Functional and structural properties of lipid-associated apolipoprotein J (clusterin)

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Apolipoprotein J (apoJ, clusterin) is a multifunctional protein normally associated with lipids in plasma and cerebrospinal fluid, and secreted as lipoparticles by hepatocytes and astrocytes. To investigate whether the structural and functional properties of apoJ are modulated upon binding to lipids, we prepared apoJ high-density lipoprotein (HDL)-like particles employing either synthetic or plasma HDL-derived lipids. The majority of the resulting lipoparticles contained one molecule of apoJ per particle and exhibited the same α_2 electrophoretic mobility characteristic of apoJ-containing plasma HDL. Particle size seemed to be dependent on the presence of cholesterol in the lipid mixture and ranged from diameters of 10 nm in the presence of cholesterol to 20 nm in the absence of cholesterol. CD analysis and Fourier-transform infrared spectroscopy revealed similar changes in the apoJ secondary structure induced by its incorporation into lipoparticles, namely a decrease in α -helix

content and an increase in β -turn structures. Two functional assays, the binding interaction with Alzheimer's amyloid β peptides and the inhibitory activity of the complement membrane-attack complex, did not detect any changes in apoJ activity following its lipidation ($P > 0.05$). On the contrary, the binding affinity to the cellular receptor megalin was enhanced significantly ($P < 0.01$) after the association with lipids; the K_d value decreased from 78.8 ± 10.7 nM for the delipidated form to 37.0 ± 7.3 nM for apoJ-HDL. Although it is not known whether the structural changes observed are directly responsible for the higher receptor-binding affinity, the data suggest that the complement inhibition and amyloid β -binding motifs are located in areas of the molecule different from those involved in the apoJ-megalin interaction.

Key words: Alzheimer's disease, apolipoproteins, HDL, lipoparticles.

INTRODUCTION

Apolipoprotein J (apoJ, clusterin) is a heterodimeric, highly glycosylated, protein composed of 449 amino acid residues, with an apparent molecular mass of 80 kDa [1]. The 40-kDa α and β subunits, linked by a unique five-disulphide-bond motif, are encoded by a single mRNA, which is transcribed from a single-copy gene located on chromosome 8 (8p21) [2]. ApoJ is highly conserved among species and widely expressed in different tissues, i.e. brain, ovary, testis and liver [3]. ApoJ mRNA is also heterogeneously distributed within the nervous system. A high level of expression was found in ependymal cells and neurons from hypothalamus, brain stem, habenula and spinal cord [4].

ApoJ is abundant in numerous biological fluids including semen, urine, breast milk, plasma and cerebrospinal fluid [5]. There are two major sources of apoJ in the circulation: plasma, in which it is found associated with high-density lipoprotein (HDL) particles [6]; and platelets, where it is a constituent of the α granules [7]. Approx. 30–40% of the total apoJ seems to be sequestered by the α granules, and is released upon platelet activation. In contrast to the α_1 electrophoretic mobility of the majority of the plasma HDL [8], apoJ-containing lipoparticles display α_2 mobility, defining unique subclasses of HDL that are heterogeneous in size and composition. They consist of 3–16-nm particles composed of 78–89% protein and 11–22% lipid

(31–54% total cholesterol, 42–59% phospholipids, 4–9% triacylglycerols) that exhibit a biphasic distribution into HDL₂ and HDL₃/very-high-density lipoprotein fractions [6,8,9]. Although the gene structure and the sequences of the putative amphipathic helices seem to indicate that apoJ is not a member of the apolipoprotein multigene family [10], much evidence supports the classification of apoJ as a lipoprotein: (i) it is associated with lipids in plasma and cerebrospinal fluid [6]; (ii) it is secreted by hepatocytes and astrocytes as a lipoprotein [11,12]; and (iii) it is a ligand for megalin (gp330), a member of the low-density-lipoprotein (LDL) receptor gene family [13].

Originally, apoJ was characterized as a cell-aggregating factor produced by Sertoli cells and present at high levels in ram rete testis [14], a function that dictated its designation as clusterin. Later, the human homologue of the rat protein was described as a normal serum protein that binds specifically to the nascent C5b-7 complex, thus inhibiting the cytolytic activity of the membrane-attack complex of the complement system [15–17]. More recently, numerous data suggest that, in peripheral tissues, increased levels of apoJ are related to programmed cell death. Marked increases in apoJ transcript levels have been demonstrated in adult tissues in animal models of apoptosis and cell death associated with normal development [18,19]. Interestingly, elevated levels of apoJ have been also observed in several neurodegenerative disorders and experimental brain lesions, such

Abbreviations used: HDL, high-density lipoprotein; A β , amyloid β peptide; apoA-I, apolipoprotein A-I; apoE, apolipoprotein E; apoJ, apolipoprotein J; apoJ-HDLd and apoJ-HDLp, reconstituted apoJ-containing HDL particles prepared by detergent-mediated synthesis with POPC lipids or HDL lipids, respectively; apoJ-HDLs, reconstituted apoJ-containing HDL particles prepared by sonication-mediated synthesis with POPC and cholesteryl oleate lipids; ATR, attenuated total reflection; FTIR, Fourier-transform infrared; POPC, 1-palmitoyl 2-oleoylphosphatidylcholine; RAP, receptor-associated protein; LDL, low-density-lipoprotein.

