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Effects of enzymes on elastic modulus of low-density lipoproteins were investigated using atomic force microscopy

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ABSTRACT

Oxidation of low-density lipoproteins (LDLs) induces development of cardiovascular disease. Recently, reports of studies using atomic force microscopy (AFM) have described that the elastic modulus of metal-induced oxidized LDLs is lower than the modulus before oxidation. However, the mechanisms of change of the elastic modulus have not been well investigated. We postulated that disorder of the LDL structure might decrease the elastic modulus. This study measured the elastic modulus of LDLs before and after enzyme treatment with V8 protease, α-chymotrypsin, and phospholipase A_2. After LDLs were obtained from serum by ultracentrifugation, LDLs or enzyme-treated LDLs were physically absorbed. They were crowded on a mica surface. Although V8 protease and α-chymotrypsin did not induce the elastic modulus change, treatment with PLA_2 decreased the elastic modulus. The LDL particle size did not change during the enzyme treatment. Results suggest that disordering of the lipid structure of the LDL might contribute to the elastic modulus change. Results show that AFM might be a useful tool to evaluate disorders of complex nanoscale particle structures from lipids and proteins such as lipoproteins.

1. Introduction

Physical and chemical properties of lipoproteins are known to change their role and to affect the human body. Low-density lipoproteins (LDLs) are known as lipoproteins. Their aspherical structure with 23–28 nm diameter includes triacylglycerol, cholesterol esters, phospholipids, and apolipoprotein B-100 (apoB-100) as the major apolipoprotein. In physiological conditions, LDL particles can pass through the layer of endothelial cells by transcytosis and can thereafter accumulate in side arterial walls [1]. In a side arterial wall, unsaturated lipids included in the LDLs are oxidized by oxidative stresses, followed by an auto-oxidation process and generation of many aldehydes such as malondialdehyde and 4-hydroxynonenal [2,3]. These aldehydes can modify the amino groups of the lysine moiety of the apoB-100. That modification decreases the positive charge of the LDL. These modified LDLs are designated as oxidized LDLs. Macrophages ingest the oxidized LDL via scavenger receptors, leading to foam cell formation, which is a risk factor for atherosclerosis development. Oxidized LDLs are also known to induce morphological changes in endothelial cells and to increase their stiffness and endothelial dysfunction [4,5]. Therefore, oxidized LDLs are widely regarded as a risk factor for the development of atherosclerosis.

Small dense LDLs are also positive risk factors for atherosclerosis. Small dense LDLs with diameter of less than 25.5 nm stay in the blood longer than large LDLs because of their low affinity to the LDL receptors and because of the increasing probability of passage through endothelial cells as a result of their small size [6]. Reportedly, high-density lipoprotein (HDL) surface rigidity is important for transportation of lipid-peroxide from LDLs to HDLs [7]. The thermal stability of LDLs is important to control LDL particle fusion, which can develop thermogenesis [8]. The physical properties of lipoproteins can strongly affect their important role in the human body.

Recently, atomic force microscopy (AFM) has been used to evaluate lipoprotein topography [9–11]. An earlier report of our study using AFM describes that the elastic modulus of the LDL became lower than that before oxidation [12]. Although the putative relation between the elastic modulus of the lipoprotein and its physiological role has not been well investigated, the elastic modulus change of LDL might be important. For last decade, stiffness, rigidity, and fluidity of liposomes are of interest not only for basic research of lipids but also for research into drug delivery

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systems. For instance, ethosomes, which are transdermal liposomes containing a hydro-alcoholic core, enhance transdermal drug delivery by improving permeation. Although increasing lipid multilayer fluidity by insertion of ethanol to the target lipid layer is suggested as the first step, their malleable structure also contributes to skin permeation as the second step [13,14].

We surmised that decreasing the elastic modulus of the LDLs might increase its probability of passage through endothelial cells in the case of endothelial dysfunction by slightly deforming their structure. Although oxidation of the LDLs is known to generate lipid hydro-peroxides, aldehydes that modify the apoB-100, and fragments of the apoB-10, the mechanism of change of the elastic modulus remains unclear.

The study described in this paper investigated whether fragmentation of the apoB-100 or hydrolysis of the lipid contributes to change of the elastic modulus, or not. We investigated the effects of V8 protease, α-chymotrypsin, and phospholipase A_2 (PLA_2) on the elastic modulus of the LDLs. As described herein, we measured the stiffness of LDLs immobilized as crowded on a mica substrate before and after enzyme treatment. We ascertained the elastic modulus using force curve measurements with AFM.

2. Experimental details

2.1. Materials

The following reagents were used for this study. Di-sodium hydrogen phosphate heptahydrate, sodium dihydrogen phosphate dihydrate, sodium chloride, ethylenediamine-N,N,N,N-tetraacetic acid, disodium salt (EDTA), V8 protease, α-chymotrypsin PLA_2 from *Crotalus adamanteus* and copper sulfate were purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan. Cantilevers for AFM were used (BL-AC40TS-C2; Olympus Corp., Japan). Mica and plates used for the weight of the mica were purchased from Asylum Research, Santa Barbara, USA. A 2-thiobarbituric acid reactive substance (TBARS) assay kit, free acid assay kit, and PAF-AH assay kit were purchased from Cayman Chemical, MI, USA.

