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Electronegative LDL is linked to high-fat, high-cholesterol diet–induced nonalcoholic steatohepatitis in hamsters☆,☆☆

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Abstract

The pathogenesis of nonalcoholic steatohepatitis (NASH), like that of atherosclerosis, involves lipid accumulation, inflammation and fibrosis. Recent studies suggest that oxidized LDL (oxLDL) may be a risk factor for NASH, but oxLDL levels were not directly measured in these studies. The aim of this study was to examine whether there was an association between electronegative LDL [LDL(−)], a mildly oxLDL found in the blood, and the development of NASH using two animal models. Golden Syrian hamsters and C57BL/6 mice were fed a high-fat, high-cholesterol (HFC) diet for 6 or 12 weeks, then liver lipid and histopathology, plasma lipoprotein profile and LDL(−) levels were examined. The HFC-diet-fed hamsters and mice had similar levels of hepatic lipid but different histopathological changes, with microvesicular steatosis, hepatocellular hypertrophy, inflammation and bridging fibrosis in the hamsters, but only in mild steatohepatitis with low inflammatory cell infiltration in the mice. It also resulted in a significant increase in plasma levels of LDL cholesterol and LDL(−) in hamsters, but only a slight increase in mice. Moreover, enlarged Kupffer cells, LDL(−) and accumulation of unesterified cholesterol were detected in the portal area of HFC-diet-fed hamsters, but not HFC-diet-fed mice. An *in vitro* study showed that LDL(−) from HFC-diet-fed hamsters induced TNF-α secretion in rat Kupffer cell through a LOX-1-dependent pathway. Our results strongly suggest that LDL(−) is one of the underlying causes of hepatic inflammation and plays a critical role in the development of NASH.

Keywords: Electronegative LDL (LDL(−)); Hamster; Hepatic inflammation; Nonalcoholic steatohepatitis (NASH); Lectin-like oxidized low-density lipoprotein (LDL) receptor-1 (LOX-1)

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide. Simple steatosis is usually a benign and reversible condition, but some patients progress to nonalcoholic steatohepatitis (NASH) with inflammation and fibrosis and may then progress to end-stage liver disease [1]. A two-hit hypothesis has been proposed for the transition from simple steatosis to NASH and fibrosis [2]. In this hypothesis, the first hit refers to obesity (or metabolic factor-induced accumulation of lipids in the liver) and the second to proinflammatory cytokine (or oxidative stress-induced liver injury). However, the factors that trigger the second insult remain unclear. NASH shares many features in common with cardiovascular disease, including lipid accumulation, macrophage activation and infiltration and inflammation. Results of recent animal studies have suggested that dietary cholesterol is a critical factor in induction of NASH and showed that *Ldlr*$^{-/-}$ mice are more vulnerable to cholesterol-induced NASH than wild-type mice [3–6]; this could be due to high dietary

Abbreviations: α-SMA, α-smooth muscle actin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HE, hematoxylin–eosin; HFC, high-fat, high-cholesterol; LDL(−), electronegative LDL; MPO, myeloperoxidase; nLDL, native LDL; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; oxLDL, oxidized LDL; PBS, phosphate-buffered saline; TC, total cholesterol; TG, triglyceride.

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cholesterol inducing higher circulating levels of LDL in *Ldlr*$^{-/-}$ mice than in wild-type mice [7]. Rabbits are also susceptible to dietary cholesterol-induced hypercholesterolemia and have been shown to develop NASH with extensive fibrosis as a result of a high-cholesterol diet [8]. Compared to mice, hamsters have higher plasma LDL levels and lower HDL levels, a situation closer to that in humans, and are susceptible to high-cholesterol diet–induced hypercholesterolemia [9,10]. Wang *et al*. showed that feeding hamsters a high-fat, high-cholesterol (HFC) diet results in diabetic hyperglycemia, insulin resistance and dyslipidemia [11]. We have previously observed a substantial increase in plasma LDL level and accumulation of cholesteryl ester in the liver of HFC-diet-fed hamsters [12,13]. These results suggest that the hamster might be a promising model for studying hypercholesterolemia and the development of NASH.

