Analysis of Apolipoprotein B Protein of Circulating Multiple-Modified Low-Density Lipoprotein

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Abstract

Keywords

- atherosclerosis
- low-density lipoprotein
- modified LDL
- apolipoprotein B
- desialylation

Modified low-density lipoprotein (LDL) is the main source of lipid accumulation in the arterial wall affected by atherosclerosis. We aimed to compare the properties of apolipoprotein B (apoB) from native and modified LDL. Modified (desialylated) LDL and native LDL were extracted from blood of atherosclerotic patients. We characterized apoB structure of LDL particles in total LDL preparation, circulating modified LDL (cmLDL), and native LDL. Intact cmLDL had a twofold lower content of free amino groups than native LDL. Delipidated apoB from cmLDL also had a lower content of free amino groups. The rates of tryptic hydrolysis and elastase digestion of cmLDL were twofold higher in comparison to native LDL. Therefore, cmLDL from atherosclerotic patients had altered apoB properties. Our observations strengthen the hypothesis of multiple modification of LDL in the bloodstream and underscore the importance of desialylated LDL as a possible marker of atherosclerosis.

Atherosclerosis is triggered by lipid accumulation in the arterial wall, especially in the subendothelial layer of the arterial intima. Low-density lipoprotein (LDL) circulating in human blood is regarded as a principal source of intracellular lipid accumulation. However, only modified lipoprotein, but not native LDL, causes intracellular lipid accumulation. Oxidation remains the most studied type of atherogenic modification of LDL, although detailed analysis of atherosclerotic patients' blood samples revealed the presence of other atherogenic modifications including desialylation.¹ Glycosphingolipids of LDL particles have a terminal sialic acid residue. In case of its removal, the following galactose residue becomes the terminal saccharide. This can be used for selective isolation of desialylated LDL from total LDL preparation using *Ricinus communis* agglutinin (RCA₁₂₀), which binds the terminal galactose with high affinity.¹ To isolate desialylated LDL, total LDL

preparation is applied on a column with cyanogen bromide (CNBr)-activated agarose bearing RCA₁₂₀ lectin. Normally, sialylated LDL passes through the column without significant binding to the sorbent. By contrast, desialylated LDL binds to the lectin and can later be eluted with 5- to 50-mM galactose solution. The described method allowed us to isolate subfractions of both sialylated and desialylated LDL from total LDL preparation obtained from the blood of atherosclerotic patients. Because the desialylated LDL subfraction was extracted from patients' blood, it can be referred to as circulating modified LDL (cmLDL).¹ It is important to determine physical and chemical characteristics of the atherogenic cmLDL in comparison to native LDL and to analyze its content in the blood of atherosclerotic patients and healthy subjects. In this study, we focused on chemical characteristics of apolipoprotein B (apoB) of cmLDL particles.

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Materials and Methods

Study Subjects

Study subjects included 12 men and women aged between 30 and 60 years with angiographically demonstrated atherosclerosis and 12 healthy donors aged between 25 and 55 years with no signs of ischemic heart disease according to Rose angina questionnaire.^{2,3} The study was approved by the local ethics committee of the Institute of General Pathology and Pathophysiology (Moscow, Russian Federation). Cholesterol and triglyceride in blood plasma of study subjects did not exceed 200 and 150 mg/dL, respectively. Subjects with diabetes and arterial hypertension were excluded from the study.

Preparation of Blood Plasma and Serum

Blood samples were drawn in the morning after a night of fasting using tubes with 1 mg/mL ethylenediaminetetraacetic acid (EDTA), 0.38% sodium citrate, or 10 u/mL sodium heparin (final concentrations). Samples were centrifuged for 10 minutes at 800 g in 5-mM phenylmethylsulfonyl fluoride, 1-mM ϵ -aminocaproic acid, and 0.01% sodium azide. Serum was obtained by incubation of blood samples for 1 hour at 37°C followed by centrifugation for 15 minutes at 800 g.

