Apoprotein B-48 Is a Constant Finding in Very Low Density Lipoproteins of Humans

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The results of this study indicate that very low density lipoprotein (VLDL) from the plasma of fasting normolipidemic or slightly hypertriglyceridemic subjects contains two apo B species. In SDS gel electrophoresis, the VLDL shows the presence of a major band corresponding to low density lipoprotein (LDL) apo B (apo B-100) and a minor band with the appropriate mobility of the lymph chylomicron apo B (apo B-48). The reactivity of monoclonal antibodies directed against opportunely selected human apo B suggests that the protein with the lower molecular weight corresponds to apo B-48. This finding was confirmed by using immunoabsorbants and affinity chromatography with monoclonal antibodies that react only with apo B-100. Through this method, VLDL were separated into two fractions: one that had only apo B-100 and one with both apo B-100 and apo B-48. Hepatic and intestinal VLDL may constitute different particles. The ratio of apo B-100 to apo B-48 in VLDL decreased as the mass of fasting plasma VLDL increased. This may improve our understanding of the metabolism of triglyceride-rich lipoproteins. The investigation of the new subspecies of apo B may be relevant in understanding the atherogenetic process and better defining the hyperlipidemic states. (Arteriosclerosis 5:88–92, January/February 1985)

In recent years it has become evident that human apo B is chemically and physically heterogenous. The principal species of apo B in human very low density lipoproteins (VLDL) and low density lipoproteins (LDL) is synthesized by the liver and is termed apo B-100 in a percentile system of nomenclature. An apo B form of intestinal origin, apo B-48, is predominant in human chylomicrons and is not found in normal LDL. The amino acid composition of apo B-100 and apo B-48 is different, and their synthesis seems to be under separate genetic control. Although immunological cross-reactivity exists between the two apo B species, Marcel and co-workers have described the production and characterization of a series of monoclonal antibodies against human apo B-100; five of these did not react with apo B-48. Recently, researchers have reported the presence of apo B-48 in the VLDL of subjects with familial Type III hyperlipoproteinemia, in patients with Types I and V, and in a subject with LCAT deficiency. In normal fasting VLDL or in fasting VLDL of other types of hyperlipoproteinemia, no apo B-48 has been detected. In the rat, hepatic synthesis of both apo B-100 and apo B-48 has been demonstrated, while human hepatic cells synthesize VLDL containing only apo B-100. Nevertheless, VLDL are not synthesized only by the liver; human intestine also produces VLDL. Fasting and postabsorptive human jejunal mucosa contain VLDL-sized particles, and intestinal VLDL are present in lymph. The chemical composition of intestinal VLDL is similar to that of chylomicrons; however, the increased content of surface constituents (phospholipids, free cholesterol, and proteins) and of apo B suggests that intestinal VLDL are not just small chylomicrons. Furthermore, the apo A-I isoform that predominates in lymph chylomicrons is also present in the VLDL of normal subjects.

The aim of our study was to verify the presence of apo B-48 in normal fasting VLDL by using SDS gel electrophoresis and monoclonal antibodies against human apo B.

Methods

Subjects

The plasma used for the study was derived from six Italian men aged 30 to 40 years with values of plasma triglycerides below the 90th percentile of our population (160 mg/dl) or with a mild endogenous hypertriglyceridemia (plasma triglycerides above the 90th percentile but normal LDL cholesterol levels...
and a normal cholesterol/triglyceride ratio in total VLDL. No subject showed signs of Type III hyperlipoproteinemia, as judged by the absence of beta electrophoretic mobility in the d < 1.006 g/ml lipoprotein fraction of plasma. Nor did any have a cholesterol/triglyceride ratio below 0.42 in total VLDL. The selected subjects each had a body weight index below 1.2, normal glucose tolerance, and were eating an average Italian diet (55% carbohydrate, 15% protein, and 30% fat). All were free of liver or renal disease and thyroid disfunction and there was no evidence of alcohol abuse. Patients gave informed consent to the study.

