Characterization of ApoA-IV–Containing Lipoprotein Particles Isolated From Human Plasma and Interstitial Fluid

Nicolas Duverger, Nordine Ghalim, Gerard Ailhaud, Armin Steinmetz, Jean-Charles Fruchart, and Graciela Castro

Apolipoprotein (apo) A-IV has been proposed to play a role in reverse cholesterol transport. ApoA-IV–containing lipoprotein particles (A-IVLp) were isolated from human plasma and interstitial fluid and characterized by immunoaffinity chromatography. Two major A-IVLp subpopulations, lipoprotein particles containing apoA-I with apoA-IV (LpA-I:A-IV) and lipoprotein particles containing apoA-IV without apoA-I (LpA-IV), were identified. The larger subpopulation of A-IVLp is the LpA-IV that represents 70% (protein mass) of the initial particles. Only 5.8% of apoA-I V was recovered in the retained fraction after affinity chromatography with an anti-apoA-I immunosorbent. ApoA-I, apoA-II, apoA-IV, apoB, apoC-III, apoD, apoE, apoH, lecithin: cholesterol acyltransferase (LCAT), cholesteryl ester transfer (CET) protein, proline-rich protein, and a protein of Mr 59,000 were detected in the A-IVLp. These particles contain more than 20% triglycerides (lipid mass). ApoA-IV-containing particles that were isolated from plasma are heterogeneous in size, consisting of two major populations with Stokes’ diameters of 10.3 nm and 9.3 nm. Both subpopulations of A-IVLp contain LCAT and CET activities and promote cholesterol efflux from cholesterol-preloaded adipose cells. These data support the hypothesis that A-IVLp particles may be involved in reverse cholesterol transport. (Arteriosclerosis and Thrombosis 1993;13:126–132)

KEY WORDS • immunoaffinity chromatography • gel filtration • lipoprotein particles

Human apolipoprotein (apo) A-IV is a 46-kd protein that is synthesized primarily in the intestine.1,2 This apolipoprotein is found in plasma, interstitial fluid,3 and lymph.4 In plasma, apoA-IV has been shown to be associated with lipoprotein density fractions as well as in a lipoprotein-free form.5 However, apoA-IV is easily dissociated from lipoproteins by ultracentrifugation,6,7 and therefore, its true plasma distribution among lipoprotein particles remains unknown. By nondenaturing gradient polyacrylamide gel electrophoresis (PAGE), apoA-IV was found to be associated with high density lipoproteins (HDLs) as well as comigratory with albumin.8 By gel filtration9 and immunoprecipitation,10 apoA-IV was shown to be partially associated with HDL. However, all of these techniques could potentially induce perturbations and modify apoA-IV distribution. In addition, apoA-IV is an activator of the plasma enzyme lecithin:cholesterol acyltransferase (LCAT), and cholesterol ester production can also influence the distribution of apoA-IV among lipoproteins.11

Human apoA-IV is an LCAT activator12–14 and has a specific binding site on cultured bovine aortic endothelial cells15,16 and cultured mouse Ob 1771 adipose cell surfaces.14,17 Also, reconstituted apoA-IV–dimyristoylphosphatidylcholine (DMPC) complexes, as well as apoA-IV–containing lipoprotein particles that have been isolated from plasma, promote cholesterol efflux from cholesterol-preloaded adipose cells.18 To gain further insight into the physiological role of apoA-IV, we characterized apoA-IV–containing particles that were isolated from normolipemic human plasma and interstitial fluid by immunoaffinity chromatography.

Methods

Subjects

Fresh blood was collected from six healthy normolipidemic volunteers in tubes containing 1 µM EDTA, 1.2 g/l D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone, 0.1 g/l NaN3, 80 mg/l gentamicin, 10,000 units/l aprotinin, and 0.15 M NaCl (final concentration). All subjects consented to the procedure after explanation of the purpose of the study. The subjects had fasted for 12 hours. Plasma was collected by centrifugation for 20 minutes at 2,000g at 4°C. Benzamidine was added to the plasma at a final concentration of 1 mM.

Interstitial fluid was collected from 20 healthy normolipidemic subjects by blister suction of forearm skin18
for 1 hour. The final volume was 6 ml, to which the same inhibitory cocktail was immediately added. All following isolation procedures were performed at 4°C.