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as brain tumours, epileptic foci [20], excitotoxic lesions, transient ischaemia [21], scrapie-infected hamsters [22], and Pick and Alzheimer's diseases [21–23].

ApoJ has also been found associated with parenchymal and vascular amyloid β peptide ($A\beta$) deposits in Alzheimer's disease, in conjunction with apolipoprotein E (apoE) and many other amyloid-associated proteins [24]. ApoJ and apoE have been shown to bind $A\beta$ both *in vivo* and *in vitro* [25,26]. Furthermore, apoJ seems to be implicated in the maintenance of circulating $A\beta$ in its soluble conformation and in its transport through the blood–brain barrier by a mechanism that involves megalin, the receptor for apoJ [27,28].

Some of the biological properties of apoJ, such as complement regulation, cell–cell interactions, $A\beta$ binding, blood–brain-barrier transport and neurotoxicity protection have been studied employing the non-lipidated form of the protein [13,16,26,27]. The presence of a lipid environment may potentially modulate the ability of apoJ to interact with other molecules, thereby altering the biological processes or metabolic pathways in which it is involved. In the present report, we incorporated purified apoJ into HDL-like lipoparticles generated both with synthetic lipids and with lipids isolated from circulating plasma HDL, and studied the structural and functional properties of lipidated apoJ compared with the lipid-free form of the molecule.

EXPERIMENTAL

Materials

Cholesteryl oleate, sodium cholate, and dimethyl suberimidate were purchased from Sigma (St. Louis, MO, U.S.A.). 1-Palmitoyl 2-oleoylphosphatidylcholine (POPC) was obtained from Avanti Polar Lipids (Birmingham, AL, U.S.A.). Biobeads-SM2 were from Bio-Rad (Hercules, CA, U.S.A.). ApoJ was either acquired from Quidel (Los Angeles, CA, U.S.A.) or purified from human plasma as described below. Complement components were purchased from Quidel. Human receptor-associated protein (RAP), expressed as a glutathione S-transferase fusion protein in *Escherichia coli*, was kindly provided by Dr. Robert McCluskey (Massachusetts General Hospital, Boston, MA, U.S.A.).

Peptides $A\beta$ 42 (DEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVVGGVIA) and $A\beta$ 40 (DEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVVGGVV) were synthesized at the W. M. Keck facility at Yale University, purified and characterized biochemically as described in [26]. Plasma samples were obtained from normal healthy subjects, aged 25–40 years, after a 12-h fast.

Purification of apoJ

ApoJ was isolated from fresh human plasma by immunoaffinity chromatography and further purified by reversed-phase HPLC [29]. Anti-apoJ 1532 antiserum was obtained by immunizing 12-week-old New Zealand white rabbits with synthetic peptide CRLLSNLEEAKKKKED (corresponding to positions 48–61 of the apoJ α -chain) coupled to keyhole limpet haemocyanin (Sigma). Following an initial subcutaneous injection of 250 μ g of antigen (1 mg/ml in sterile saline) emulsified with 0.5 ml of monophospholipid A-synthetic thehalose dicrynomycolate (RIBI; Immunochem Research, Hamilton, MO, U.S.A.), rabbits were boosted subcutaneously every 15 days with the same amount of antigen without the addition of adjuvant. After 8 weeks, test bleedings were obtained and the presence of specific antibodies analysed by ELISA. For affinity chromatography experiments, the antiserum was purified by chromatography on protein G–Sepharose (Pharmacia, Piscataway, NJ, U.S.A.) and

the resulting IgG fraction immobilized on to CNBr-activated Sepharose 4B (Pharmacia) in accordance with the manufacturer's specifications.

For the apoJ isolation, 50 ml of pooled normal human plasma was added to an equal volume of PBS, containing 5 mM EDTA (PBS/EDTA) and a cocktail of protease inhibitors (Complete, Boehringer Mannheim, Mannheim, Germany), and incubated with the anti-apoJ affinity matrix. The resin was washed with PBS/EDTA containing 1% Triton X-100, and bound proteins were eluted with 1 M acetic acid, pH 2.3. This crude preparation was applied on to a C_4 -reversed-phase preparative column (22 $\times$ 250 mm; Vydac, Hesperia, CA, U.S.A.) and eluted with a 60-min linear gradient (20–80% acetonitrile in 0.1% trifluoroacetic acid). Final purification was achieved by HPLC rechromatography on a semi-preparative C_4 -reversed-phase column (10 $\times$ 250 mm; Vydac) under the conditions described above. Protein purity was assessed by SDS/PAGE and protein identification determined by amino acid sequence analysis (477A protein sequencer, Applied Biosystems, Foster City, CA, U.S.A.).

