Biochimica et Biophysica Acta 1778 (2008) 2594–2603

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Cytotoxicity of lipid-free apolipoprotein B

Shin-ya Morita [a], Yuko Deharu [b], Eriko Takata [b], Minoru Nakano [b,*], Tetsurou Handa [b]

[a] Laboratory of Pharmaceutical Technology, Kobe Pharmaceutical University, Motoyamakita-machi, Higashinada-ku, Kobe 658-8558, Japan
[b] Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

ARTICLE INFO

Article history:
Received 3 April 2008
Received in revised form 19 August 2008
Accepted 19 August 2008
Available online 27 August 2008

Keywords:
Apolipoprotein B
Cytotoxicity
J774 macrophage
Apoptosis
Plasma membrane
Atherosclerosis

ABSTRACT

To investigate the effect of apolipoprotein B (apoB) on cell viability, we used lipid-free apoB as a model for denatured apoB. Lipid-free apoB had cytotoxicity to J774 macrophages, CHO cells and HepG2 cells, whereas apoB bound to low density lipoprotein (LDL) and lipid-free apolipoprotein A-I had no effect on cell viability. Lipid-free apoB induced apoptosis in J774 macrophages assessed by caspase-3 activation and annexin V binding. LDL receptor, heparan sulfate proteoglycans, and class A scavenger receptor were involved in the binding/uptake of lipid-free apoB, but lipid-free apoB binding/uptake by the cells did not correlate with cytotoxicity. Lipid-free apoB disrupted the lipid bilayer of large unilamellar vesicles containing calcein. We evaluated the interaction between apoB and cellular membrane by monitoring the change in intracellular Ca^{2+} concentration using Fura-2, and found that lipid-free apoB rapidly disrupted the cellular membrane in the absence or presence of the inhibitors for cellular binding/uptake mediated by the receptors. Therefore, it is suggested that lipid-free apoB induces cell death by disturbance of the plasma membrane. In addition to other lipid component in modified LDL, apoB itself has an ability to induce apoptosis and plays a crucial role in the development of atherosclerotic lesions.

1. Introduction

The subendothelial retention of atherogenic lipoproteins, low density lipoprotein (LDL) and triglyceride-rich lipoproteins is a critical step in the initiation of atherosclerosis. Denatured LDL is accumulated abundantly in atherosclerotic lesions [1,2]. This accumulation of denatured LDL induces the apoptosis of vascular endothelial cells and monocyte-derived macrophages [3,4]. Numerous studies have identified apoptosis as a prominent feature of atherosclerosis [5–8]. All cell types present in atherosclerotic plaques, including endothelial cells, smooth muscle cells, lymphocytes and macrophages, are known to undergo apoptosis. It has been suggested that macrophage apoptosis in early lesions decreases macrophage burden and slows lesion progression [9–11]. On the other hand, macrophage apoptosis in late lesions causes necrotic core formation, which may promote inflammation, plaque rupture, and thrombosis [11]. Therefore, the macrophage apoptosis is a crucial determinant of lesion development.

In early atherosclerotic lesions, even before atheroma appears, symptomatic patients have significantly more arterial apolipoprotein B (apoB) deposits than patients without cardiovascular events, and large amounts of apolipoproteins are found in advanced atherosclerotic lesions [12]. ApoB is a 4536-residue polypeptide that comprises the major protein component of very low density lipoprotein and its metabolite LDL. ApoB has a particular role in maintaining the structural integrity of LDL particles and controlling their interaction with LDL receptors [13]. The peak size of human LDL can be separated into two phenotypes: large buoyant LDL of 24–25 nm and small dense LDL of 18–20 nm (sdLDL) [14]. Although arguments have been raised for the casual relationship between sdLDL and cardiovascular disease, the predominance of sdLDL has been indicated as an emerging cardiovascular risk factor and a consistent characteristic of familial combined hyperlipidemia [15,16]. Recently, a low resolution model of apoB was revealed by small angle neutron scattering in combination with advanced shape reconstitution algorithms showing that the lipid-free protein adopts an expanded curved shape composed of distinct domains connected flexible regions [17]. The radius of gyration of 15 nm implies that lipid-free or lipid-poor apoB possibly constitutes a part of sdLDL.