**Preparation and Purification of Anti-ApoA-IV Antibodies**

Antisera to apoA-IV were produced in rabbits by intraperitoneal administration of antigen. The procedure was in accordance with the guidelines of the French Department of Health and Protection of Animals. After precipitation with 40% (wt/vol) (NH₄)₂SO₄, immunoglobulins were passed over a column of cyanogen bromide–activated Sepharose 4B (Pharmacia) to which human apoA-IV had been coupled. Specific antibodies against apoA-IV were retained in the column and then eluted. First, an elution with 0.1 M acetic acid and 0.15 M NaCl (pH 3.5) was carried out to elute low-affinity antibodies. Second, an elution with 3 M NaSCN was performed to select high-affinity antibodies. Both eluted fractions were immediately dialyzed against 10 mM Tris and 0.15 M NaCl, pH 7.4 (referred to as Tris buffer), and passed over a column to which human apoA-I had been coupled. The unbound fractions were concentrated in Amicon Diaflo cells by using a PM 10 membrane (Amicon, Danvers, Mass.).

**Lipoprotein Particle Isolation**

Immunosorbents with polyclonal antibodies to apoA-IV (low affinity) and a mixture of monoclonal antibodies to apoA-I (A05-A17-A30) were prepared by coupling the antibodies to cyanogen bromide–Sepharose 4B according to the procedure of the manufacturer (Pharmacia). Plasma or interstitial fluid was applied at a flow rate of 10 ml/hr in Tris buffer to the anti-apoA-IV immunosorbent column until the absorption at 280 nm of the eluate was below 0.01 optical density units. After a wash with Tris buffer containing 0.5 M NaCl at a flow rate of 60 ml/hr to remove nonspecifically bound particles, the specifically bound particles, referred to as A-IVLp, were eluted with 3 M NaSCN and immediately filtered through a Sephadex G25 column to remove the thiocyanate.

An aliquot of A-IVLp was applied to an anti-apoA-I immunosorbent under the same conditions. To analyze simple lipoprotein particles and to avoid interactions with apoE and B/E receptors, both retained and unretained fractions were passed through a heparin–Sepharose column in 10 mM Tris (pH 7.4) to remove apoE. No apoE was detectable by a specific apoE enzyme-linked immunosorbent assay (ELISA) in the apoA-IV–without–apoA-I–containing lipoproteins (LpA-IV) and in the apoA-IV–with–apoA-I–containing lipoproteins (LpA-I:A-IV) that were obtained after this process. Finally, isolated particles were dialyzed against Tris buffer in a multiple Micro-Prodicon (Bio-Molecular Dynamics) equipped with a PM 10 membrane and filtered through a 0.22-μm Millipore filter.

**Analytical Methods**

ApoE and apoA-IV phenotypes were determined as described. Protein content was determined by the method of Lowry et al. with bovine serum albumin as the standard. The apolipoprotein concentrations were measured by specific noncompetitive ELISA with monoclonal antibodies to apoA-I, apoA-II, and apoE and polyclonal antibodies to apoB and apoC-III. For apoA-IV determination, the high-affinity apoA-IV antibodies were used at a concentration of 10 mg/ml for plate coating. The same antibodies were conjugated to horseradish peroxidase and used at a 1:5,000 dilution to develop the assay. Calibration curves were prepared with a secondary standard that was determined according to previously described methods.

Total cholesterol, free cholesterol, triglycerides, and phospholipid contents were measured by using kits from Boehringer Mannheim.

**Sodium Dodecyl Sulfate Electrophoresis and Immunoblotting**

Protein composition was analyzed by sodium dodecyl sulfate (SDS) 5–19% acrylamide gradient electrophoresis. Eighty micrograms of particle proteins was applied to each sample well. Protein bands were visualized by staining with 0.03% Coomassie blue R-250 that was dissolved in a methanol/acetic acid/water (25:10:45, vol/vol/vol) solvent and destained in the same solvent. For immunoblotting, proteins were electrophoretically transferred onto nitrocellulose paper at 4°C (30 minutes at 50 V, then 90 minutes at 100 V). The paper was cut into strips and saturated with 1% nonfat dry milk in Tris buffer for 1 hour at room temperature. The strips were incubated with the appropriate antibodies (10 μg/ml; 1 hour at room temperature for polyclonal antibodies or overnight at 4°C for monoclonal antibodies). After extensive washing with Tris buffer, the strips were incubated with a peroxidase-labeled second antibody for 1 hour at room temperature. Excess labeled antibody was washed away with Tris buffer. Enzymatic activity was developed by addition of 4-chloro-1-naphthol and hydrogen peroxide.