The ApoJ molar absorption coefficient was calculated by UV spectroscopic analysis in a Beckman DU 640 spectrophotometer employing a preparation of known concentration of apoJ, determined independently by amino acid analysis using a Waters Accutag system (Waters, Milford, MA, U.S.A.).

Preparation of reconstituted apoJ-HDL particles

By detergent-mediated synthesis

(a) Reconstituted from synthetic lipids (apoJ-HDLd, reconstituted apoJ-containing HDL particles prepared by detergent-mediated synthesis with POPC lipids). ApoJ-HDLd particles were prepared by a modification of the method described previously for reconstituted apolipoprotein A-I (apoAI) HDL [30]. POPC and sodium cholate were combined to a final concentration of 20 mM POPC/27 mM sodium cholate in Tris-buffered saline, vortexed and incubated for 90 min at 37 °C, prior to the addition of apoJ (5 mg) and further incubation for 1 h at 37 °C. The detergent was removed from the emulsion by incubation with Biobeads-SM2 and the reconstituted lipoparticles isolated by sequential ultracentrifugation within the 1.063–1.21 g/ml density range, dialysed extensively against 20 mM ammonium bicarbonate, and stored at 4 °C.

(b) Reconstituted from lipids extracted from plasma HDLs (apoJ-HDLp, prepared by detergent-mediated synthesis with HDL lipids). Plasma HDLs were purified from a pool of normal plasma by density ultracentrifugation and the total lipids isolated by chloroform/methanol extraction. Purified lipids were dried under a nitrogen stream, dissolved in chloroform and stored at –70 °C. Total cholesterol, triacylglycerols and phospholipids in the HDL fraction and in the reconstituted apoJ-HDL particles were determined enzymically using Sigma diagnostic kits.

Preparation of apoJ-containing particles with the HDL-extracted lipids was assessed as described above by the detergent-mediated synthesis, the only difference being that 10 mg of the extracted lipids, consisting of a mixture that contained 18.7% cholesterol, 9% triacylglycerols and 72.3% phospholipids, were employed instead of POPC.

By sonication-mediated synthesis (apoJ-HDLs, prepared by sonication-mediated synthesis with POPC and cholesteryl oleate lipids)

Spherical apoJ-HDL particles were prepared as described previously for the preparation of apoAI-containing HDL [31]. POPC (0.9 mg) and cholesteryl oleate (0.6 mg), were suspended in Tris-buffered saline and sonicated at 49–52 °C under nitrogen

for 30 min. Upon cooling to 39–40 °C, 1 mg of apoJ in 2.5 M urea was slowly added to the solution followed by sonication for another 10 min. Reconstituted spherical HDL particles were further purified by sequential ultracentrifugation, as above.

Cross-linking of apoJ-containing lipoparticles

To determine the number of apoJ molecules incorporated into the reconstituted HDL, the lipoparticles were cross-linked using dimethyl suberimidate and the molecules of apoJ per particle assessed by SDS/PAGE [32].

Electron microscopy

An aliquot of each apoJ-HDL preparation was deposited on to a 400 mesh Formvar carbon-coated copper grid, negatively stained with 2 % phosphotungstic acid [33] and observed using a Zeiss EM10 electron microscope.

Secondary structure determination

CD spectroscopy

Spectra in the far UV (195–260 nm) were recorded at 24 °C with a Jasco J-720 spectropolarimeter (Jasco, Tokyo, Japan), using a cell path of 0.1 cm at a protein concentration of 0.15 mg/ml. Correction for the lipid contribution to the CD spectra of the reconstituted apoJ-HDL particles was achieved by subtracting the CD values of an identical concentration of lipids as those in the particle, measured in a chloroform solution [34]. The percentages of the different secondary structure motifs were calculated based on the CD data by the Selcom algorithm [35].

Fourier-transform infrared (FTIR) spectroscopy

Samples were subjected to 1 h deuteration and attenuated total reflection (ATR) FTIR spectra recorded at room temperature on a Bio-Rad FTS6000 infrared spectrophotometer equipped with a horizontal Golden Gate single reflection diamond ATR cell (Specac, Smyrna, GA, U.S.A.). Individual components of the amide I mode were resolved from the deconvoluted spectra (resolution-enhancement factor of $k = 2$ and a peak half-width of 12 cm⁻¹) by least-squares iterative curve fitting using Lorentzian–Gaussian curves. Assignment of the different components of amide I to secondary structure was performed as described in [36].