Delipidated apoB is insoluble in aqueous buffers in the absence of detergents or high concentrations of denaturants [18]. A LDL particle contains a single apoB molecule together with about 3000 lipid molecules. Walsh and Atkinson developed a method to delipidate and purify apoB on LDL by using 10 mM sodium deoxycholate (NaDC) [19]. In this study, we investigated the cytotoxic effect of lipid-free apoB (free apoB) solubilized with NaDC, as a model for denatured apoB, and compared it with apoB bound to LDL (LDL-apoB) and lipid-free, exchangeable apolipoprotein A-I (apoA-I). We also examined the

Abbreviations: apoB, apolipoprotein B; LDL, low density lipoprotein; NaDC, sodium deoxycholate; free apoB, lipid-free apoB; LDL-apoB, apoB bound to LDL; apoA-I, apolipoprotein A-I; BSA, bovine serum albumin; SRA, class A scavenger receptor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LDH, lactate dehydrogenase; HSPG, heparan sulfate proteoglycan; LUV, large unilamellar vesicles; EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid

* Corresponding author. Tel.: +81 75 753 4555; fax: +81 75 753 4601.
E-mail address: mnakano@pharm.kyoto-u.ac.jp (M. Nakano).

0005-2736/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2008.08.012

relationship between cytotoxicity and cellular uptake, and investigated the interaction of free apoB with cellular membrane to clarify its cytotoxic mechanism.

2. Materials and methods

2.1. Materials

Human apoA-I was purchased from Chemicon International (Tamecula, CA). Human plasma LDL (d=1.02–1.063) was obtained from Calbiochem (San Diego, CA). Heparin, bovine serum albumin (BSA), egg yolk phosphatidylcholine (PC), EGTA and thapsigargin were obtained from Sigma Chemical Co. (St. Louis, MO). Rat anti-mouse class A scavenger receptor (SRA) (2F8) was purchased from Serotec, Inc. (Raleigh, NC) and mouse anti-murine CD36 IgA (clone 63) was from Cascade Biosciences (Winchester, MA). Calcein and Fura-2-AM were purchased from Dojindo Laboratories (Kumamoto, Japan). All other chemicals used were of the highest reagent grade.

2.2. Free apoB

Human free apoB, provided by Chemicon, was purified from plasma LDL isolated by density gradient ultracentrifugation followed by size exclusion chromatography in 10 mM NaDC, 50 mM NaCl, and 50 mM Na_2CO_3 (pH 10). The stock solution of free apoB (~1.5 mg protein/ml in 10 mM NaDC) was stored at 4 °C. Because free apoB was purified from plasma LDL, free apoB was mainly apoB100 but not apoB48. Most of free apoB showed a major high-molecular-mass band (>500 kDa) on a 5–20% SDS-PAGE gel detected by 0.1% Coomassie Brilliant Blue R-250 staining, whereas a minor broad band (200–500 kDa) was also shown on the gel, probably due to incomplete denaturation or cleavage of a small number of apoB. The apoB preparation did not contain detectable amount of phospholipids, triglycerides, free cholesterol or cholesteryl ester tested by the enzymatic assay kits purchased from Wako Pure Chemical Industries (Osaka, Japan), indicating that the apoB solubilized in NaDC was free of lipid, whereas the concentrations of phospholipids, triglycerides, free cholesterol and cholesteryl ester in the LDL-apoB preparation were 1.26, 0.389, 0.959 and 2.51 μmol/mg protein, respectively. The apoB concentration was determined by the method of Lowry.

