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The binding of apolipoprotein H (β_2-Glycoprotein I) to lipoproteins

Roberto Gambino[a], Gianluca Ruiu[b], Gianfranco Pagano[a], Maurizio Cassader[a,*]

[a]*Dipartimento di Medicina Interna, Universitá di Torino, Corso A.M. Digliotti 14, 10126 Torino, Italy*
[b]*Laboratorio Centrale Baldi e Riberi, Azienda Ospedaliera S. Giovanni Battista, 10126 Torino, Italy*

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Abstract

β_2-Glycoprotein I has a high affinity for triglyceride-rich particles, activates lipoprotein lipase, and is also defined as an apolipoprotein H. Previous studies have shown that apolipoprotein H is a regular structural component of the major classes of lipoproteins. In view of these findings, we analyzed the interactions of apolipoprotein H with lipoproteins in the fasting plasma of eight normal, seven hypertriglyceridemic, and seven hypercholesterolemic subjects. After rate-zonal, density gradient ultracentrifugation, apolipoprotein H was little distributed among the different density fractions, and most of it was recovered in the last fraction that contained the lipoprotein-free plasma. A small percentage (4–13%) of the apolipoprotein H associated with plasma lipoproteins was detected at the density ranging from 1.090 to 1.225 g/ml. This result means that apolipoprotein H is little associated with lipoproteins.

Keywords: Apolipoprotein H; β_2-Glycoprotein I; Human serum lipoproteins

1. Introduction

β_2-Glycoprotein I is a plasma glycoprotein that has a molecular weight ranging from 43 000 to 50 000 daltons [1,2]. It binds to platelet membranes; interacts with heparin;

* Corresponding author. Tel.: +39-11-696-7864; fax: +39-11-663-4751.
E-mail address: pagano@molinette.unito.it (M. Cassader)

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modulates the activity of adenylate cyclase; and because it inhibits the prothrombinase activity on human platelets, may be involved in blood coagulation. Compared to other human plasma proteins, β_2-Glycoprotein I has a high content of cystein (6.2%) and proline (8.3%) [1].

Because β_2-Glycoprotein I is isolatable on plasma lipoprotein, has a high affinity for triglyceride-rich lipoprotein particles, and activates lipoprotein lipase, it is also defined as an apolipoprotein (apo)H [5]. ApoH shows a genetically determined structural polymorphism caused by the presence of three common allelic forms at a single structural locus that determine six different phenotypes [6,7]. In our previous work, we found an association between hyper-triglyceridemia and apoH polymorphism, especially with the H*3 allele [8].

Although its physical and chemical characteristics are well studied, its physiological functions remain unknown. ApoH also displays anticoagulant properties, and interference with its function by anti-phospholipid antibodies could explain the thrombotic diathesis seen in association with these antibodies: apoH is the target to which antibodies are directed [9–11].

According to Polz and Kostner [12], apoH is a regular structural component of chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). It has been reported that apoH has a high affinity for triglyceride-rich particles, causing their selective precipitation by detergents such as sodium lauryl sulfate or sodium dodecyl sulfate [13], and activates lipoprotein lipase in the in vitro hydrolysis of artificial lipid emulsions [14]. In view of these findings, we analyzed the interactions of apoH with lipoproteins in fasting plasma of eight normal subjects, seven hypertriglyceridemic subjects, and seven hypercholesterolemic subjects.

2. Materials and Methods

2.1. Materials

Tris, urea, Tween 20, acrylamide, and N,N'-methylene-bis-acrylamide, analytical grade, were purchased from Bio-Rad (Richmond, CA, USA). Nitrocellulose sheets were also from Bio-Rad. Rabbit polyclonal antibody to apoH was kindly supplied by Behring (Scoppito, Italy). Goat anti-rabbit IgG (alkaline phosphatase conjugated), the alkaline phosphatase conjugate substrate kit, molecular weight standards were from Bio-Rad, and Prep-Cell was bought from Bio-Rad. Centricon tubes were from Amicon (Beverly, MA, USA). The cyanogen bromide activated Sepharose 6B column was purchased from Pharmacia Biotech (Uppsala, Sweden)

2.2. Subjects

We investigated the binding of apoH to plasma lipoproteins. We enrolled seven hypertriglyceridemic subjects (three males and four females, age range 32–60 years, triglycerides level $>$250 mg/dl), seven hypercholesterolemic subjects (three males and four females, age range 27–58 years, total cholesterol $>$240 mg/dl), and eight normal subjects (four males and

