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Chylomicron remnant model emulsions induce intracellular cholesterol accumulation and cell death due to lysosomal destabilization

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ABSTRACT

Chylomicron remnants, which carry dietary fats and cholesterol, play a role in promoting atherosclerosis. Chylomicron remnants are characterized by high cholesterol content at the surface, different from low-density lipoproteins (LDLs) containing high amounts of esterified cholesterol (CE) in the core. We prepared cholesterol-rich emulsions (TO–PC/cholesterol emulsions) as models for chylomicron remnants and compared their effects on J774 macrophages with acetylated-LDL (ac-LDL). Internalization of TO–PC/cholesterol emulsions into macrophages reduced cell viability, whereas ac-LDL did not. Surprisingly, there was no difference in intracellular free cholesterol content between cells incubated with TO–PC/cholesterol emulsions and with ac-LDL. Furthermore, cholesterol in TO–PC/cholesterol emulsions and ac-LDL both were internalized into J774 macrophages; however, incubation with TO–PC/cholesterol emulsions induced leakage of lysosomal protease, cathepsin-L, to cytosol, which was not observed for incubation with ac-LDL. Inhibition of the activity of cathepsin-L recovered the viability of macrophages that ingested TO–PC/cholesterol emulsions. We suggest an alternative fate of cholesterol-rich emulsions taken up by macrophages, which is different from other atherogenic lipoproteins rich in CE; internalization of TO–PC/cholesterol emulsions into macrophages induces rapid free cholesterol accumulation in lysosomes and cell death due to lysosomal destabilization.

1. Introduction

Accumulation of excess cholesterol derived from lipoproteins in arterial macrophages is one of the early events in the progression of atherosclerosis [1–3]. It is well known that macrophages storing a large amount of esterified cholesterol (CE), called 'foam cells', are present in atherosclerosis lesions [4]. Dietary fats and cholesterol are incorporated into chylomicrons, which are synthesized by intestinal mucosal cells and contain apolipoprotein B48 (apoB48) [5]. Chylomicrons undergo hydrolysis of triglycerides by lipoprotein lipase in the capillary beds of peripheral tissue [6,7], and as a result, smaller particles, referred to as chylomicron remnants, are produced. Unlike LDL, which is rich in CE [8], the most remarkable feature of chylomicron remnants is represented by containing a large amount of free cholesterol at the surface [5,6].

Epidemiological studies have shown that patients with postprandial hyperlipemia have premature clinical signs of atherosclerosis [9–13]. Delayed clearance of chylomicrons is a cause of hypertriglyceridaemia and increases its atherogenicity [14]. Weinstein et al. have demonstrated that mice manifesting severe chylomicronemia develop progressive aortic atherosclerosis [15]. ApoB48 carrying lipoproteins are present in human atherosclerotic plaques with denatured or degraded structure [16]. Apolipoprotein E (apoE) plays a crucial role in the metabolism of remnant lipoproteins through specific interactions with cell-surface heparan sulfate proteoglycans (HSPGs) and receptors including LDL receptor and LDL receptor-related protein (LRP) [17,18]. The major route of chylomicron remnant uptake into macrophages occurs via LRP, while LDL receptor and scavenger receptors may play minor roles [19]. HSPGs participate in the remnant uptake by associating with LRP or acting alone as receptors [20]. Similar to scavenger receptors, HSPGs and LRP are not suppressed by intracellular cholesterol content [21]. Chylomicron remnants are cytotoxic to macrophages and smooth muscle cells [22]. Numerous studies have identified apoptosis as a prominent feature of atherosclerosis. It has been suggested that macrophage apoptosis is a crucial determinant of lesion development and causes necrotic core formation, which promotes inflammation,

Abbreviations: ACAT, acyl CoA:cholesterol acyltransferase; ac-LDL, acetylated LDL; apoE, apolipoprotein E; BSA, bovine serum albumin; CE, esterified cholesterol; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; FBS, fetal bovine serum; HSPGs, heparan sulfate proteoglycans; LDL, low density lipoprotein; LRP, low density lipoprotein receptor-related protein; NBD-cholesterol, 22-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol; NPC1, Niemann–Pick C1; PBS, phosphate-buffered saline; PC, phosphatidylcholine; TO, triolein; VLDL, very low density lipoprotein; Z–FF–FMK, benzyloxycarbonyl–phenylalanine–phenylalanine–fluoromethylketone

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plaque rupture, and thrombosis [23]. Macrophages in advanced atherosclerotic lesions accumulate large amounts of free cholesterol, which is a potent inducer of apoptosis [23–25]. We have previously reported that denatured apoB induces macrophage apoptosis by disturbance of the plasma membrane [26]. It has been shown that chylomicron remnant-like particles downregulate the pro-inflammatory chemokine and cytokine secretion by monocytes and macrophages [27,28]. More recently, it has been reported that ezetimibe and simvastatin increase the removal of chylomicron-like emulsions from plasma in coronary heart disease subjects [29]. However, the atherogenic mechanisms of chylomicron remnants are not clear.

To investigate the mechanism of chylomicron remnant-promoted atherosclerosis, we prepared triolein (TO)–phosphatidylcholine (PC)/cholesterol emulsion as a model for chylomicron remnants. In the previous study, we have shown that cholesterol enrichment on the surface of emulsions enhances the binding of apoE, but not apoC-III, by increasing separation between PC headgroups [30,31]. It has been shown that apoE-containing emulsion particles are taken up by HSPGs and LRP [30–32]. The apoE-dependent uptake of TO–PC/cholesterol emulsions into J774 macrophages is much higher than that of cholesterol-free (TO–PC) emulsions [30]. In addition, we have confirmed that uptake of massive TO–PC/cholesterol emulsions is cytotoxic [30]. In this study, we compared the effects of TO–PC/cholesterol emulsions with those of acetylated LDL (ac-LDL), with respect to the cellular content and distribution of cholesterol, and discussed their cytotoxic action.

