Proteolysis of Apoprotein B-100 Impairs Its Topography on LDL Surface and Reduces LDL Association Resistance

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 140, No. 11, pp. 530-534, November, 2005
Original article submitted September 14, 2004

Serine proteinases (trypsin and chymotrypsin) cause destruction of apolipoprotein B-100 on the surface of human blood LDL. Incubation of LDL with these enzymes increases the mean size of LDL particles. Proteolysis of apolipoprotein B-100 induces changes in surface structure, destabilizes LDL particles, and reduces their association resistance. Presumably, this proteolytic modification of LDL with subsequent association of these particles plays an important role in accumulation of cholesterol in the vascular wall and in the development of early stages of atherosclerosis.

Key Words: *low density lipoproteins; trypsin; chymotrypsin; lipoprotein aggregation; atherosclerosis*

Initial stages of atherosclerosis are characterized, among other things, by the appearance of extra- and intracellular lipid incorporations 100-400 nm in diameter in the arterial intima [3,13]. It is hypothesized that these lipid formations are LDL associations circulating in the blood. The cause of LDL association remains unknown. Most probably, the loss of association resistance is a result of LDL modifications in the blood flow and cell wall due to the action of some enzymes and non-enzymatic factors detected in atherosclerotic intima (proteolytic enzymes plasmin, kallikrein, thrombin, lysosomal proteinases, serine proteinases, metalloproteinases, *etc.*) [6].

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In vitro experiments showed that degradation of apolipoprotein B-100 (apo B-100), the main LDL protein, modified surface characteristics of LDL particles and led to their association. These associations were intensely absorbed by macrophages and were detected in the extracellular space [7].

We studied disorders in the topography of apo B-100 on the surface of LDL particles by enzyme immunoassay with monoclonal antibodies to this protein after treatment with proteolytic enzymes and cleared out whether apo B-100 proteolysis modified LDL association resistance.

MATERIALS AND METHODS

Total LDL fraction was isolated by two-step ultracentrifugation in NaBr density gradient [5]. Native LDL were separated from circulating repeatedly modified LDL by lectin chromatography on a column with *Ricinus communis* agglutinin agarose (Boehringer Mannheim) [12].

Native LDL were subjected to proteolysis: incubated with trypsin (Serva) or chymotrypsin (Sigma) for 3.5 h at 37°C in isotonic phosphate buffer

0007-4888/05/1405-0521

(IPB; GIBCO, Paisley) of the following composition: 0.2 g/liter KCl, 0.2 g/liter KH_2PO_4, 8 g/liter NaCl, 1.15 g/liter Na_2HPO_4 (pH 7.2). LDL subjected to proteolysis were separated from the enzyme by recentrifugation at 41,000 rpm (Ti 50 rotor, Beckman) for 2 h in a NaBr density gradient. The resultant LDL were dialyzed against IPB for 12 h and used for enzyme immunoassay. The degree of apo B-100 proteolysis was evaluated by electrophoresis in polyacrylamide gel after Laemmli. The gels were stained with Coumassie brilliant blue R-250.

Mouse monoclonal antibodies to human apo B-100 were obtained by hybridization [1]. Eight antibodies, produced by clones 2G8, 3C8, 4C11, 2E3, 5F8, 2G1, 7C2, and 3G4 were used in the study. The former 5 antibodies are monoclonal to the native LDL apo B-100 (Table 1), while antibodies 2G1, 7C2, and 3G4 are monoclonal to MDA-modified LDL apo B-100 [7].

For evaluation of LDL binding with monoclonal antibodies 96-well plates (Nunc) were incubated with goat polyclonal antibodies (100 μl; 1 μg/ml in IPB) to human apo B-100 (IMTEK) for 24 h at 4°C. After each incubation the wells were washed in IPB containing 0.2% BSA. Then 100 μl IPB with 2% BSA was added into wells and incubated for 1 h at ambient temperature. LDL in concentrations of 0.2-200.0 μg protein/ml were added into wells and incubated for 2 h at 7°C. Then murine monoclonal antibodies to human apo B-100 (100 μl) were added and incubated for 1 h at ambient temperature. The concentrations of monoclonal antibodies were selected in preliminary experiments and were 10-50 μg/ml. Peroxidase-labeled goat polyclonal antibodies (100 μl; 1 μg/ml) were added into wells and incubated for 1 h at ambient temperature. Subsequent development was carried out by adding 0.1 M citrate buffer (pH 4.5) containing 0.04% orthophenylenediamine and 0.003% H_2O_2. Incubation was carried out for 20-30 min at 37°C. The reaction was stopped by adding 20 μl 50% H_2SO_4. Optical density was measured at λ=492 nm on a Multiscan Bichromatic multichannel spectrophotometer (Labsystems OY).