Determination of the biological activities of apoJ and apoJ-containing lipoparticles

Binding to the megalin receptor on F9 kidney cells

Radiolabelled apoJ was used to study the binding of apoJ to F9 cell-culture monolayers, either in its non-lipidated form or incorporated into lipoparticles. ¹²⁵I-ApoJ with a specific radioactivity of 6 µCi/µg was prepared using Iodobeads (Pierce, Rockford, IL, U.S.A.) and separated from free iodine by chromatography over a Sephadex G-25 Superfine column (0.7 × 30 cm, Pharmacia) as described in [28]. The absence of free ¹²⁵I was tested by trichloroacetic acid precipitation. ¹²⁵I-ApoJ-HDLp particles were prepared using ¹²⁵I-apoJ (6 µCi/µg) and plasma HDL lipids by the detergent-mediated synthesis described above. Mouse embryonic carcinoma F9 cells (A.T.C.C. CRL 1720) were grown as described previously [13], and stimulated with retinoic acid and dibutyryl cAMP for 7 days. Prior to the receptor-binding assays, the cells were reseeded on to gelatin-coated 12-well plates at 5 × 10⁵ cells per well, grown for 18 h and acid-stripped with

culture media at pH 3.0 for 30 s. After incubation with increasing concentrations (1–95 nM) of ¹²⁵I-labelled apoJ or apoJ-containing lipoparticles for 4 h at 4 °C, bound radiolabelled ligands were determined by counting the radioactivity in a gamma counter upon treatment with 0.2 M NaOH/1 % SDS.

For the inhibition experiments, the F9 cells were incubated with a constant amount (30 nM, 6 µCi/µg) of ¹²⁵I-apoJ-HDL and increasing concentrations of RAP (0.1–1562.5 nM) for 4 h at 4 °C. Bound radio-iodinated apoJ-containing HDLs were determined as above.

Inhibition of the complement haemolytic activity

The ability of apoJ to inhibit the haemolytic activity of the complement system was assessed by determining the amount of apoJ necessary to inhibit by 50 % the activity of the C5b-9 complex to induce lysis of guinea pig erythrocytes [37]. The C5b-6 complex [38] at a concentration that produced 50–80 % lysis of guinea pig erythrocytes (1.5 × 10⁷ cells) was incubated with C7 and increasing amounts of apoJ or apoJ-containing HDL particles (0–10 µg) for 15 min at 30 °C, followed by the addition of C8 and C9 and incubation at 37 °C for 60 min. The haemolytic reaction was stopped with ice-cold Gelatin Veronal Buffer (Sigma) and the haemoglobin released assessed by absorbance at 412 nm. The haemolytic activity of the C5b-9 complex was determined for each concentration of added apoJ as percentage lysis compared with the control of 100 % lysis, in which an identical concentration of guinea pig erythrocytes was totally lysed by the addition of distilled water.

Binding of apoJ to Aβ

The dissociation constants of non-lipidated apoJ, and apoJ-HDL particles for the binding interaction with Aβ, were estimated by ELISA using Aβ40- or Aβ42-coated (400 ng/100 µl per well) microtitre plates as described [26].

Analysis of the data

Non-linear regression analysis, estimation of dissociation constants and half-maximal inhibition values, and comparison of protein-binding data for statistical significance by calculation of the *F* ratios were assessed employing GraphPad Prism software (GraphPad, San Diego, CA, U.S.A.).

RESULTS AND DISCUSSION

ApoJ was purified from human plasma by a combination of immunoaffinity chromatography and reversed-phase HPLC. Normal plasma was incubated with an affinity matrix that contained a polyclonal anti-apoJ antibody that recognizes residues 48–61 of the apoJ α-chain. Non-specifically retained proteins were removed from the affinity matrix by washing extensively with PBS/EDTA. Additional washes with PBS/EDTA containing 1 % Triton X-100 [29] allowed the disruption of the apoJ interaction with apoAI, the main component of human HDL and a major contaminant in all apoJ preparations [9], therefore decreasing the concentration of apoAI in the affinity purified material. Proteins that remained bound to the anti-apoJ matrix were eluted with 1 M acetic acid, pH 2.3 (Figure 1A). Two major bands of 40 and 25 kDa (Figure 1A, inset) that represented approx. 50 and 25 % of the total proteins in the eluent were observed in SDS/PAGE under reducing conditions and identified by sequence analysis as apoJ and apoAI, respectively. ApoJ was purified further by HPLC on a C₄-reversed-phase preparative column, and eluted as a distinct component at 50 % acetonitrile