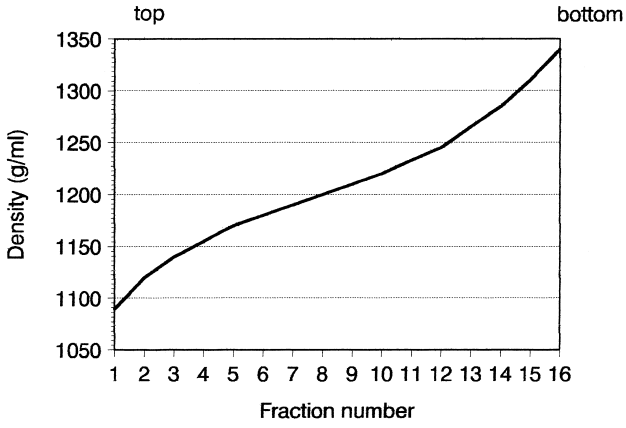

Fig. 1. Density profile of NaBr gradient after ultracentrifugation. The x axis represents the number of the fractions collected from the gradient. The y axis represents the density of each fraction. The bottom of the sample is on the right, and the top is on the left. The gradient was centrifuged and fractionated as indicated in Methods and materials.

four females, age range 30–55 years). Plasma samples and the ultracentrifugation fractions were assayed for their content of apoH with an enzyme-linked immunosorbent assay (ELISA) method.

2.3. Separation of lipoproteins

After 12 h of fasting, blood was drawn from each subject and placed in a vacutainer tube containing K_3 ethylene-diaminetetraacetic acid (EDTA) as an anticoagulant. Plasma was obtained by low speed centrifugation at 3000 $\times$ *g* at 4°C for 15 min and, lipoproteins were separated by preparative ultracentrifugation. Plasma was adjusted to the density of 1.220 g/ml by adding solid KBr (0.352 g/ml) and brought to a final volume of 2 ml with a salt solution of the same density. The samples were sealed in Quick-Seal tubes and ultracentrifuged at 60 000 $\times$ *g* for 48 h at 12°C in a TL-100 ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA, USA). The floating lipoproteins were removed by tube slicing.

To confirm the poor binding of apoH to lipoproteins, plasma from one subject from each group was subjected to a density gradient ultracentrifugation [15]. The rate-zonal, density gradient ultracentrifugation used was originally developed for the separation of the high density lipoprotein subclasses HDL_2 and HDL_3 from human plasma. (VLDL and LDL were not separated from each other by this procedure.) This method of separation is based on the difference in the floation rate of the high density lipoprotein subclasses. The density profile of a NaBr gradient after ultracentrifugation is displayed in Fig. 1. The gradient fractions were analyzed for cholesterol, triglycerides, and the distribution of apoH.

Plasma was adjusted to a density of 1.40 g/ml by adding solid KBr. Aliquots (2-ml) were pipetted on the bottom of Beckman Ultra-Clear SW-40 tubes and over-layered with 2.5 ml NaBr-1 mM EDTA (pH 7.4), density 1.25 g/ml; 7.5 ml NaBr-1 mM EDTA (pH 7.4), density 1.19 g/ml; and 2.0 ml 1 mM EDTA (pH 7.4). The gradients were centrifuged at 15°C for 21 h

at 40 000 × *g* in a Ti SW-40 swinging bucket rotor using a Beckman LM-70 ultracentrifuge. After the run, the tubes were punctured from the bottom, and 0.75 ml fractions were collected (Density Gradient Fractionator, Model 185, ISCO Inc., Lincoln, NE, USA). Absorbance was measured at 254 nm. Fractions were assayed for cholesterol and triglycerides content.

2.4. Dot blot analysis

Rapid screening of the fractions for apoH content after density gradient ultracentrifugation was conducted in a Bio-Dot micro-filtration apparatus (Bio-Rad). A sheet of nitrocellulose was clamped between a gasket and a 96-well sample template. Sample (5 μl) was allowed to filter through the membrane. After the antigen was immobilized, the nitrocellulose was incubated in 4% albumin-blocking solution. The nitrocellulose was subsequently incubated with rabbit IgG anti-apoH, at a 1:2000 dilution at room temperature for 1.5 h, and then with goat IgG anti-rabbit IgG labeled with alkaline phosphatase at a 1:6000 dilution (Sigma, Milan, Italy). Dots were visualized with Immun-Blot Assay kit (Bio-Rad) by following the manufacturer's instructions.