Preliminary experiments were carried out for all monoclonal antibodies in order to rule out the possibility of their nonspecific binding to goat polyclonal antibodies to human apo B-100.

The significance of differences in monoclonal antibody binding with lipoproteins was evaluated by ANOVA. The differences were considered significant at $p<0.05$.

The degree of LDL association was evaluated by recording the transmission fluctuation of a laser beam (λ=860 nm) [11]. The method is based on the following phenomenon: relative dispersion of optical density fluctuations caused by stochastic changes in the number of particles in optical path reflects their deviations from the mean size (degree of their association). LDL aggregation was studied by incubating LDL at 37°C in IPB with and without proteolytic enzymes. In some experiments proteinase inhibitor (phenylmethane sulfonylfluoride; PMSF) in a concentration of 0.5 mM was added into the incubation medium together with the enzymes. After certain periods of time the cuvette with incubated sample was placed into measuring cell of a two-channel laser aggregometer (LA220, BIOLA Firm) and light transmission fluctuations were recorded. The increment in light transmission fluctuation was expressed in percent of the initial value.

RESULTS

LDL pretreated with trypsin more effectively than native LDL reacted with clone 2G8 antibodies (Fig. 1). Native LDL better bound to clones 3C8 and 4C11 antibodies. Experiments with other clones showed no appreciable differences in their binding to native and trypsin-treated LDL.

Clones 2G8, 3C8, and 2E3 more effectively bound to LDL subjected to enzyme proteolysis. Clone 4C11 better reacted with native LDL. Results for clones 2E3, 4C11, and 3G4 are shown in Fig. 2. Clones 5F8, 2G1, 7C2, and 3G4 exhibited no appreciable difference in binding to native and chymotrypsin-modified LDL. Antigenic differences in native and proteolytic enzyme-treated LDL are summed up in Table 1.

Electrophoresis showed that the enzyme concentrations used in the study induced proteolysis of apo B-100 in LDL. The presence of PMSF in the incubation medium protected LDL main protein from proteolysis.

Hence, proteolytic enzymes modified the topography of apo B-100 on the surface of an LDL particle. Presumably, this modification in apo B-100 location should modify surface characteristics of LDL particles, which can be essential for their association resistance.

During 6-h incubation without enzymes fluctuation of light transmission of native LDL suspension virtually did not change, which attested to the absence of LDL association (curves *2* and *4* in Fig. 3, *a* and *b*, respectively). Addition of trypsin or chymotrypsin to LDL increased light transmission fluctuation, which reflected enlargement of LDL particles. Increasing enzyme concentration (in experiments with chymotrypsin) led to an increase in LDL suspension light transmission fluctuation

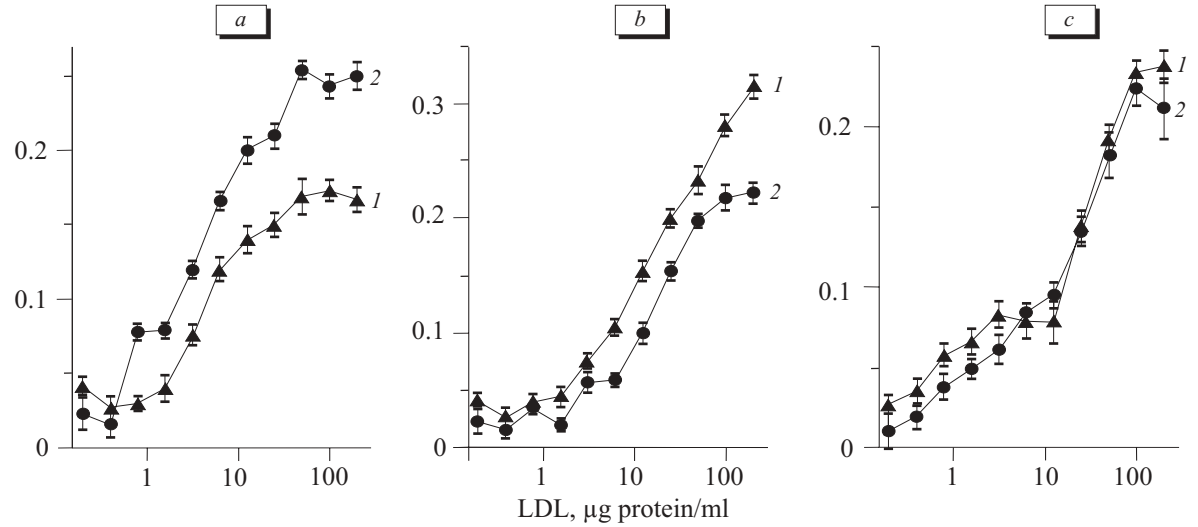

Fig. 1. Concentration dependence of reactions between native (*1*) and trypsin-treated LDL (*2*) with monoclonal antibodies to human apolipoprotein B-100 (apo B-100). *a*) 2G8; *b*) 4C11; *c*) 5F8. Here and in Fig. 2: ordinate: optical density at λ=492 nm.