2.2. Preparation of native LDLs, ox-LDLs, and enzyme-treated LDLs

Native LDLs (n-LDLs) and ox-LDLs were prepared as described below. The n-LDLs were isolated from normal adult sera (5 men, ages 22, 23, 25, 47, 48) by ultracentrifugation based on the density difference of lipoproteins, other proteins, and biomolecules according to procedures reported in the literature [15]. Before using n-LDLs, EDTA was removed from them using an ultra-filtration device with phosphate-buffered saline (PBS) at pH 7.4. The concentration of the n-LDLs was determined using a modified Lowry method as the apoB-100 protein concentration. The concentration was adjusted to 0.3 mg/ml. Oxidation of the n-LDLs was done by incubation with 1 μM $CuSO_4$ at 37 °C for 3 h. The oxidation level of oxidized LDLs was determined as the malondialdehyde (MDA)-equivalent TBARS concentration [16].

Effects of darapladib, a specific inhibitor of the lipoprotein associated PLA_2 (Lp-PLA_2), were assessed at concentrations of 1–100 μM. The activities of the Lp-PLA_2 were measured using a PAF-AH assay kit according to the manufacturer's instructions.

Regarding preparation of the protease-treated LDLs, 0.01 mg/ml solution of V8 protease or α-chymotrypsin was added to the LDL solution and incubated at 37 °C for 2 h. In the case of PLA_2, a 0.01 mg/ml solution of PLA_2 was added to the LDL solution and incubated at 25 °C for 3 h. The activities of the PLA_2 were evaluated by measuring the free acid concentration using a free acid assay kit according to the manufacturer's instructions. Free fatty acid and TBARS released from LDL to the bulk solution were obtained by filtration using an ultracentrifuge device with the molecular cut-off of 100 kDa. The filtrate of TBARS and free fatty acid were measured.

These LDL solutions were immobilized on the freshly cleaved mica surface by dropping 20 μL of the solution for 3 min at 20 °C, followed by removal of the LDL solution. Then they were washed four times with 30 μL PBS at pH 7.4 to remove excess LDLs. A substrate with LDLs was used immediately for AFM measurements. The LDL sizes were also measured using direct light scattering (DLS) with a Zetasizer Nano ZS (Malvern Panalytical). A size analysis was done using software equipped with the apparatus using size distribution analysis. Ethical approval: No. 15-89-6, The Ethical Committee, Faculty of Health Sciences, Hokkaido University.

2.3. Measurement of topography and force curves of LDLs using AFM

Topography and force curves of LDLs were measured using an AFM system (MFP-3D; Asylum Research, Santa Barbara, USA). Topography of the LDL was measured in PBS using AC mode. The scan size and scan rate were, respectively, 500 × 500 nm and 0.7 Hz.

Force curves were obtained by changing the tip position after measuring the topography using contact mode. In brief, an AFM tip was moved closer to the surface at 105 nm/s until the tip contacted the surface. Then the deflection value of the cantilever increased 0.02–0.05 V from the deflection before contact. The cantilever spring constant was measured using a thermal method program included with the MFP-3D apparatus.

The elastic moduli of the LDLs, which indicate the LDL stiffness, were estimated from the force curves of the approach process using software equipped with the MFP-3D with a Hertz model modified by Sneddon [17]. For the case in which the tip contacted to the mica surface or contaminant materials adsorbed onto the mica substrate, the indentation length was observed as less than 3 nm. In that case, the elastic modulus was calculated as greater than 10 MPa. These data accounted for about 8% of the obtained data. They were excluded from analysis in this study. Analyses of the distribution of the elastic modulus data were carried out using Mann–Whitney tests.

3. Results and discussion

We immobilized the n-LDL, n-LDL treated by α-chymotrypsin, V8 protease, and PLA_2 on the mica surface and measured their topographies using AFM. α– Chymotrypsin is a digestive enzyme and it cleaves C-terminal of amide bonds where the side chain of the amino acid include aromatic residues such like tyrosine, tryptophan, and phenylalanine. V8 protease from *Staphylococcus aureus* is one of the endoproteinase that specifically hydrolyzes peptide at the carboxylic side of Glu and Asp. These enzymes expected to hydrolyze apoB-100. Typical topographies and typical force-indentation curves are presented in Fig. 1. As these figures show, LDLs were immobilized: crowded on the mica surface. Their diameters were estimated from the image as 20–30 nm. No significant difference was found in the size of the LDLs before and after the protease and the lipase treatment. We measured the force curves by changing the tip position horizontally. The elastic modulus of the LDL was estimated from the force-indentation curves calculated from the force curves using software included with the instrument. The elastic modulus was estimated from the region of the contact point to 6 nm of indentation using the Hertz model as the elastic modulus. A typical trace and retrace force indentation curve of LDL before enzyme treatment is portrayed in supplemental Fig. 1a). The elastic modulus was calculated as 1.2 MPa and the value was consistent with those reported from