**Gel Filtration Chromatography**

Particle size was determined by gel filtration chromatography on a fast protein liquid chromatography system (Pharmacia) with a Superose 6B 10/30 column equilibrated in Tris buffer at a flow rate of 0.2 ml/min. All of the isolated lipoproteins were applied to the column, which was calibrated for particle size with very low density lipoprotein (VLDL), low density lipoprotein (LDL), and HDL, as well as the following protein standards: thyroglobulin (17 nm), ferritin (12.2 nm), and bovine serum albumin (7.5 nm). When one sample of each isolated subpopulation was applied three times to the column, identical profiles were obtained.

**Cellular Cholesterol Efflux Studies**

The characterization of the Ob 1771 preadipocyte cell line was reported previously. [3H]Cholesteryl linoleate was transferred to LDL by the method of Craig et al, and the [3H]cholesteryl linoleate–labeled LDL was used for cholesterol loading of the cells. To promote cholesterol efflux at 37°C, differentiated cells were maintained for 48 hours in 7% lipoprotein-deficient serum and then exposed to [3H]cholesteryl linoleate–rich LDL for 48 hours (0.15 mg LDL cholesterol per milliliter) in the same buffer. Subsequently, cells were rinsed in 0.1 M phosphate-buffered saline, maintained in serum-free medium, and incubated with particles (50 μg protein
TABLE 1. Plasma Lipid Concentrations and Apolipoprotein Concentrations of Study Subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>F</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>155</td>
<td>176</td>
<td>148</td>
<td>201</td>
<td>154</td>
<td>161</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>91</td>
<td>90</td>
<td>57</td>
<td>81</td>
<td>115</td>
<td>102</td>
</tr>
<tr>
<td>Phospholipid (mg/dl)</td>
<td>195</td>
<td>179</td>
<td>183</td>
<td>207</td>
<td>196</td>
<td>212</td>
</tr>
<tr>
<td>Apolipoprotein A-I (mg/dl)</td>
<td>116</td>
<td>166</td>
<td>123</td>
<td>225</td>
<td>118</td>
<td>120</td>
</tr>
<tr>
<td>Apolipoprotein A-II (mg/dl)</td>
<td>55</td>
<td>33</td>
<td>65</td>
<td>36</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>Apolipoprotein A-IV (mg/dl)</td>
<td>10.5</td>
<td>9.6</td>
<td>9.5</td>
<td>15</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>Apolipoprotein B (mg/dl)</td>
<td>85</td>
<td>96</td>
<td>90</td>
<td>87</td>
<td>76</td>
<td>90</td>
</tr>
<tr>
<td>Apolipoprotein C-III (mg/dl)</td>
<td>3.4</td>
<td>6</td>
<td>4</td>
<td>3</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>Apolipoprotein E (mg/dl)</td>
<td>4.5</td>
<td>7</td>
<td>5</td>
<td>3.4</td>
<td>9</td>
<td>4</td>
</tr>
</tbody>
</table>

per milliliter) or only 50 ng of DMPC per milliliter as a control. Cells were washed with phosphate-buffered saline at 4°C to stop the reaction and solubilized in 0.1N NaOH. The remaining cellular cholesterol was determined by radioactivity counting.

LCAT Activity Assay

The LCAT activity of the particles was measured by the method of Chen and Albers. ApoA-I-lecithin [14C]cholesterol complexes were incubated with 20-60 µg particle protein in a shaking water bath for 15 hours at 37°C. The esterification rate was linear during this time. The reaction was then stopped, and lipids were extracted. Esterified and unesterified cholesters were separated by thin-layer chromatography, and the radioactivity of the bands was counted.

CET Activity Assay

Cholesterol ester transfer (CET) activity of the particles was measured by the method of Albers et al. In short, isolated particles were mixed with 0.1 mg of a [14C]cholesterol ester–HDL donor and 0.1 mg of an LDL acceptor and incubated at 37°C in a shaking water bath for 5 hours. The reaction was stopped by chilling the tubes on ice. Donor and acceptor lipoproteins were separated by the dextran sulfate/magnesium chloride precipitation procedure.

Results

Plasma Lipid and Apolipoprotein Profile

The general plasma lipid and apolipoprotein profiles of the donor subjects are shown in Table 1. All subjects were healthy normolipidemic adults with apoE-3/3 and apoA-IV-1/1 phenotypes.

Lipid and Protein Composition of Particles

The lipid and apolipoprotein compositions of the A-IVLP isolated from plasma and interstitial fluid and the LpA-I: A-IV and LpA-IV isolated from plasma are shown in Table 2. The protein mass ratio between LpA-IV and LpA-I: A-IV is 7:3. All isolated particles have a high protein to lipid ratio and also a high triglyceride content compared with apoA-I-containing particles.