Figure 1 Purification of human apoJ

(A) Immunoaffinity chromatography. ApoJ was purified from human plasma by immunoaffinity chromatography using a polyclonal antibody (1532) that recognized the α -chain of apoJ immobilized on Sepharose 4B. Normal human plasma was allowed to interact with the affinity matrix for 16 h at room temperature. After washing off the unbound proteins with PBS/EDTA containing 1% Triton X-100, apoJ was eluted with 1 M acetic acid, pH 2.3. Inset indicates 10% Tris/Tricine SDS/PAGE under reducing conditions (0.1 M dithiothreitol) of the acetic acid-eluted fraction (lane a). Arrowheads show the protein bands corresponding to apoJ and apoAI identified by amino acid sequence analysis. (B) Reversed-phase HPLC. The immunoaffinity Sepharose-bound fraction, dissolved in 20% acetonitrile/0.1% trifluoroacetic acid, was injected subsequently into a C_4 -reversed-phase HPLC preparative column (22 $\times$ 250 mm; Vydac) and eluted using a 60-min linear gradient from 20 to 80% acetonitrile in 0.1% trifluoroacetic acid. Final purification was achieved by rechromatography on a semi-preparative C_4 -reversed-phase column (10 $\times$ 250 mm; Vydac) under identical conditions. Protein separation was monitored by absorbance at 280 nm. Insets show the 10% Tris/Tricine SDS/PAGE analysis under reducing conditions of the apoJ pool following chromatography on the preparative (lane a) and semi-preparative (lane b) HPLC columns.

(Figure 1B). Minor contaminants were removed by a repurification step on a semi-preparative C_4 -reversed-phase column. The purity of the apoJ preparation (99%) was estimated based on SDS/PAGE (Figure 1B, inset, lane b) and amino acid sequence analysis. The molar absorption coefficient at 280 nm employing this apoJ preparation resulted in the experimental value of $70073 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

To incorporate apoJ into reconstituted lipoparticles, we followed three different strategies previously used to incorporate apoAI into lipoparticles [30,32]. The first approach was to obtain apoJ-HDL particles from synthetic lipids by detergent-mediated synthesis (apoJ-HDLd), which relies on the interaction of the apolipoprotein with micellar dispersions of synthetic phospholipids in the presence of a detergent and was reported to render discoidal particles. ApoJ was added to POPC dispersed with sodium cholate, and the resulting lipoparticles purified by ultracentrifugation within a density range of 1.063–1.21 g/ml.

Figure 2 Electron microscopy of apoJ-HDL particles

Electron microscopy of negatively stained apoJ-HDL lipoparticles. Left-hand panel, apoJ-HDLd. Middle panel, apoJ-HDLs. Right-hand panel, apoJ-HDLp. Particle size (given below each panel) was determined as the mean of 100 particles. Original magnification, $\times 30\,000$.

Electron microscopy showed that the apoJ-HDLd preparation consisted of a relatively homogeneous population of large particles (diameter 15–20 nm), as indicated in Figure 2, left-hand panel. After covalent cross-linking, SDS/PAGE demonstrated that the majority of the reconstituted particles ($\approx 95\%$) contained one apoJ molecule per particle, whereas a small population ($\approx 5\%$) seemed to contain two apoJ molecules (Figure 3A). The electrophoretic mobility of the apoJ-HDLd particles assessed by agarose-gel electrophoresis was located in the α_2 region (Figure 3B). When lipids extracted from human plasma HDL fractions were used to prepare apoJ-containing lipoparticles by the detergent-mediated method (apoJ-HDLp), the particle size obtained was similar (12–15 nm diameter; Figure 2, right-hand panel). The majority of them ($\approx 92\%$) also contained one molecule of apoJ per particle (Figure 3A) and had an electrophoretic mobility in the α_2 region (Figure 3B).

In a different approach, we followed a method based on sonication of the apolipoprotein with phospholipids and cholesterol esters that was reported to yield spherical HDL-like particles (apoJ-HDLs). Analysis of the preparation by electron microscopy after purification of the lipoparticles by ultracentrifugation showed a more homogeneous population of smaller particles (10–12 nm diameter; Figure 2, middle panel). Regardless of the smaller size, the number of apoJ molecules per particle (Figure 3A) and the α_2 electrophoretic mobility of the HDLs lipoparticles (Figure 3B) remained almost identical to those obtained with the detergent-mediated synthesis.

Table 1 summarizes the physicochemical properties of the different lipoparticles prepared by incorporation of purified apoJ into a lipid environment. All three approaches yielded reconstituted apoJ-containing lipoparticles that resemble those present *in vivo* under physiological conditions. Particles were 10–20 nm in diameter and exhibited electrophoretic mobilities in the α_2 region, whereas the majority of them ($\approx 95\%$) contained one molecule of apoJ per particle. Particle size seemed to be related to the presence of cholesterol in the lipid mixture; it ranged from 10 nm in the presence of cholesterol to 20 nm in its absence. These results are in agreement with the biochemical properties described for circulating apoJ-containing lipoparticles. They

Figure 3 Charge and composition of apoJ-HDL particles

(A) Analysis of dimethyl suberimidate cross-linked apoJ-HDL particles by 10% Tris/Tricine SDS/PAGE. Arrows indicate the position of the monomer (M) and dimer (D) of the apoJ molecule. Nomenclature of the apoJ-HDL particles is as in Figure 2. (B) Agarose electrophoresis of lipid-free apoJ and reconstituted lipoparticles was carried out at 100 V for 30 min on precast 0.5% agarose gels (Beckman, Paragon, Palo Alto, CA, U.S.A.) employing barbital buffer, pH 8.6. The gels were stained with 0.15% Coomassie Brilliant Blue in water/methanol/acetic acid (55:40:5, by vol.). Commercially available apoJ (Quidel; apoJ*) is shown for comparison.