2. Experimental procedures

2.1. Materials

Recombinant human apoE (isoform E3) and trypan blue were purchased from Wako Pure Chemicals (Osaka, Japan). Egg yolk PC, TO, cholesterol and serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO). 22-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3-ol (NBD-cholesterol) was purchased from Life Technologies (Eugene, OR). Human plasma LDL and cathepsin L inhibitor benzyloxycarbonyl–phenylalanine–phenylalanine–fluoromethylketone (Z–FF–FMK) were purchased from Calbiochem (San Diego, CA). All other chemicals used were of the highest reagent grade.

2.2. Preparation of emulsions

Emulsion particles were prepared using a high-pressure emulsifier (Nanomizer System YSNM-2000AR; Yoshida Kikai Co., Nagoya, Japan) by a method slightly modified from the previous report [33]. Briefly, a mixture of TO, PC, and cholesterol was suspended in 20 mM Hepes solution containing 5% glucose (pH 7.4), and successively emulsified under 100 MPa of pressure at 40–60 °C. Depending on the purpose of the experiment, NBD-cholesterol was added to the lipid mixture at a ratio of 0.75 mol% of cholesterol. After the removal of contaminating vesicles by ultracentrifugation, Hepes solution containing 5% glucose was substituted by Hepes buffer (137 mM NaCl, 6 mM KCl, 6.1 mM D-glucose, and 10 mM Hepes; pH 7.4), and emulsion particles were concentrated by ultrafiltration. The mean particle diameter of TO–PC and TO–PC/cholesterol emulsions was about 120 nm, determined from dynamic light scattering measurements using Photal FPAR-1000 (Otsuka Electronic Co., Osaka, Japan). The concentrations of TO, PC, and cholesterol were estimated using enzymatic assay kits purchased from Wako Pure Chemicals. The lipid composition of each emulsion was as follows: TO:PC = 5:1 (in mol) for TO–PC emulsions, and TO:PC:cholesterol = 15:3:2 for TO–PC/cholesterol emulsions. These emulsions were incubated with apoE for 30 min at 37 °C prior to loading to the cells [30].

2.3. Acetylation of LDL

LDL was acetylated (ac-LDL) by the method of Basu et al. [34]. One ml of 0.15 M sodium chloride solution containing LDL (2 mg protein/ml) was added to a saturated solution of sodium acetate (1 ml). Next, acetic anhydride (50 μl) was added dropwise under continuous stirring in an ice-water bath. The reaction solution was then dialyzed against Hepes buffer for 24 h. The resulting ac-LDL was passed through a syringe-operated filter (0.22 μm) purchased from Millipore (Bedford, MA). Depending on the purpose of the experiment, NBD-cholesterol was incorporated at a ratio of 0.75 mol% of total cholesterol by incubation at 37 °C.

2.4. Cell cultures

J774 macrophages were grown in a humidified incubator (5% CO_2) at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin. FBS was replaced with 0.1 or 1% BSA 15 min before each experiment. Experiments were performed in DMEM containing 0.1 or 1% BSA.

2.5. Cell viability assay

Cell viability was assayed by trypan blue dye exclusion [35]. Cells were incubated with emulsions (5 mM TO, 8 μg/ml apoE), LDL or ac-LDL (100 μg/ml) at 37 °C for 18 h. After incubation, emulsions were removed and replaced with 0.4% trypan blue solution. Macrophages were immediately examined for trypan blue uptake using an inverted microscope (CKX41; Olympus Co., Tokyo, Japan).

2.6. Cellular cholesterol quantification

After incubation with emulsions (5 mM TO, 8 μg/ml apoE), LDL or ac-LDL (100 μg/ml) at 37 °C for 18 h, cells were chilled on ice and washed twice with cold Hepes buffer containing 0.2% BSA and then washed twice with cold Hepes buffer alone. Cellular lipids were extracted by isopropanol directly added to culture dishes. The isopropanol containing the extracted lipids was treated by the method of Smart and Anderson [36]. Briefly, the extract was dried and dissolved in chloroform solution of 1% Triton X-100. It was dried again and suspended in water to evaluate total and free cholesterol content by Amplex® Red assay kits purchased from Life Technologies. The fixed cell monolayers were dissolved in 0.2% Triton X-100 and protein concentration was determined by the method of Lowry et al. [37].

2.7. Confocal fluorescence microscopy

To determine intracellular distribution of emulsion- or ac-LDL-derived cholesterol, NBD-cholesterol-labeled emulsions (0.5 mM TO, 8 μg/ml apoE) or NBD-cholesterol-labeled ac-LDL (100 μg/ml) was added to J774 macrophages grown on coverglasses, and incubation was carried out at 37 °C for 18 h. After incubation, the cells were chilled on ice and washed twice with cold Hepes buffer containing 0.2% BSA and then washed twice with cold Hepes buffer alone. The cells were then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) on ice and washed twice with Hepes buffer.

For the detection of cathepsin-L activity, cresyl violet-linked substrate ((Phenylalanine–Arginine)$_2$) in a cathepsin-L detection kit obtained from Biomol (Plymouth Meeting, PA) was used. Emulsions (5 mM TO, 8 μg/ml apoE) or ac-LDL (100 μg/ml) was added to J774 macrophages grown on coverglasses, and incubation was carried out at 37 °C for 18 h. After incubation, the cells were chilled on ice and washed twice with cold Hepes buffer containing 0.2% BSA and then washed twice with cold Hepes buffer alone. The cells were then fixed with 4% paraformaldehyde in PBS on ice and washed twice with Hepes buffer.