2.5. Isolation and purification of apoH

ApoH was isolated from human plasma and purified through a combination of affinity chromatography and continuous-elution electrophoresis as previously described [16]. Briefly, rabbit anti-human apoH antibodies were immobilized on a cyanogen-bromide activated Sepharose 6B column (Pharmacia Biotech), and normal plasma samples were applied. The apoH bound to the column was eluted with 0.1 M glycine, 0.05% NaN_3, pH 2.5, and concentrated to a final volume of ~1 ml. To achieve maximum purification of the apoH, the concentrated sample was then subjected to continuous-elution electrophoresis in a Prep-Cell (Bio-Rad) at 40 mA through a cylindrical gel made with 8.5% polyacrylamide. The samples containing apoH were subjected to 12% sodium dodecyl sulfate polyacrilamide gel electrophoresis and immunoblotted after to a nitrocellulose sheet electrophoretic transfer. The purified apolipoprotein migrated as a single band on the sodium dodecyl sulfate gel electrophoresis and reacted with the specific antibody. The amino acid analysis of our purified apoH was similar to that reported by Steinkasserer et al. [17]. ApoH, in its pure form, was used for both horse-radish-peroxidase (HRP) labeling and as the standard for the ELISA.

The Bio–Rad protein assay was used to measure the purified apoH concentration. An Ouchterlony plate analysis against purified apoH, apoE, albumin, transferrin, α-1 acid glycoprotein, and β2-microglobulin showed only one precipitating arc between anti-apoH and purified apoH.

2.6. ELISA method

ELISA was performed as previously described [18] with some modifications. The two-step glutaraldehyde method was used. Briefly, glutaraldehyde was diluted in 0.1 M phosphate buffer, pH 6.8, to a final concentration of 1.25%. HRP (5 mg) was dissolved in 0.1 ml glutaraldehyde solution and allowed to incubate overnight at room temperature. The HRP-

glutaraldehyde mix was added to an apoH solution (400 μg in 200 μl 0.1 M carbonate/bicarbonate buffer, pH 9.5) and incubated overnight at room temperature. The remaining sites were blocked with 0.2 M ethanolamine, pH 7, for 2 h at 4°C. Labeled apoH was dialyzed in 0.05 M Tris 0.15 M NaCl, pH 7.4, overnight at 4°C. The unconjugated HRP molecules were removed by gel filtration.

Rabbit anti-apoH antibodies were diluted 1:8000 with 0.05 M carbonate/bicarbonate buffer, pH 9.5, and 100 μl were pipetted into each well of a 96-well microtiter plate. This was then sealed and left at 37°C for 1 h. Washing with 0.02 M sodium phosphate, pH 7.8, 0.15 M NaCl (phosphate-buffered saline) to remove unbound antibodies was followed by a blockade of the remaining sites by incubation with 350 μl 4% bovine serum albumin in phosphate-buffered saline for 1 h at 37°C.

Unknown plasma samples and standards were diluted in assay buffer solution before being assayed. The optimum ELISA conditions were 50 μl standards or plasma diluted 400-fold and 50 μl purified apoH labeled with HRP diluted 1000-fold. Samples were pipetted into the wells of pre-coated microtiter plate, which was then covered and incubated for 2 1/2 h at 37°C.

The plate was washed six times with assay buffer solution. A freshly prepared enzyme substrate solution (100 μl), made by dissolving an o-phenylendiamine dihydrochloride tablet in 0.05 M phosphate-citrated buffer, pH 5.0 to a final concentration of 1 mg/ml was added to the wells. The plate was briefly agitated, covered, and left in the dark at room temperature for 20 min. The reaction was then stopped by adding 50 μl 3M HCl to each well, and the plate was agitated to ensure thorough mixing. Well absorbance was measured with a Bio-Rad 3550 reader at 490 nm. Plasma concentrations were expressed in mg/dl. The standard curves were constructed with apoH isolated from human plasma. The working range was from 1.9 to 120 mg/dl.

The intra-assay coefficient of variation (CV) for each sample calculated from the results of 10 pairs of wells in a single assay was 3.8–5.0%. The inter-assay CV for each sample calculated from the results of pairs of wells in five assays was 6.2–8.8%. In the absence of appropriate apoH reference plasma, accuracy was determined from the recovery that was obtained after the addition of our isolated apoH in known concentrations to normal plasma.