For A-IVLP and LpA-IV, apoA-IV is the major apolipoprotein, whereas apoA-I is the major apolipoprotein of LpA-I: A-IV. From the composition data of A-IVLP, we estimate that the amount of plasma apolipoproteins associated with apoA-IV is as follows: 0.9±0.4% apoA-I, 2.1±1.4% apoA-II, 1.9±0.7% apoB, 0.8±0.4% apoC-III, and 11.7±4.1% apoE. In addition to the assayed apolipoproteins, other proteins could also be detected in the A-IVLP that was isolated from plasma (Figure 1). The diffuse band around 200 kd is apoB-48. ApoD, apoH, proline-rich protein, LCAT, and CET protein were also detected by immunoblotting with specific antisera. In addition, a single band of 59 kd that has not yet been identified was found in the A-IVLP particle.

Gel Filtration of Isolated Particles From Plasma

When apoA-IV–containing particles were separated by gel filtration chromatography, distinct size subpop-
ulations could be seen (Figure 2). A-IVLp consisted of two major subpopulations with mean hydrated Stokes' diameters of approximately 10.3 and 9.3 nm and two minor ones of 20 and 29.9 nm. Similar profiles were obtained with LpA-I:A-IV and LpA-IV but without the 20-nm subpopulation, and an additional minor subpopulation with a hydrated Stokes' diameter of 16 nm for the LpA-IV was also found. The peaks corresponding to the 29.9-, 10.3-, and 9.3-nm-Stokes'-diameter subpopulations of A-IVLp were collected and their protein components analyzed by SDS-PAGE after immunoblotting. By using ELISA and immunoblotting, apoA-IV was found in all fractions of A-IVLp as expected. In the largest subpopulation of 29.9 nm, apoB-48 and a very low amount of apoA-I were visualized (Figure 3). In the subpopulation of 10.3 nm, strong bands corresponding to apoA-I, apoD, and apoH were observed, along with some apoA-II. In the subpopulation of 9.3 nm, only apoA-I was visualized in addition to apoA-IV. As expected, apoA-I was found in these three fractions of A-IVLp because identical peaks were found in LpA-I:A-IV.

**Cholesterol Efflux From Cholesterol-Preloaded Ob 1771 Cells**

Incubation of [3H]cholesterol-preloaded adipose cells with LpA-IV and LpA-I:A-IV promoted a cholesterol efflux from cells, whereas no efflux was promoted by the control DMPC liposomes (Table 3). There was a trend toward greater cholesterol efflux promotion by LpA-IV, but the two were not statistically different (nonparametric Wilcoxon test).

**LCAT and CET Activities of Isolated Particles From Plasma**

Incubation of LpA-I:A-IV and LpA-IV with reconstituted [14C]cholesterol-containing phospholipid complexes resulted in the generation of 2 and 1.7 nmol, respectively, of esterified cholesterol per milligram protein particle per 15 hours. Incubation of LpA-I:A-IV and LpA-IV with [14C]cholesteryl ester-HDL donor and LDL acceptor induced a transfer of 8.4% and 18.3%, respectively, of [14C]cholesteryl ester per 10 μg

---

**FIGURE 1.** Sodium dodecyl sulfate (5–19%) gel electrophoresis of A-IV lipoprotein particle isolated from the plasma of subject 1. apo, apolipoprotein; PRP, proline-rich protein.

---

**FIGURE 2.** Representative example of a separate gel filtration chromatogram of apolipoprotein A-IV-containing particles isolated from plasma (subject 2) on a Superose 6 column using fast protein liquid chromatography. Panel A presents A-IV lipoprotein particle. Panel B shows lipoprotein particle A-I:A-IV, and panel C represents lipoprotein particle A-IV. Fifty micrograms of particle protein was applied and eluted at a flow rate of 0.2 ml/min in tris(hydroxymethyl)aminomethane buffer. VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; O.D., optical density.
protein particle per hour. These results showed that both particles have the potential to esterify cholesterol and the capacity to transfer it to a cholesterol acceptor.

Discussion

We describe here the characterization of apoA-IV-containing lipoprotein particles from human plasma and interstitial fluid. Given the poor lipid-binding capability of human apoA-IV, our aim was to isolate native particles while minimizing the possibility of dissociation of this apolipoprotein. We used immunoaffinity chromatography with affinity-purified polyclonal antibodies against human apoA-IV.