2.3. CD spectral measurements

CD spectra of LDL-apoB and free apoB in 0.5 mM NaDC were recorded on a JASCO J-820 spectropolarimeter, using a 0.1 cm quartz cuvette, in the peptidic region (200–250 nm). Free apoB (20 μg/ml) was solubilized in 0.5 mM NaDC. To increase the signal-to-noise ratio, eight spectra were averaged for each measurement, and the blank was subtracted. The cell holder compartment was thermostatically maintained at 37 °C. The spectra were analyzed for the secondary structure contents using the software accompanying the spectropolarimeter.

2.4. Cell cultures

J774 macrophages and HepG2 cells 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. CHO cells were grown in a humidified incubator (5% CO_2) at 37 °C in Ham's F12 supplemented with 10% heat-inactivated FBS, L-glutamine, penicillin, and streptomycin. The FBS was changed to 1% BSA 15 min before each experiment. Experiments were performed in DMEM containing 1% BSA for J774 macrophages and HepG2 cells, and Ham's F12 containing 1% BSA for CHO cells.

2.5. Cell viability assays

Cells were incubated for 6 h with various concentrations of free apoB, LDL-apoB or apoA-I in the presence of 0.5 mM NaDC. 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 dissolved in 0.2% Triton X-100. Cell viability was estimated by measuring the lactate dehydrogenase (LDH) activity in the media and total cells using a CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit purchased from Promega (Madison, WI). The LDH-mediated conversion of the tetrazolium salt into the red formazan product was measured at 490 nm. The fraction of LDH release was an index of irreversible injury or necrosis. %LDH release=LDH activity in media/(LDH activity in media+LDH activity in total cells)×100%.

2.6. Differential interference-contrast microscopy

Cells grown on culture dishes were incubated for 6 h with 0.5 mM NaDC in the presence or absence of free apoB, LDL-apoB or apoA-I, and washed twice with cold Hepes buffer containing 0.2% BSA and with cold Hepes buffer. The cells were then viewed with an Olympus IX70 microscope.

2.7. Quantification of caspase-3 activity

J774 macrophages were incubated for 3 h with 0.5 mM NaDC in the presence or absence of 20 μg/ml free apoB. 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 dissolved in 0.2% Triton X-100. The caspase-3 activity in the cell lysate was measured using Caspase-3 Fluorometric Assay Kit purchased from BioVision, Inc. (Mountain View, CA). The substrate for the assay was Ac-Asp-Glu-Val-Asp-[7-amino-4-trifluoromethyl coumarin]. Cleavage of the substrate by caspase-3 resulted in the emission of a fluorescent signal. The fluorescence intensity was measured using a fluorescence microplate reader equipped with a 390-nm excitation filter and a 535-nm emission filter.

2.8. Annexin V-fluorescein and propidium iodide (PI) staining

J774 macrophages grown on culture dishes were incubated for 6 h with 0.5 mM NaDC in the presence or absence of 20 μg/ml free apoB, and washed twice with cold Hepes buffer containing 0.2% BSA and with cold Hepes buffer. Phosphatidylserin externalization was assayed by binding of fluorescein-labeled annexin V using Annexin-V-FLUOS Staining Kit obtained from Roche Applied Science (Penzberg, Germany) according to the manufacturer's instructions. Briefly, cells were incubated with annexin V-fluorescein in a Hepes buffer containing PI for 15 min at room temperature. Cells were immediately viewed with an Olympus IX70 inverted fluorescence microscope. Eight fields of cells for each condition (~1200 cells) were counted.

2.9. ApoB binding/uptake assays

Free apoB and LDL-apoB were labeled using Fluorescein Labeling Kit-NH_2 provided by Dojindo. The cells were incubated with fluorescein-labeled free apoB or LDL-apoB at 37 °C for 2 h in the presence of 0.5 mM NaDC. 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 dissolved in 0.2% Triton X-100. The amount of apoB in the cell lysate was determined from the fluorescence intensity of fluorescein (excitation 490 nm, emission 520 nm) measured with a Hitachi