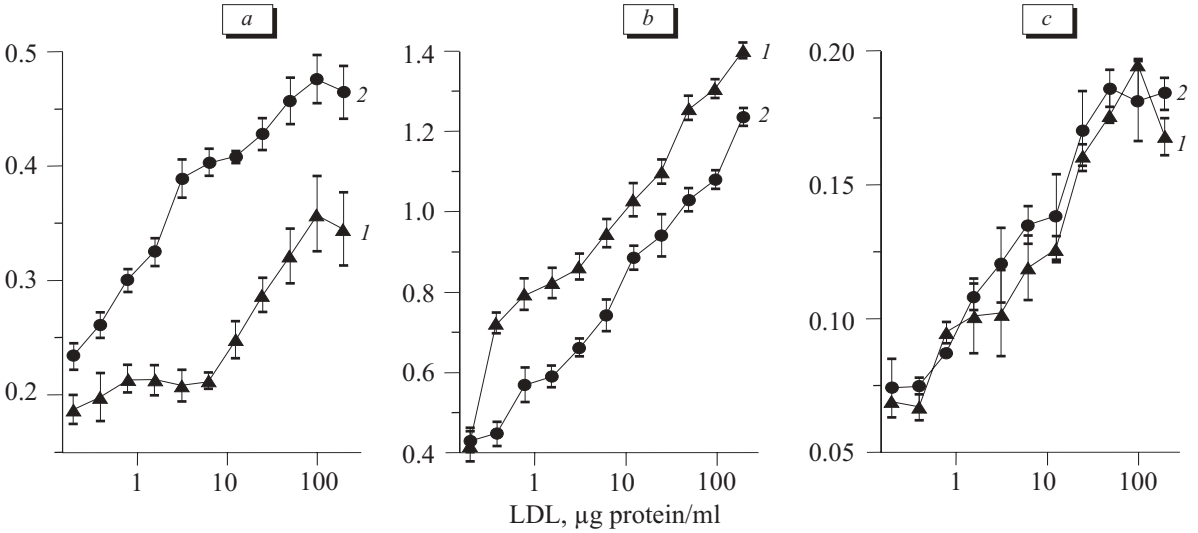

Fig. 2. Concentration dependence of reactions between native (*1*) and chymotrypsin-treated LDL (*2*) with monoclonal antibodies to human apo B-100. *a*) 2E3; *b*) 4C11; *c*) 3G4.

TABLE 1. Antigenic Differences of Apo B-100 in LDL Treated and Not Treated with Proteolytic Enzyme Treated

Clone	Chymotrypsin	Trypsin	Clone Characteristics
5F8	0	0	Apo B-100 amino acid residues 1-1297
4C11	N	N	Apo B-100 amino acid residues 2377-2658
2G8	M	M	Apo B-100 amino acid residues 3728-4306
3C8	M	N	Apo B-100
2E3	M	0	Apo B-100
2G1	0	0	MDA modified apo B-100
7C2	0	0	MDA modified apo B-100
3G4	0	0	MDA modified apo B-100

Note. M: antibodies significantly better bind enzyme-modified LDL; N: antibodies better bind native LDL; 0: negligible difference in antibody binding to native and enzyme modified LDL.

