were considered high. **(B+C)** oxLDL content and modulation of *in vitro* Fluc-Jc1 infectivity of serum samples from **(B)** healthy (n = 29) and **(C)** and chronically HBV infected (n = 36) individuals. Heat inactivated sera were added at a 1:10 dilution together with Fluc-Jc1 to Huh-7.5 cells. Individuals were grouped according to their oxLDL levels into a low, middle and high third and infectivity was compared between groups.

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