**Samples**

Venous blood, drawn after a 14-hour fast, was collected in test tubes containing ethylene diamine tetraacetic acid (EDTA; 1 mg/ml), E-amino-n-caproic acid (EACA; 1.3 mg/ml), reduced glutathione (GSH; 0.5 mg/ml), and chloramphenicol (CAF; 0.05 mg/ml); this was immediately centrifuged at low speed. The plasma thus obtained was adjusted to d < 1.006 g/ml by adding 0.15 M NaCl containing the same preservative components. The VLDL were then separated by ultracentrifugation at 105,000 g at 5°C for 25 hours with a Beckman L5-65 ultracentrifuge and a Ti-70 rotor. The floating lipoproteins were removed by the tube-slicing technique and recentrifuged under similar conditions to purify the d < 1.006 g/ml fraction. No chylomicrons were detected in the samples when checked by agarose electrophoresis. Aliquots of plasma samples were stored at 4°C in a refrigerated box and carried to the Clinical Research Institute of Montreal, Canada, where the VLDL for the experiments was prepared 24 hours later for a study using monoclonal antibodies. As standard samples, we used chylomicrons from thoracic duct lymph which contained only apo B-48, VLDL from a Type III subject and equal amounts of pooled LDL were applied to the columns. Using 0.015 M phosphate-buffered saline (pH 7), we eluted the unbound lipoproteins (nonretained fraction). We eluted the bound lipoproteins (retained fraction) with 0.05 M citric acid containing 0.02% sodium azide and 0.01% EDTA. The nonretained fraction was applied again to both columns, then subjected to alternating washes of 0.1 M sodium acetate (pH 4) and 0.1 M sodium carbonate (pH 8-9). An 8 ml column of 5E11 Sepharose and an 8 ml column of 4G3-Sepharose were disposed in serial sequence.

VLDL (2 mg of protein) isolated from a normal subject, from a patient with endogenous hypertriglyceridemia, and from a Type III subject and equal amounts of pooled LDL were applied to the columns. Using 0.015 M phosphate-buffered saline (pH 7), we eluted the unbound lipoproteins (nonretained fraction). We eluted the bound lipoproteins (retained fraction) with 0.05 M citric acid containing 0.02% sodium azide and 0.01% EDTA. The nonretained fraction was applied again to both columns, then chromatographed twice, and re-eluted. The eluted fraction was immediately dialyzed against 0.015 M phosphate-buffered saline containing 0.02% sodium azide and 0.01% EDTA; the immunoreaction of apo B after SAS gel electrophoresis and transfer to nitrocellulose paper were performed.

**Monoclonal Antibodies**

Details of preparation and characterization of the monoclonal antibodies against apo B have been described in previous papers by Marcel's group. Briefly, from a cell fusion between cells of the plasmocytoma cell line, SP2-0, and isolated spleen cells from BALB/c mice previously immunized with normal human LDL (1.030–1.050 g/ml), hybridomas were obtained that secreted monoclonal antibodies that reacted positively with LDL. Of these, seven were selected for detailed study. These antibodies, (designated 1F1, 2D8, 3A8, 3A10, 3F5, 4G3, and 5E11) reacted with delipidated, denatured apo B, but showed no reactivity with apo A-I, apo A-II, apo C-II, apo C-III, or apo E. Some antibodies (3F5, 4G3, 3A8, 3A10, 5E11) recognized determinants of apo B close to the LDL-receptor recognition site and reacted only with apo B-100; others (1D1, 2D8) were specific for different regions of apo B and reacted with both apo B-100 and apo B-48. Monoclonal antibodies, 4G3 and 5E11, which did not react with the apo B of chylomicrons (apo B-48), and 1D1, which showed cross-reactivity with apo B-100 and apo B-48, were used in the study.