ApoA-IV-containing particles that were isolated from human plasma and interstitial fluid were found to have a low lipid to protein ratio compared with apoA-I-containing particles. No significant differences were observed between A-IVLp isolated from human plasma and from interstitial fluid. This result indicates that the A-IVLp composition is similar in these two compartments. The high triglyceride content of the apoA-IV-containing lipoprotein particles compared with the apoA-I-containing lipoprotein particles is consistent with the intestinal origin of human apoA-IV and the correlation between plasma levels of apoA-IV and triglycerides.

Although apoA-IV is the principal apolipoprotein of the A-IVLp particles, other proteins were also detected on SDS-PAGE. A protein band of 59 kd was associated with the A-IVLp particles. A similar protein was first described in association with apoA-IV in rat chylomicrons and was also identified in the apoA-IV-containing fraction that was isolated from human plasma by Ohta et al. In the latter study, apoH was also found in association with apoA-IV. As the observed particle sizes of apoA-IV-containing particles are not large enough to accommodate even a single copy of all of these proteins, apoA-IV-containing particles must be heterogeneous. Moreover, the distribution of apoA-IV in plasma must be more complex, as apoA-IV is also associated with apolipoproteins in subclasses other than the HDL and VLDL subfractions.

When the A-IVLp particles were applied to the anti-apolipoprotein A-I immunosorbent, 70% of the total protein in the particles was not retained by the immunosorbent. This fraction, referred to as the LpA-IV particle, contains mainly apoA-IV. This result disagrees with the data from Malmedier et al. and Lagrost et al., who found that the majority of apoA-IV was associated with apoA-I. This difference may be partially explained by the preparation of antibodies used for isolation. Our antibodies against apoA-IV were affinity purified and were passed through an apoA-I immunosorbent to remove antibodies that could recognize apoA-I as well as apoA-IV. Furthermore, when plasma aliquots were directly applied to an anti-apolipoprotein A-I immunosorbent, only 5.8% of the total apoA-IV was retained by the immunosorbent. This result is in agreement with the apoA-IV proportion in the two A-IVLp subpopulations, LpA-IV and LpA-1:A-IV. The apolipoprotein molar ratio in LpA-1:A-IV is approximately 7 apoA-1, 2 apoA-II, and 1 apoA-IV. The LpA-1:A-IV may be a mixture of apoA-I and apoA-IV-containing particles and of apoA-I-, apoA-II-, and apoA-IV-containing particles.

ApoA-IV-containing lipoprotein particles isolated from plasma are heterogeneous in size. All consist of two major populations, a larger one with a Stokes' diameter of 10.3 nm and a smaller one with a Stokes' diameter of 9.3 nm. These two apoA-IV-containing particle subpopulations have a distinct apolipoprotein composition. In A-IVLp, the presence of apoA-IV in each subpopulation and also apoA-I, apoA-II, apoD, or apoH was confirmed by using specific antibodies. Further investigation is required to understand the compo-
sition and metabolic effects of the subpopulations of apoA-IV-containing lipoprotein particles.

LpA-I:A-IV and LpA-IV particles are effective in promoting cholesterol efflux from cholesterol-preloaded adipose cells as well as A-I:VLP. In all three particle subpopulations, the content (on a molar basis) of apoA-II, however, is too far outnumbered by apoA-I and apoA-IV. Thus, the apoA-II in these apoA-IV-containing particles may not exert its inhibitory function on cholesterol efflux at this concentration and molar ratio. LpA-I:A-IV and LpA-IV particles also contain LCAT and CET activities. ApoA-IV-containing particles exist in high levels in human interstitial fluid. The lipid and protein composition of apoA-IV-containing particles that have been isolated from interstitial fluid seems to be similar to that of apoA-IV-containing particles that have been isolated from plasma.

ApoA-IV-containing particles have a different composition than apoA-I-containing particles but have several common physiological properties, i.e., they promote cholesterol efflux from adipose cells and have LCAT and CET activities. Thus, apoA-IV-containing particles may serve as a “surrogate” for apoA-I-containing particles in promoting reverse cholesterol transport in conditions characterized by a low plasma apoA-I level without premature coronary heart disease, such as Tangier disease, LCAT deficiency, and fish eye disease and apoA-I mutations.

Acknowledgments

We wish to thank Dr. Diepninger for providing the LCAT and CET protein antibodies, Dr. Kostner for the apoD antibodies, Dr. Havel for the PRP antibodies, and Dr. Minscha for the apoH antibodies. We are indebted to M. Kindt and Dr. D. Rader for a critical reading of the manuscript.

References


N Duverger, N Ghalim, G Ailhaud, A Steinmetz, J C Fruchart and G Castro

doi: 10.1161/01.ATV.13.1.126
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1993 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/13/1/126

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/