Total Low-Density Lipoprotein Preparation

Total LDL fraction was prepared as described previously⁴ or using the method established by our group. A total of 8 mL of plasma or serum was overlaid by 2 mL of sodium bromide (NaBr) solution with density 1.019 g/mL in isotonic phosphate buffer in a centrifuge tube and centrifuged first for 18 hours at 12°C (40,000 rpm using 50 Ti Rotor, Beckman Coulter, Brea, CA). A total of 2 mL of the upper fraction was accurately collected and the remaining volume was brought to the density of 1.070 g/mL with solid NaBr. Next, 2 mL of NaBr solution with density 1.065 g/mL was accurately layered on top of the resulting solution, and the tube was centrifuged for 24 hours at 12°C (40,000 rpm). Then 2.5 mL of solution containing LDL was collected from the top of the tube and dialysed against 6,000 volumes of phosphate buffer with 10- μ M EDTA for 24 hours. The solution was filter-sterilized and stored in plastic tubes at 4°C for 1 to 2 weeks. We have developed a faster (4.5–5 hours) method of total LDL extraction. Blood plasma was brought to density of 1.390 g/mL with solid NaBr (2 g/mL of plasma), and 4 mL of plasma was put into a centrifuge tube. Then, 6 mL of NaBr solution with density 1.019 g/mL was layered on top, and the tube was centrifuged for 2 hours (40,000 rpm). The top 2 cm of liquid containing floating LDL was collected, 2 g/mL of NaBr was added to the solution, and the tube was centrifuged again in identical conditions. The obtained LDL samples were dialyzed and stored as described previously. The LDL fractions obtained by the two methods had similar physical and chemical properties.⁵

Lectin Chromatography of Modified Low-Density Lipoprotein

ApoB contains two types of oligosaccharide conjugates, oligomannoside and terminally sialylated.^{6,7} Glycosphingolipids in LDL also have a terminal sialic acid residue.⁸ Desialylation will result in the exposure of the next residue of the

carbohydrate chain, which is galactose. Correspondingly, we hypothesized that desialylated LDL will interact with galactose-specific lectins, such as *Ricinus communis* agglutinin (RCA₁₂₀).⁹ To prepare the affinity columns, RCA₁₂₀ was immobilized on CNBr-activated agarose as described previously.¹⁰ The columns were equilibrated with 10 to 15 mL of isotonic phosphate buffer, pH 7.2. LDL samples (0.5 to 5 mL containing 0.2–10 mg of protein) were loaded on the columns. The major part of unbound LDL was washed from the column in the first 10 volumes of phosphate buffer. Desialylated LDL was washed from the column with galactose solutions in phosphate buffer (5, 10, 20, 50, and 100 mM). Washing with 50-mM galactose resulted in virtually complete elution of LDL in the first 5 volumes, and this concentration was used most often.¹¹ Bound and unbound LDL fractions were brought to the density of 1.070 g/mL with NaBr, concentrated by ultracentrifugation and dialyzed against 6,000 volumes of isotonic phosphate buffer as described previously.

Trinitrobenzenesulfonic-Acid-Reactive Lysine Residues Level Determination in LDL

Method for trinitrobenzenesulfonic acid-reactive lysine residues level determination in LDL was described elsewhere.¹² To 10 μ L of LDL or delipidated apoB containing 5 to 20 μ g of protein, 150 μ L of 0.1 M, pH 10.3, borate buffer was added. Later on, 35 μ L of 2-mg/mL trinitrobenzenesulfonic acid solution was added. The mix was incubated for 40 minutes at room temperature, and the reaction was stopped by adding 35 μ L of 0.23% sodium sulfite in 2-M sodium dihydrophosphate solution. Optical density was measured at 415 nm on automatic spectrophotometer Multiskan MCC (LabSystems Titertek Multiskan MCC/340 MKII Type 347 Microplate Reader, located in Russian Cardiology Research and Production Complex, Moscow, Russia). 1mM glycine solution was used as a standard.