Table 1 Physicochemical parameters of apoJ-HDL particles

Percentages of secondary structure obtained from CD data were analysed by the Selcom algorithm [35]. Percentages of secondary structure from ATR-FTIR spectra were obtained after assignment of the different components of amide I according to Goormaghtigh and collaborators [36]. ND, not determined; MAC, membrane-attack complex; Ch, cholesterol; PL, phospholipids; TG, triacylglycerols.

	ApoJ-HDLd	ApoJ-HDLs	ApoJ-HDLp	ApoJ
Particle size (nm)*	15–20	10–12	12–15	–
Initial molar composition (apoJ:Ch:PL:TG)	1:0:320:0	1:74:94:0	1:22:150:15	–
Final composition (apoJ:Ch:PL:TG)	1:0:120:0	1:60:83:0	1:11:105:21	–
ApoJ molecules/particle	1.05	1.03	1.08	–
Electrophoretic mobility†	α2	α2	α2	α2
Secondary structure (% from CD data)				
α-Helix	36.1	37.3	34.5	36.3
β-Structure‡	33.2	34.2	35.7	34.4
Random coil	30.7	28.5	29.8	29.3
Secondary structure (% from FTIR data)				
α-Helix	ND	ND	29.7	34.2
β-Structure‡	ND	ND	45.4	38.3
Random coil	ND	ND	22.7	23.1
Binding to F9 cellular receptor, $K_d \pm$ S.D. (nM)	ND	ND	37.0 ± 7.3	78.8 ± 10.7
Half-maximal inhibition of MAC lytic activity, mean $\pm$ S.D. (μ g)	ND	ND	8.4 ± 0.2	8.8 ± 0.2
Affinity to Aβ, $K_d \pm$ S.D. (nM)				
Aβ40	ND	ND	4.0 ± 0.6	3.1 ± 0.4
Aβ42	ND	ND	2.7 ± 0.3	1.9 ± 0.2

* Particle size was determined as the average diameter of 100 particles from an electron micrograph at $\times 50\,000$ magnification.

† Electrophoretic mobility in agarose gel.

‡ β-Structure consists of β-sheets plus β-turns.

consist of a unique subclass of HDL particles of heterogeneous size (3–16 nm), containing a sole molecule of apoJ per particle, with an α2 electrophoretic mobility and a biphasic distribution into HDL₂ and HDL₃/very-low-density lipoprotein fractions

[6,8,9,11,12]. Since the three approaches rendered very similar lipoparticles, the structural and functional studies that follow were performed with particles prepared with plasma HDL lipids (apoJ-HDLp), which are alike in lipid composition to the natural circulating apoJ-HDL.

CD spectra in the far-UV region were used to assess modifications in the apoJ secondary structure induced by its incorporation into a lipidic environment. As indicated in the top half of Figure 4(A), the secondary structure calculated by the Selcom algorithm for the lipid-free protein yielded 36.3% α-helix, 17.4% β-sheet, 17% β-turns and 29.3% random-coil structures, in agreement with the theoretical values calculated by the Garnier and Chou-Fassman methods [3]. Although the spectra recorded before and after lipid association were similar in shape, a decrease in the α-helix structures and an increase in the β-turn content were observed upon lipidation (Figure 4A, bottom half). Comparative results were obtained by FTIR spectroscopy on samples subjected to deuteration for 1 h. Partial deuteration of the sample allows for discrimination between the relative contributions of random-coil and α-helix conformers. As illustrated in Figure 4(B), self-deconvolution and curve fitting of the spectra in the amide I region showed maxima centred at wave numbers characteristic of β-turns (1670 cm⁻¹), α-helices (1650–1655 cm⁻¹), random coils (1640 cm⁻¹) and β-structures (1629 cm⁻¹). The two major changes observed previously in the CD experiments were also detected by FTIR, namely a decrease in the α-helix content and an increase in the β-turn structures. In addition, the spectrum of the non-lipidated apoJ (Figure 4B, top half) revealed two extra features, a strong band at 1615 cm⁻¹ and a weaker band at 1684 cm⁻¹, both frequencies characteristic of anti-parallel β-sheet structures and/or aggregated strands, probably reflecting the high tendency of apoJ to polymerize and aggregate in the absence of lipids. Confirming this assumption, upon incorporation to the lipid particles, these bands diminished drastically and even disappeared (Figure 4B, bottom panel).

