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 M_r 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. This apolipoprotein is found in plasma, interstitial fluid, and lymph. In plasma, apoA-IV has been shown to be associated with lipoprotein density fractions as well as in a lipoprotein-free form. However, apoA-IV is easily dissociated from lipoproteins by ultracentrifugation, 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. By gel filtration and immunoprecipitation, 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. Human apoA-IV is an LCAT activator and has a specific binding site on cultured bovine aortic endothelial cells and cultured mouse Ob adipose cell surfaces. 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. 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 NaNO₃, 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 skin.

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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**

Immunosorbsents 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 immunoaffinity 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 unretracted 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-

**Cellular Cholesterol Efflux Studies**

The characterization of the Ob 1771 preadipocyte cell line was reported previously. Cholesterol efflux was transferred to LDL by the method of Craig et al, and the [3H]cholesterol-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]cholesterol–labeled 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 cholesterols 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 [14C]cholesterol, complexes were incubated with 20-60 μg of a 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/I 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:

<table>
<thead>
<tr>
<th>Subject</th>
<th>Plasma</th>
<th>A-IVLP</th>
<th>LpA-I: A-IV</th>
<th>LpA-IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (weight %)</td>
<td>86.9</td>
<td>78.8±4.9</td>
<td>79.2±4.5</td>
<td>86.0±5.3</td>
</tr>
<tr>
<td>Total cholesterol (weight %)</td>
<td>5.4</td>
<td>5.9±0.7</td>
<td>5.2±0.4</td>
<td>3.2±1.9</td>
</tr>
<tr>
<td>Free cholesterol (weight %)</td>
<td>0.6</td>
<td>2.8±0.7</td>
<td>1.0±0.6</td>
<td>1.2±0.7</td>
</tr>
<tr>
<td>Triglyceride (weight %)</td>
<td>2.6</td>
<td>5.5±3.1</td>
<td>4.5±1.1</td>
<td>6.0±2.7</td>
</tr>
<tr>
<td>Phospholipid (weight %)</td>
<td>4.9</td>
<td>9.7±3.8</td>
<td>10.1±4.5</td>
<td>4.6±3.6</td>
</tr>
<tr>
<td>Apolipoprotein A-I (mole %)</td>
<td>17.8</td>
<td>12.7±0.8</td>
<td>82.3±3.7</td>
<td>...</td>
</tr>
<tr>
<td>Apolipoprotein A-II (mole %)</td>
<td>18.9</td>
<td>8.2±5.1</td>
<td>5.7±3.5</td>
<td>3.5±2.6</td>
</tr>
<tr>
<td>Apolipoprotein A-IV (mole %)</td>
<td>41.2</td>
<td>70.4±8.0</td>
<td>10.8±3.4</td>
<td>95.3±3.0</td>
</tr>
<tr>
<td>Apolipoprotein B (mole %)</td>
<td>2.1</td>
<td>1.4±0.6</td>
<td>0.5±0.4</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>Apolipoprotein C-III (mole %)</td>
<td>9.5</td>
<td>0.7±0.5</td>
<td>0.6±0.6</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>Apolipoprotein E (mole %)</td>
<td>10.3</td>
<td>6.6±2.4</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>


*Subjects 1–5.*
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**


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**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-apoA-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 Malmendier 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-apoA-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-

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**TABLE 3. Cholesterol Efflux From [3H]Cholesterol-Preloaded Ob 1771 Cells**

<table>
<thead>
<tr>
<th>Incubation time (hours)</th>
<th>[3H]Cholesterol efflux (percent of initial cell-associated cholesterol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LpA-1:A-IV</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>27.0±4.2</td>
</tr>
<tr>
<td>3</td>
<td>29.9±4.4</td>
</tr>
<tr>
<td>LpA-IV</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>32.0±7.1</td>
</tr>
<tr>
<td>3</td>
<td>38.8±6.7</td>
</tr>
<tr>
<td>DMPC</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.0±0.7</td>
</tr>
<tr>
<td>3</td>
<td>3.1±0.5</td>
</tr>
</tbody>
</table>

LpA-1:A-IV, lipoproteins with apolipoproteins A-I and A-IV; LpA-IV, lipoproteins with apolipoprotein A-IV only; DMPC, dimyristoyl phosphatidylcholine. Values are mean±SD. The radioactivity appearing in the medium was expressed as a percentage of the initial cell-associated [3H]cholesterol. Measurements were performed in duplicate on parallel dishes. Mean values from duplicate dishes are reported (<10% from the mean) and are representative of three different experiments performed on two independent series of cells. The initial cholesterol content (mean±SD) of cholesterol-preloaded cells was 50±6 μg/mg cell protein.
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 in 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/or 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.

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