3. Results

Total plasma levels of triglycerides, cholesterol, and apoH in normal, hypercholesterolemic, and hypertriglyceridemic subjects who were recruited in accordance with the reported parameters are described in Table 1. This table also reports the percentage of apoH bound to the lipoproteins that were separated by preparative ultracentrifugation, expressed as the top and bottom of samples that were raised at 1.220 g/ml density. Our results show that apoH is little bound to the fraction containing the lipoproteins (Table 1).

After the rate-zonal, density gradient ultracentrifugation, the gradient fractions were analyzed for cholesterol, triglycerides, and the distribution of apoH. The cholesterol and triglyceride concentrations were in accordance with the chemical composition of plasma lipoproteins. As for the apoH, most of it (dot blot of plasma from one subject from each

Table 1
Total plasma levels of triglycerides, cholesterol, and apoH in normal (n = 8), hypercholesterolemic (n = 7), and hypertriglyceridemic (n = 7) subjects

	Triglycerides (mg/dl)	Cholesterol (mg/dl)	apoH (mg/dl)	Density Top = 1.220 (% total)	Bottom = 1.220 (% total)
Normals (n = 8)	128 ± 56	189 ± 26	13 ± 4.4	3.6	96.4
Hyper-Tg (n = 7)	357 ± 61	199 ± 34	42 ± 13	10.4	89.6
Hyper-Chol (n = 7)	147 ± 45	281 ± 18	29 ± 10	13.2	84.9

Reported are the percentage of apoH bound to the lipoproteins separated by preparative ultracentrifugation and expressed as top and bottom of samples raised at 1.220 g/ml density. Values are mean ± SD.

group) was recovered in the last fractions that contained the lipoprotein-free plasma (Fig. 2). A small percentage of the apoH associated with plasma lipoproteins was detected at density ranging from 1.090 to 1.220 g/ml. This result means that apoH was predominantly found in the fractions that had a density above 1.230 g/ml.

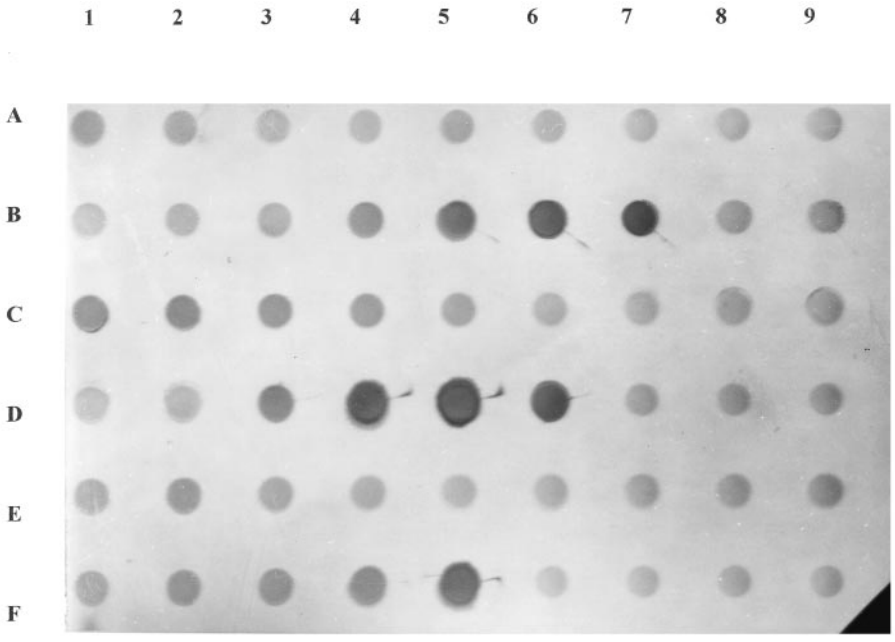

Fig. 2. Dot blot of the fractions collected from the gradient after rate-zonal ultracentrifugation of the plasma from one subject from each group. Dots A–B refer to one hypercholesterolemic subject; dots C–D refer to one hypertriglyceridemic subject; dots E–F refer to normal subject. Dots A1–3, C1–3, E1–3 show the presence of apoH in VLDL and LDL. Dots A4–10, C4–10, E4–10 show the presence of apoH in HDL_2 and HDL_3. Dots B1–10, D1–10, F1–9 shows the presence of apoH in the lipoprotein-free plasma. Dot-blotting analysis shows that most of apoH is lipoprotein-free as indicated by the positive dots B5–6–7, D4–5–6, and F6.