To evaluate the physiological competence of apoJ incorporated into reconstituted lipoparticles we characterized the binding of apoJ and apoJ-HDL to the endocytic receptor megalin. Megalin, the only known receptor for apoJ [13], binds *in vitro* to a variety

Figure 4 Secondary structure analysis of apoJ incorporated into HDL-like particles

(A) The CD spectra of non-lipidated apoJ (top panel) and reconstituted apoJ-HDL particles (bottom panel) were recorded from 195 to 260 nm at 24 °C with a Jasco J-720 spectropolarimeter at a protein concentration of 0.15 mg/ml. Correction for the lipid contribution to the CD spectra of the reconstituted apoJ-HDL particles was achieved by subtracting the CD values of an identical concentration of the lipids as those in the particle, measured in a chloroformic solution. The percentages of the different secondary structure motifs based on the CD data were calculated by the Selcom algorithm [35]. (B) ATR-FTIR spectra of non-lipidated apoJ (top panel) and reconstituted apoJ-HDL particles (bottom panel) were recorded at a nominal resolution of 0.5 cm⁻¹ and 256 scan accumulations on a Bio-Rad FTS6000 infrared spectrophotometer equipped with a Golden Gate diamond ATR cell (Specac, Smyrna, GA, U.S.A.). Fourier self-deconvolution of the spectra in the amide I region was performed using a Bessel apodization function with a resolution-enhancement factor of $k = 2$ and a peak half-width of 12 cm⁻¹, in the Win-IR Pro system (Bio-Rad). Assignment of the different components of amide I after Fourier self-deconvolution to secondary structure was performed as described [36].

of different ligands, among them the RAP, lipoprotein lipase, apoE-enriched very-high-density lipoprotein, complexes of the tissue-type plasminogen activator and plasminogen activator inhibitor-1 [39]. Mouse embryonal carcinoma F9 cells on which megalin expression level was induced with retinoic acid and dibutyl cAMP [13], were used to quantify the binding of lipidated and non-lipidated apoJ. When ¹²⁵I-apoJ or ¹²⁵I-apoJ-HDL was allowed to interact with the cell-surface receptor megalin at 4 °C, similar dose-response curves were obtained. Non-linear regression analysis of the specific binding data of four independent experiments performed in triplicate fitted to a rectangular hyperbola and allowed the calculation of mean dissociation constants ($K_d \pm \text{S.D.}$) of 78.8 ± 10.7 and 37.0 ± 7.3 nM for ¹²⁵I-apoJ and ¹²⁵I-apoJ-HDL, respectively (Figure 5A). Analysis of the binding data by calculation of the F ratios indicated a statistically significant increase in the megalin-binding affinity of lipidated apoJ ($P < 0.01$), suggesting that the

presence of lipids facilitates the interaction with the cellular receptor, perhaps by altering the conformation of apoJ. The K_d value observed for the interaction of apoJ with megalin on F9 kidney cells, although in the same range, is lower than data reported previously estimated for the binding of apoJ to purified megalin by ELISA ($K_d = 14.2$ nM [13]) and values recorded in vascular brain-perfusion experiments at the blood-brain barrier and the choroid epithelium *in vivo* ($K_m = 0.2$ and 0.5 nM, respectively [28]). The differences observed are likely to depend on different methodologies, on the cells used to estimate the binding affinities, and on the number of megalin receptors per cell. The enhancing effect of lipidation on receptor-binding affinity may have significant effect on apoJ transport across the vascular barrier of the central nervous system. Inhibition experiments employing the RAP, which acts to antagonize binding of all known ligands for megalin, showed that the displacement of the binding of apoJ-HDL to the receptor megalin followed a

Figure 5 Binding of apoJ-HDLp to mouse embryonal F9 cells

(A) Receptor binding: mouse embryonal carcinoma F9 cells were stimulated with $0.1 \mu\text{M}$ retinoic acid and $0.2 \mu\text{M}$ dibutyryl cAMP for 7 days. The cells were re-seeded subsequently on to gelatin-coated well plates at 5×10^5 cells/well and grown for 18 h at 37°C and 5% CO_2 . Prior to the addition of radiolabelled ligands, F9 monolayers were acid-stripped at pH 3 for 30 s. Increasing concentrations (0–95.5 nM) of ^{125}I -labelled apoJ ($\square$) or ^{125}I -labelled apoJ-HDL particles ($\blacksquare$) were incubated with the cells for 4 h at 4°C . After the incubation, the cells were washed, solubilized with 0.2 M NaOH/1% SDS and the radioactivity assessed with a gamma counter. Data obtained from four independent triplicate experiments are expressed as means $\pm$ S.D. (B) Inhibition experiments: retinoic acid-differentiated F9 cells were seeded on to gelatin-coated wells and incubated with a constant amount (30 nM) of ^{125}I -apoJ-HDL followed by increasing concentrations of RAP (0.1–1562.5 nM) for 4 h at 4°C . Bound radiolabelled apoJ-containing particles (not displaced by RAP) were estimated as above. IC_{50} values (means $\pm$ S.D.) were calculated from three independent duplicate experiments.