**Immunoadsorbants and Affinity Chromatography**

Immunoadsorbants and affinity chromatography according to the method of Milne et al. were used. The IgG were precipitated from the ascitic fluid of mice bearing the hybridomas, 4G3 and 5E11, by using 40% saturated ammonium sulphate. The precipitated proteins were redissolved and dialyzed against 0.1 M sodium bicarbonate (pH 8). Activation of Sepharose 4B (Pharmacia Incorporated, Uppsala, Sweden) and the coupling of 200 mg protein (5 mg/ml) of activated Sepharose 4B (16 hours at 4°C, with agitation) were carried out according to the method of March et al. The Sepharose was saturated with 1 M glycine (pH 8.5) overnight at 4°C and then subjected to alternating washes of 0.1 M sodium acetate (pH 4) and 0.1 M sodium carbonate (pH 8.5). An 8 ml column of 5E11 Sepharose and an 8 ml column of 4G3-Sepharose were disposed in serial sequence.

For SDS electrophoresis, the total VLDL and the fractions obtained by using immunoadsorbants and affinity chromatography were delipidated with ethanol/diethyl-ether and were dried under nitrogen. Protein (35–50 µg) was then dissolved in 50 mM sodium phosphate (pH 7.2) containing SDS (10 mg/ml of protein). This was heated at 100°C for 2 minutes. The samples were cooled, and the fractions containing 30 µg of proteins were used for electrophoresis.

Electrophoresis was performed in 3% polyacrylamide gel tubes (0.6 x 10.5 cm) and in gel slabs (1.5 cm).
Electrophoresis was conducted in Tris glycine buffer containing 0.1% SDS at 3% A/gel until the tracing dye migrated to 2 mm from the bottom of the tubes. The gels were removed, fixed in 10% trichloroacetic acid for 5 to 6 hours, and then stained with a solution containing 0.5 g of Coomassie R 250 in 10% isopropyl alcohol for 12 hours. They were then stained for 5 hours with 0.05 g of Coomassie R 250 in 10% isopropyl alcohol and 10% acetic acid at 55°C. After destaining in 7% acetic acid at 55°C, the protein bands were evaluated at 575 nm by a Chromoscan (Carlo Erba, Milano).

Immunoreaction of Apo B after Electrophoresis

After migration in SDS gel slabs, the separated protein bands were transferred electrophoretically at constant current (300 mA/3 hours) to nitrocellulose paper (Millipore) and processed as described by Marcel et al. The nitrocellulose paper was saturated for 1 hour at 35°C in 10 mM Tris-HCl buffer (pH 7.4), 150 mM NaCl, 0.1% Na azide buffer (Buffer A) containing 3% bovine serum albumin (BSA). The solution was made up to 3% normal rabbit serum. Appropriate monoclonal antibodies against apo B or normal mouse IgG (control) were added at dilutions ranging from 1/150 to 1/500 to provide an excess of antibody relative to the apo B transferred to the nitrocellulose paper. Incubation with the antibodies lasted for 4 hours at 37°C. The paper was washed three times with buffer A, and then incubated for 4 hours at 37°C with rabbit 125I-antimouse antiserum (106 cpm) in 15 ml of Buffer A containing 3% BSA and 3% normal rabbit serum. The bands were washed three times with Buffer A and dried before autoradiography on XAR-5 Kodak films with an intensifier screen (Cronex, Dupont).

Chemical Analyses

Proteins were assayed by the method of Lowry et al. with human albumin used as the standard. Triglycerol and total cholesterol were determined enzymatically by using reagents from Menarini in Florence and Carlo Erba in Milan, respectively. Phospholipids were quantitated with a specific test kit from Poly Diagnostics in Milan.
Results

The study of apo B by SDS polyacrylamide gel electrophoresis proved the presence in the VLDL of all the subjects of a major band corresponding to LDL apo B (apo B-100) and a minor band with the mobility of lymph chylomicrons of apo B-48 (Figure 1). The same pattern in SDS gel electrophoresis was repeatedly obtained with the VLDL from these and other subjects. Scanning of the gels showed the apo B-100/apo B-48 ratio tending toward a negative correlation with the levels of triglycerides and of VLDL mass. The lowest value of the ratio was found in the VLDL of the Type III patient (Figure 1).