There is a growing body of evidence that oxidized LDL (oxLDL) may play a role in the pathophysiology of NASH. Yimin *et al*. demonstrated that iv injection of oxLDL to mice fed a high-fat diet induces liver injury, inflammatory cell infiltration and fibrosis, typical histological features of NASH [14]. Another group reported that induction of IgM antibodies against oxLDL ameliorates HFC-diet-induced NASH in *Ldlr*$^{-/-}$ mice [15] and that genetic deletion of two scavenger receptors, SR-A and CD36, which are responsible for oxLDL uptake in macrophages, reverses hepatic inflammation, apoptosis and fibrosis in *Ldlr*$^{-/-}$ mice [16]. However, oxLDL levels were not measured in these studies. Electronegative LDL [LDL(−)], a mildly oxidized form of LDL found in the blood, shares many features with oxLDL. Both induce release of inflammatory cytokines by macrophages, monocytes and endothelial cells [17,18]. LDL(−) levels in the blood are higher in patients with hypercholesterolemia, diabetes, severe renal disease and coronary syndromes than in healthy individuals, and LDL(−) is therefore considered a biomarker of cardiovascular risk [18]. Since NASH is associated with increased incidence of cardiovascular disease [19], it is possible that LDL(−) is involved in the development of NASH.

In the present study, we compared development of NASH in hamsters and mice fed an HFC diet. The results showed that HFC-diet-fed hamsters developed severe NASH with bridging fibrosis, while HFC-diet-fed mice developed steatosis with mild inflammation, and that the HFC-diet-fed hamsters had significantly higher plasma levels of cholesterol and LDL(−) than the HFC-diet-fed mice. We also demonstrated accumulation of LDL(−) in the portal region in HFC-diet-fed hamsters, but not HFC-diet-fed mice. These results suggest that LDL(−) acts as a link between atherogenic lipoproteins and the development of NASH.

2. Materials and methods

2.1. Animals and diets

For the *in vivo* studies, 8-week-old male C57BL/6 mice and golden Syrian hamsters, obtained from the National Laboratory Animal Center (Taipei, Taiwan), were housed in colony cages (3 per cage) on a 12-h light cycle (0800–2000 h). The procedures for the animal study were reviewed and approved by the National Taiwan University Institutional Animal Care and Use Committee. The mice and hamsters were assigned to either a control group fed with standard chow diet (Test Diet, IN, USA) or a group fed an HFC diet (chow diet supplemented with 11.5% coconut oil, 11.5% corn oil and 1% cholesterol) according to Wang *et al*. [11]. All animals were given free access to the experimental diet and tap water for 6 or 12 weeks, then food was withheld for 14 h. Blood samples were collected in tubes containing EDTA and centrifuged at 1400*g* at 4°C for 10 min, and the plasma was transferred to new tubes and stored at 4°C. The liver was excised and divided into three parts, one of which was fixed in 4% paraformaldehyde and embedded in paraffin, while the other two were either embedded in optimum cutting temperature compound (Sakura, NL, USA) or snap-frozen in liquid nitrogen, then both were stored at −80°C for further analysis.

2.2. Plasma biochemistry

Plasma levels of total cholesterol (TC), triglyceride (TG), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using enzymatic assay kits (Randox, Crumlin, UK).

2.3. Lipoprotein isolation and analysis of negatively charged LDL

The plasma lipoprotein profile in the mice and hamsters was determined by gel-filtration chromatography on a Superose 6HR 10/30 column at a flow rate of 0.25 ml/min in phosphate-buffered saline (PBS) and 0.5-ml fractions were collected and the cholesterol concentration in each fraction was measured enzymatically as described above. oxLDL has been shown to be negatively charged and can be isolated using this characteristic, as described in Ref. [20]. To isolate plasma LDL and LDL(−), plasma LDL was first isolated by NaBr density gradient ultracentrifugation, first at a density of 1.030 kg/L to remove VLDL then at 1.063 kg/L to isolate LDL [21], as described previously [21]. For LDL(−) isolation, LDL was dialyzed for 24 h against buffer A [0.02 mol/L Tris–HCl (pH 8.0) and 0.5 mmol/L EDTA], then 1 ml of LDL solution was loaded onto a UnoQ6 column (BioRad, CA, USA) preequilibrated with buffer A and eluted at a flow rate at 0.5 ml/min with a multistep gradient of buffer B (1 mol/L NaCl in buffer A) using two P-500 pumps controlled by an AKTA system (GE Pharmacia, USA) as described in Ref. [20]. The effluent was monitored at 280 nm, and chromatography was carried out in a 4°C cold room.