Figure 6 Inhibition of the complement haemolytic activity

The ability of apoJ-HDL to inhibit the haemolytic activity of the complement system was assessed by determining the amount of apoJ necessary to inhibit by 50% the activity of the C5b-9 complex to induce lysis of guinea pig erythrocytes as described above. The haemolytic activity of the C5b-9 complex was determined for each concentration of added apoJ ($\blacksquare$) or apoJ-HDL ($\square$) as percentage lysis compared with the control of 100% lysis in which an identical concentration of guinea pig erythrocytes was totally lysed by the addition of distilled water. Means $\pm$ S.D. of three independent duplicate experiments are shown.

one-site competition curve, with a calculated value for the half-maximal inhibition of 8.2 ± 0.1 nM (mean $\pm$ S.D.; Figure 5B), confirming the specificity of the binding.

To further characterize the biological functions of lipidated apoJ, we determined its inhibitory activity on the haemolytic efficiency of the membrane-attack complex of the complement system and compared it with the inhibition induced by the non-

lipidated protein. As shown in Figure 6, purified apoJ inhibits guinea pig erythrocyte haemolysis by the cytolytic complex in a concentration-dependent manner. The half-maximal inhibition value obtained ($8.8 \pm 0.2 \mu\text{g}$), in agreement with data reported previously [40], reflects the potent inhibitory activity of apoJ for the terminal complement complex, which was originally attributed to the competition exerted by hydrophobic regions of the molecule to the insertion of C5b-7 in the membrane lipid bilayers [40]. A similar value ($IC_{50} = 8.4 \pm 0.2 \mu\text{g}$) was obtained for the lipidated protein, indicating no influence of the lipid moieties on the inhibitory effect of apoJ. Interestingly, three other complement regulatory proteins have been found associated with lipoproteins: C₄-binding protein and the human factor H-related protein 4, which are associated with triacylglycerol-rich lipoproteins, and CD59 (protectin) found in HDL particles [41]. The distribution of these complement regulatory proteins among diverse lipoparticles, together with our data on the effect of lipidation on apoJ suggest that the association with lipoparticles does not modulate their activity, but rather provides a more physiological access of complement inhibitors to different environments.

Another well-characterized biological function of apoJ is the binding to Alzheimer's A β [26,42]. We evaluated the binding affinity of lipidated and non-lipidated apoJ for the A β 40 and A β 42 peptides via ELISA experiments. In all cases, a dose-response curve that reached saturation was obtained. The binding data fitted to a rectangular hyperbola corresponding to a single binding site with K_d values of 4.0 ± 0.6 and 2.7 ± 0.3 nM for the interaction of apoJ-HDL with A β 40 and A β 42, respectively, versus 3.1 ± 0.4 and 1.9 ± 0.2 nM, calculated for the binding of non-lipidated apoJ to A β 40 and A β 42 respectively (Figure 7). No statistically significant difference was observed between lipidated and non-lipidated samples ($P > 0.05$), indicating that the lipid environment does not affect the binding affinity. This is in agreement with our previous demonstration that the competition of

Figure 7 Binding of apoJ-HDL to A β *in vitro*

Variable concentrations of apoJ ($\circ$, $\diamond$) and apoJ-HDL ($\bullet$, $\blacklozenge$) were incubated with either A β 40 ($\circ$, $\bullet$) or A β 42-coated ($\diamond$, $\blacklozenge$) wells for 3 h at 37 °C. Bound apoJ was detected with specific monoclonal anti-apoJ antibody (Quidel; 1:2000), followed by horseradish peroxidase-labelled anti-mouse IgG (Amersham; 1:4000). Means $\pm$ S.D. of three independent duplicate experiments are shown.

apoJ-containing plasma HDL particles in solution with biotin-labelled apoJ for binding to immobilized A β 42 ($IC_{50} = 66$ nM) and A β 40 ($IC_{50} = 86$ nM) is very similar to that of delipidated apoJ ($IC_{50} = 80$ and 77 nM for A β 42 and A β 40, respectively) [42].

In summary, the data presented herein indicate that apoJ can be incorporated successfully into lipoprotein particles. Although structural changes in the apoJ molecule were evidenced by spectroscopic methods, neither its complement inhibitory properties nor its A β -binding activity were modified significantly. However, the presence of lipid moieties clearly enhances the binding affinity to the cell-surface receptor megalin. It is noteworthy that the association with lipids seems to have an important role in the interaction of another apolipoprotein, apoE, with some of its ligands. Lipidated apoE was found to express significantly higher affinity for A β compared with non-lipidated apoE [43]. In addition, the absence of lipids resulted in the lack of interaction between the N-terminal 22-kDa fragment of apoE3 and the LDL receptor on fibroblasts, whereas lipid association restored the receptor-recognition properties [44,45]. It has been postulated that lipid binding induces a conformational change in this domain that confers receptor-recognition properties, although CD and FTIR measurements did not detect real changes in the secondary structure induced by lipidation [44]. In the case of apoJ, it is not known whether the structural changes observed are directly responsible for the higher receptor-binding affinity. Nonetheless, the data suggest that the complement inhibition and A β -binding motifs are located in areas of the molecule different from those involved in the apoJ-megalin interaction.

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