Monoclonal antibody 1D1, which showed a cross-reactivity with either apo B-100 or apo B-48, and antibody 4G3, which only reacted against apo B-100, were tested for their reactivity with VLDL apo B provided by a normal subject (2 in Figure 1), a hypertriglyceridemic subject (5 in Figure 1), and a Type III patient, and with apo B from pooled LDL. The reactivity of these antibodies was evaluated after SDS gel electrophoresis and transfer to nitrocellulose paper (see Figure 2). Whereas antibody 4G3 reacted only with apo B-100, Antibody 1D1 reacted both with apo B-100 and with a lower weight apo B, apo B-48. These are species present in the VLDL of normal and Type III subjects and are not detectable in LDL.

We used immunoadsorbants and affinity chromatography by 4G3 and 5E11-Sepharose columns in duplicate experiments on the fasting VLDL of a normal subject and a hypertriglyceridemic subject (2 and 5 in Figure 1) to elute the unbound lipoproteins. In this nonretained fraction, both apo B-48 and an appreciable amount of apo B-100 were present and reacted with the 1D1 antibody. In the retained fraction, only apo B-100 was detected (Figure 3); in this fraction, about 55% of the seeded VLDL protein was recovered.

Discussion

Plasma VLDL in fasting men results from a dual contribution from liver and intestine. The study of the intestinal species of apo B appears to provide useful information, since this is the marker through which lipoproteins from intestine may be detected. Kane et al. have recorded the intestinal apo B-48 in VLDL of Type III patients only. According to those authors, in normal fasting VLDL, as well as in fasting VLDL of endogenous hypertriglyceridemia, no apo B-48 is detectable when plasma triglyceride levels are below 600 to 700 mg/ml. (Only in rare instances have trace quantities of apo B-48 been observed in the VLDL of subjects with lower triglyceride values.) Recently Meng et al. have observed the presence of apo B-48 in the d <1.006 lipoprotein fraction of some patients with Type I and V hyperlipidemia; moreover, no intestinal apo B-48 was detected in this lipoprotein fraction from normal subjects or from Type IIa, IIb, IV patients; surprisingly, there was none in 35% of Type I and V patients, despite the presence of fasting plasma chylomicrons.

In contrast, we have recorded an appreciable amount of apoprotein with the appropriate mobility in SDS gel of lymph chylomicrons apo B-48 in the VLDL of both normolipidemic and slightly hypertriglyceridemic subjects. It is hard to explain these different findings; they may be due to a technical detail, possibly the load of VLDL proteins in the gels (50% higher in our experiments than in those by Meng et al.). Nevertheless, the use of different monoclonal antibodies against human apo B confirms that the
band moving in the SDS gel of VLDL with the mobility of apo B-48 of lymph chylomicrons has the same immunoreactivity and corresponds to this apoprotein. The possibility that our samples contained a protein with a molecular weight similar to apo B-48, probably a proteolytic fragment of apo B-100 (designated as apo B-50 by Marcel et al.3) may be excluded. This protein, in fact, is not recognized by the 1D1 monoclonal antibody;2 furthermore, no reactivity in the region of apo B-50 was found in our VLDL samples when we used the 4G3 antibody, which exhibits a strong reaction against apo B-50.3

The different behavior of the apo B-100/apo B-48 ratio in the various VLDL samples, as well as the trend toward lower ratio values with increasing mass of plasma VLDL, suggest that the determination of apo B-48 in this lipoprotein class may offer important information for the study of triglyceride-rich lipoprotein metabolism.

Due to the special role played by apo B in atherogenesis, it is likely that investigation of the new sub-species of apo B may be relevant in understanding the atherogenetic process and for a better definition of the hyperlipidemic states.

Acknowledgments

Part of this work was performed in the Clinical Research Institute of Montreal, Canada. We are indebted to Yves L. Marcel and thoracic duct lymph that were kindly supplied by Professor Gerhard Kostner of Graz.

References


Index Terms: very low density lipoproteins • apo B-48 • apo B-100 • monoclonal antibodies • immunoadsorbers • affinity chromatography
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_Arterioscler Thromb Vasc Biol._ 1985;5:88-92
doi: 10.1161/01.ATV.5.1.88

_Arteriosclerosis, Thrombosis, and Vascular Biology_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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