2.4. Hepatic lipid analysis

Liver lipids were extracted using the method of Folch *et al*. [22], then aliquots of the extract were transferred to glass test tubes and dried under nitrogen and levels of TG and TC determined using enzymatic kits as described above. To examine accumulation of unesterified cholesterol in the liver, cryosections were incubated for 2 h at room temperature with 0.25 mg/ml of filipin as described by Ioannou *et al*. [23] and photographed using a digital camera (SPOT Imaging Solutions, Diagnostic Instruments Inc.).

2.5. Histological and immunohistochemical analyses

Paraffin sections of liver tissue (4 μm) were stained with hematoxylin–eosin (HE) or Masson's trichrome stain to evaluate histopathological changes and tissue fibrosis and were processed for immunohistochemical staining for α-smooth muscle actin (α-SMA), as follows. The sections were deparaffinized and rehydrated, endogenous peroxidase was quenched using 3% hydrogen peroxide solution and antigen retrieval was performed in boiling Tris–EDTA buffer for 30 min. The sections were then blocked by incubation at 37°C for 1 h with citrate buffer [2 mM sodium citrate and 8.2 mM trisodium citrate dehydrate (pH 7.4)] containing 10% FBS (Hyclone Laboratories, Logan, UT, USA) and 1% normal horse serum and then were incubated overnight at 4°C with monoclonal antibody against α-SMA (DAKO, Glostrup, Denmark) then with biotinylated horse antimouse Ig (Vectorstain, CA, USA) and visualized using the avidin biotin complex method (Vectorstain, CA, USA).

Analysis of oxLDL and Kupffer cells was performed by immunofluorescence staining of cryosections. After fixation with acetone, the sections were incubated overnight at 4°C with rat monoclonal antibodies against CD68 (marker for hepatic macrophages; Serotec, Oxford, UK) or ApoB (Academy Biomedical, Houston, USA) or polyclonal goat antibodies against oxLDL (Merck, NJ, USA) or rabbit antibodies against myeloperoxidase (MPO) (Abcam, Cambridge, UK). Appropriate secondary antibodies used in various experiments included Alexa Fluor 594 donkey antirat IgG antibodies (Molecular Probes, Oregon, USA), Alexa Fluor 488 donkey antirabbit IgG antibodies or Alexa Fluor 594 donkey antigoat IgG antibodies (Molecular Probes, Oregon, USA). DAPI was used to stain nuclei.

2.6. Liver perfusion

To detect ApoB and LDL(−) in Fig. 5, the liver in the HFC-fed hamsters was perfused at 37°C for 5 min *via* the portal vein with PBS to remove residual blood in the hepatic sinusoids before removal, as described previously [24].

2.7. Isolation and culture of rat Kupffer cells

Kupffer cells were isolated from 8- to 10-week-old male F344 rats (about 250 g). Rat was anesthetized with intraperitoneal (ip) injection of ketamine (87 mg/kg body weight) plus xylazine (13 mg/kg body weight). The abdomen of anesthetized rat was opened to reveal the location of the portal vein. The liver was perfused *in situ* with perfusion buffer (0.9 mM $MgCl_2$, 0.5 mM EDTA and 25 mM Hepes in HBSS) at a flow rate of 15 ml/min for 10 min followed by collagenase solution (1000 U in 300 ml perfusion buffer) through the portal vein. Then, the liver was excised, cut into small pieces and digested by collagenase type IV in perfusion buffer (Sigma, St. Louis, MO, USA) at 37°C. After incubation for 30 min, the liver homogenate was filtered with a cell strainer (100 μm) and the filtrate was transferred into new tubes and washed and centrifuged twice at 600*g* for 5 min at 4°C and the supernatant was collected to obtain nonparenchymal cells. Kupffer cells were isolated by sedimentation of the supernatant in a two-step Percoll gradient at 1000*g* for 15 min. The middle layer was collected and washed with HBSS (5.33 mM KCl, 0.44 mM KH_2PO_4, 138 mM NaCl, 4 mM $NaHCO_3$, 0.3 mM Na_2HPO_4 and 5.6 mM glucose). Cells were centrifuged and resuspended with complete culture medium (RPMI-1640 medium supplemented with 10% FBS, 1% penicillin and 1% streptomycin) and seeded at 5×10^5 cells/well in 24-well plates. Following incubation for 2 h, the cells were gently washed with fresh culture medium.