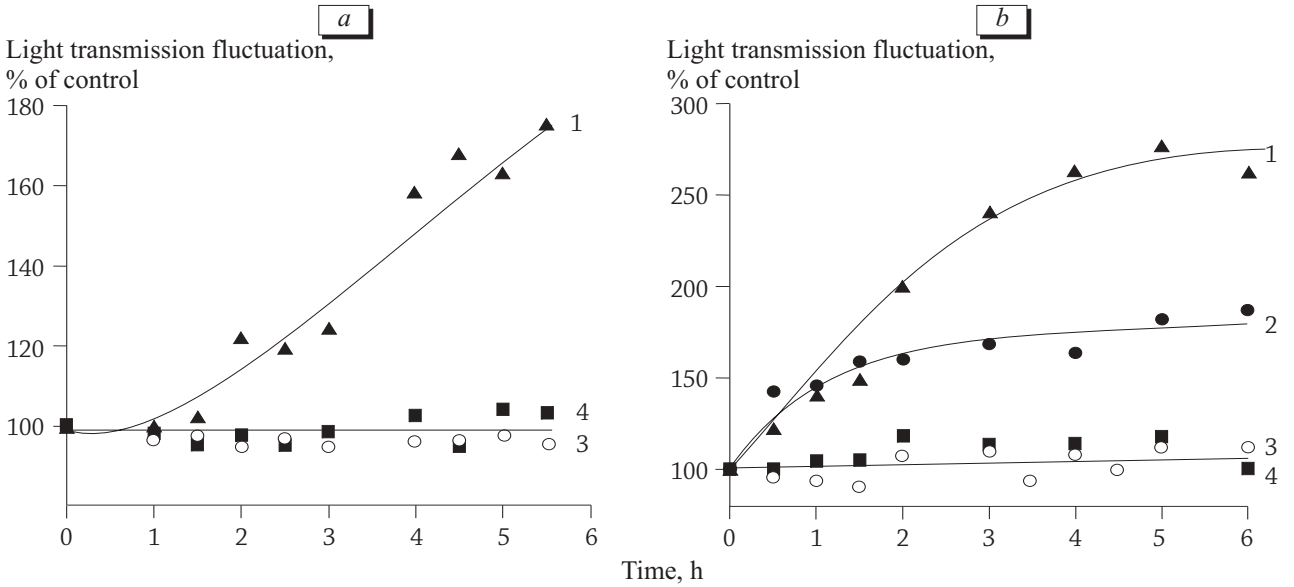

Fig. 3. Changes in LDL (0.2 mg protein/ml) suspension light transmission fluctuation at 37°C with trypsin (*1*, *3*; *a*; 1 U/ml) and without it (*4*) or with chymotrypsin (*b*). For *b*: chymotrypsin concentration 1 U/ml (*1*), 0.5 U/ml (*2*). For *a* and *b*: *3*) 1 U/ml trypsin or chymotrypsin+0.5 ml phenylmethane sulfonylfluoride.

(Fig. 3, *b*) and hence, caused more intense association of LDL particles.

Trypsin and chymotrypsin inhibitor sharply decreased apo B-100 proteolysis and prevented the increase in LDL suspension light transmission fluctuation (curves *3*, Fig. 3, *a*, *b*). This result indicates that increased association of LDL particles observed in our experiments is caused by changes in apo B-100 conformation on LDL surface as a result of proteolysis.

In the majority of cases the studied antibody clones similarly reacted to modification of apo B-100 induced by trypsin or chymotrypsin proteolysis. Binding of clone 2G8 antibodies, specific to apo B-100 site with amino acid residues 3728-4306 [1] was significantly more effective with LDL treated with trypsin or chymotrypsin compared to native LDL (Table 1). It seems that proteolysis induced by both enzymes increased availability this epitope of apo B-100 on LDL surface for clone 2G8 antibodies. Clone 4C11 antibodies bound to LDL subjected to trypsin or chymotrypsin proteolysis less intensely than to native LDL (Table 1). Presumably, after proteolysis apo B-100 epitope with amino acid residues 2377-2658, to which this clone is specific [1], was destroyed or appreciably changed its conformation, and hence, antibody binding to apo B-100 on LDL surface was deteriorated. Clone 5F8 antibodies, specific to apo B-100 epitope with amino acid residues 1-1297 [1] exhibited no appreciable differences in binding to native LDL or LDL subjected to trypsin or chymotrypsin proteolysis (Table 1). Binding of clones 2G1, 7C2, and 3G4 antibodies to LDL did not change after incubation with trypsin or chymotrypsin. These antibodies are monoclonal to LDL modified by MDA, one of the main products of lipid peroxidation. It seems that changes in apo B-100 on LDL surface caused by incubation with proteolytic enzymes have nothing to do with changes under the effect of MDA.

Clone 3C8 antibodies more effectively bound to LDL treated with chymotrypsin than to native LDL. Trypsin treatment deteriorated binding of this antibody clone on LDL surface (Table 1). Clone 2E3 antibodies also better bound to LDL treated with chymotrypsin than to native LDL, but LDL incubation with trypsin virtually did not modify binding of this antibody clone (Table 1).

Both proteinases differing by specificity modify LDL topography and reduce association resistance of LDL particles.

Hence, apo B-100 proteolysis induced by different proteolytic enzymes modified the location of this protein on LDL surface. This violation of LDL particle surface structure destabilizes it, reducing association resistance. Various proteolytic agents are detected in vascular regions damaged by atherosclerosis [5,6], and we therefore hypothesize induction of LDL association *in vivo* by proteolysis. This hypothesis is confirmed by the fact that fragmented apo B-100 was not once detected in LDL isolated from atherosclerotic areas of arteries [4,8], but never in arteries without visible foci of athero-

sclerosis [14]. LDL isolated from atherosclerotic vessels were characterized by low density, indicating partial loss of protein as a result of its proteolysis [2,8].

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