Iodine-125 (¹²⁵I)-labeled LDL preparation was conducted according to Bilheimer et al.¹³ To 300 μ L of LDL previously dialyzed against 0.1-M potassium dihydrophosphate, pH 7.6, labeled sodium iodide (17 μ Cu/mmol) was added, 100 μ Cu per mg of LDL protein. After that, 10 μ L of chloramine-T solution was added to the same buffer (2.6 mg/mL), and the tube's lid was tightly closed and vigorously shaken for 60 seconds. After that, 25 μ L of 0.75% of sodium sulphite solution at the same buffer was held for 5 minutes with an open lid. Resulting labeled LDL substrate was then dialyzed against 2,000 volumes of phosphate buffered saline for 24 hours at 4°C with triple change of dialyze medium. Effectiveness of ¹²⁵I isotope incorporation in LDL protein was 30 to 35%. Label amount in LDL samples was controlled using automatic gamma-counter 1,272 Clinigamma (LKB, Bromma, Sweden).

Low-Density Lipoprotein Proteolysis

Tryptic degradation of LDL was conducted during 12 hours at 37°C in pH 8.0 ammonium bicarbonate buffer using porcine pancreas trypsin (Sigma-Aldrich, MO) with trypsin:lipoprotein ratio held at 1:50 (by mass) according to Croft.¹⁴ Elastase (Promega, WI) digestion was conducted at Tris-HCl buffer

Fig. 1 Contents of free amino groups in native and circulating modified low-density lipoprotein (LDL). LDL was delipidated by treatment with chloroform-methanol mixture, and apolipoprotein B was dissolved in 0.2-M sodium hydroxide. Presented are means of three independent experiments $\pm$ standard deviation. *Significant difference from the corresponding fraction obtained from healthy donors. **Significant difference from total LDL. ***Significant difference from the corresponding fraction obtained from healthy donors, significant difference from total LDL, and significant difference from native LDL. ****Significant difference from the corresponding fraction obtained from healthy donors and significant difference from intact LDL. *****Significant difference from the corresponding fraction obtained from healthy donors, significant difference from intact LDL, significant difference from total LDL, and significant difference from native LDL. $P < 0.05$ (two-tailed Student's *t*-test).

(pH 9.0) during 6 hours at 37°C with elastase:lipoprotein ratio held at 1:80 (by mass) according to Gertler et al.¹⁵

Results

Analysis of Free Amino Groups of Lysine Residues

The major part of free amino groups present in LDL belongs to lysine residues of apoB. No significant difference in the amount of free amino groups could be observed between the subfractions of native and cmLDL from healthy donors. Delipidated apoB samples from these subfractions also had similar amounts of amino groups. Total LDL samples from atherosclerotic patients had a 1.4-fold decrease of the amount of free amino groups in comparison to healthy donors (**Fig. 1**). The amount of amino groups in delipidated samples of apoB was significantly higher than in intact LDL particles. In native LDL, levels of free amino groups from both intact and delipidated particles were similar to values observed for LDL

fractions from healthy donors. In cmLDL, the amount of amino groups in intact particles was 1.5-fold and twofold lower than in total and native LDL, respectively (**Fig. 1**).

Resistance to Proteolysis

Another method of studying the protein's tertiary structure is proteolytic degradation. Native and cmLDL labeled with ¹²⁵I was incubated at 20°C for 1 hour in the presence of trypsin and elastase. The rate of tryptic hydrolysis of cmLDL was increased by twofold in comparison with native LDL (**Table 1**). The degradation rate of cmLDL treated with elastase was also twofold higher than that of native LDL (**Table 1**). These results indicate the possibility of altered apoB conformation in cmLDL in comparison with native LDL.

Conclusion

To conclude, we demonstrated altered structure of apoB in cmLDL isolated from the blood of atherosclerotic patients. Circulating modified LDL was characterized by decreased amino group content and altered protein tertiary structure. The observed changes should be further evaluated for their clinical relevance as another marker of atherosclerosis.

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Table 1 Rates of trypsin and elastase proteolysis of cmLDL compared to native LDL, measured by degradation of ¹²⁵I-labeled LDL

	Type of proteolytic enzyme used	
	0.001% trypsin	0.002% elastase
Degradation rate, % from native LDL	202 $\pm$ 8 ^a	206 $\pm$ 4 ^a

Abbreviations: cmLDL, circulating modified low-density lipoprotein; LDL, low-density lipoprotein.

Note: Labeled LDL was incubated with indicated enzymes for 1 hour at 20°C. Presented are means of three independent experiments $\pm$ standard deviation.

^aSignificant difference from controls.

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