4. Discussion

Because β_2-Glycoprotein I is isolatable on plasma lipoprotein, has a high affinity for triglyceride-rich lipoprotein particles, and activates lipoprotein lipase, it is also defined as apoH [5]. ApoH has been identified as a component of circulating plasma lipoproteins [2,5]. Despite being a component of plasma, this apolipoprotein displays behavior that is substantially different from other apolipoproteins that form the integral components of plasma lipoproteins [19]. Whereas the conformation of these apolipoproteins changes in the presence of lipid, the secondary and tertiary organization of apoH, at neutral pH, may be similar in the lipidated and lipid-free states [5]. Moreover, apoH associates with plasma lipoproteins through protein-protein interactions rather than protein-lipid interactions, and the secondary structure of apoH is different from other apolipoproteins [5].

Chylomicrons and VLDL, secreted by the intestine and liver, respectively, are large lipid-protein complexes that are involved in the transport and metabolism of triglycerides, cholesterol, and cholesterol esters. The nascent particles, which are composed primarily of apolipoprotein B and lipids, gain additional apolipoproteins on entering the plasma [20,21]. Polz and Kostner [12] found apoH in chylomicrons, VLDL, LDL and HDL. Approximately 16% by weight of plasma apoH was found with chylomicron and VLDL, 2% with LDL, 17% with HDL, and the remainder (65%) in the 1.21 g/ml density infranatant.

In this work, we performed a similar experiment and plasma apoH levels were quantified with a competitive ELISA method [18]. Sensitivity and specificity of the method was satisfactory, and the performance of subsequent assays was not affected by the inter-assay CV. Cassader et al. [18] reported the mean value obtained in a normal population (22.5 ± 7.7 mg/dl) was similar to that observed, in a smaller study using a (RIA) method [22] and to that observed in a study comparing Laurell rocket immunoelectrophoresis with the ELISA technique [23]. This method was chosen to rule out the possibility of interference by proteins possessing some degree of affinity with the polyclonal antibody employed. Interference of various kinds, in fact, as well as technical problems, were encountered when a sandwich ELISA was used to measure urinary apoH levels [24].

After the removal of all lipoproteins from the plasma at a density of 1.230 g/ml, the largest amount of apoH was found in the bottom fraction. Just a small percentage of apoH was associated with lipoproteins. Our results differ from those reported by [12]. The percentage of lipoprotein-bound apoH they found was higher than the percentage we found.

The rate-zonal, density gradient ultracentrifugation used was originally developed for the separation of the high density lipoprotein subclasses HDL_2 and HDL_3 from human serum. (VLDL and LDL were not separated from each other by this procedure.) This method of separation is based on the difference in the flotation rate of the high density lipoprotein subclasses. The gradient fractions were analyzed for cholesterol, triglycerides, and the distribution of apoH. The cholesterol and triglyceride profiles were in accordance with the chemical composition of plasma lipoproteins.

Most of the apoH was recovered in the last fractions that contained the lipoprotein-free plasma. Small percentages (4–13%) of the apoH associated with plasma lipoproteins were detected. This result means that apoH is predominantly found in the fractions with a density above 1.230 g/ml. This observations agrees with the work of Polz and Kostner [12], who

found most of the apoH in the bottom fraction, too, but conflicts with their results in the percentages of free and lipoprotein-associated apoH. We report here that >95% of the apoH was not associated with lipoproteins in normal subjects and that apoH becomes more associated with lipoproteins in hypertriglyceridemic and hypercholesterolemic subjects. This increase of associated apoH could reflects a higher production of VLDL and LDL. This discrepancy could be due to an overestimation of the apoH in lipoproteins classes probably from a cross-reaction of the antibody used in the radial immunodiffusion. As a matter of fact, apoH belongs to a wide family of proteins that share a common repeat unit [25].

Our result is in agreement with our previous report [8] where we found only traces of apoH bound to plasma lipoproteins. Moreover, the sample collected from the affinity column contained apoH and other contaminating proteins. No lipoproteins were found in the eluate. One can assume that the apoH is masked on the lipoprotein surface and not recognized by the antibody. But the apolipoproteins were located on the outer surface of a lipoprotein and should be accessible to antibody. This observation led us to study the distribution of apoH in the major classes of lipoproteins after removal of lipoproteins from plasma through ultracentrifugation. Isolation of lipoproteins did not affect the binding of apoH to lipoproteins because the protein sediments slowly during ultracentrifugation, and any loss of protein from lipoproteins should be excluded [12]. This was supported by the fact that no classes of plasma lipoproteins could be isolated by affinity chromatography through an anti-apoH column as described above.

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