Structure and Stability of Apolipoprotein J-Containing High-Density Lipoproteins†

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ABSTRACT: Apolipoprotein J (apoJ) defines a heterogeneous subclass of human plasma high-density lipoproteins (HDL) having a bimodal distribution of molecular mass of 70–90 kDa (~50%) and 200 kDa or larger (~50%). ApoJ-HDL are unstable in stored plasma, and must be evaluated within 24 h. All apoJ-HDL in freshly obtained plasma have α_2 electrophoretic mobility and are distinct from a minor subpopulation of apoAI-HDL which electrophorese in the preβ region. Although apoAI is not associated with the majority of plasma apoJ-HDL, a small fraction of these particles also containing apoAI. There is little variation in the apoJ/apoAI mole ratio of apoJ-HDL immunoaffinity purified from the same individual on different days. In addition, there is a constant ratio among individuals, assessed for five volunteers, of 4.9 ± 0.6. Purified apoJ added directly to apoJ-depleted plasma can interact with apoAI or with apoAI-containing lipoproteins, as evidenced by the association of apoAI with apoJ that is reisolated by immunoaffinity chromatography. The amount of apoAI associated with apoJ increases linearly with increasing amount of apoJ added, over the range of apoJ concentrations tested. No other known apolipoprotein is associated with apoJ. By two-dimensional electrophoretic analysis, the lipoproteins containing both apoJ and apoAI have approximate molecular masses of 350–400 kDa. Taken together, the results suggest that the interaction between apoJ and apoAI is physiologically important and that lipoproteins which contain both apoJ and apoAI can be produced in the plasma. ApoJ-HDL and apoJ/apoAI-HDL may have different functions and metabolic fates or may represent different stages of apoJ catabolism.

High-density lipoproteins (HDL)[1] comprise a class of human plasma lipid transport particles of considerable structural and functional heterogeneity. In spite of the inverse correlation between HDL cholesterol and premature atherosclerosis and coronary heart disease (CHD) [reviewed in Miller (1987) and Reichl and Miller (1989)], little is known about the origins, interrelationships, or catabolic fates of the various HDL subclasses. Immunoaffinity chromatography has made possible the identification and tracking of subclasses of HDL that contain a specific apolipoprotein. We (de Silva et al., 1990a,b) and others (James et al., 1991; Jenne et al., 1991) recently identified a lipid-binding protein, apolipoprotein J (apoJ) in plasma, associated primarily with lipid-poor subclasses of HDL and very high density lipoproteins (VHDL). ApoAI, the major protein of HDL, is consistently isolated with apoJ-HDL (de Silva et al., 1990a; James et al., 1991; Jenne et al., 1991).

ApoJ's association with lipid and apoAI suggests that it functions in extracellular lipid transport. However, a specific role of apoJ in lipid and lipoprotein metabolism has not been elucidated. Moreover, apoJ is an intriguing protein which, in addition to lipid transport, is thought to function in such diverse processes as sperm maturation, complement-mediated cell lysis, and programmed cell death (Sylvester et al., 1991b). ApoJ and its homologs have been identified in various tissues, e.g., liver, brain, testis, ovary, kidney, stomach, and heart (de Silva et al., 1990c; Sylvester et al., 1991a), of many species. ApoJ is homologous with rat sulfated glycoprotein 2 (SGP-2) (Collard & Griswold, 1987), testosterone repressed protein message 2 (Léger et al., 1987; Bettuzzi et al., 1989), ram clusterin (Cheng et al., 1988), human serum protein 40,40 (SP-40,40, also termed complement lysis inhibitor) (Blaschuk et al., 1983; Murphy et al., 1988; Jenne & Tschopp, 1989), canine glycoprotein 80 (Hartmann et al., 1991), and bovine glycoprotein III (Palmer & Christie, 1990). The broad tissue distribution of the apoJ message and the conservation of its structure among species support the possibility that apoJ has an important function(s).

As a prerequisite to elucidating the function of apoJ in lipid metabolism, we have defined further the macromolecular structure of apoJ-HDL in human plasma. We focus here on the relationship between apoJ and apoAI in apoJ-containing lipoproteins, since the metabolism or function of lipoprotein particles containing both apoJ and apoAI may be significantly different from that of lipoproteins containing only apoJ or only apoAI. Our results show that apoJ defines unique subclasses of α_2-HDL and that, in plasma of normolipemic individuals, most of the apoJ is not associated with apoAI. However, in spite of significant variation in the amount of plasma apoAI between individuals, the plasma apoJ/apoAI ratio, and the levels of LDL- and HDL-cholesterol, the apoJ/apoAI mole ratios in immunoaffinity-purified apoJ-HDL are reproducible and constant. This constancy suggests strict metabolic control of a physiologically important apoJ–apoAI association.

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[1] Abbreviations: apo, apolipoprotein; CBB, Coomassie brilliant blue; CHD, coronary heart disease; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); HDL, high-density lipoprotein(s) (d = 1.063–1.21 g/mL); LCAT, lecithin: cholesterol acyltransferase; LDL, low-density lipoprotein(s) (d 1.063–1.21 g/mL); mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PDB, plasma density buffer; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; SGP-2, sulfated glycoprotein 2; SP-40,40, serum protein 40,40; TFA, trifluoroacetic acid; VHDL, very high density lipoprotein(s) (d = 1.21–1.25 g/mL); VLDL, very low density lipoprotein(s) (d < 1.006 g/mL).

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EXPERIMENTAL PROCEDURES

Isolation of ApoJ. Plasma was collected from healthy male donors who had fasted for 12 h. Heparin (14.3 USP units/mL) was used as anticoagulant. Plasma, maintained at 4 °C, was diluted 1:3 with plasma density buffer (PDB: 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.02% NaN_3, and 1 mM Na_2EDTA), and protease inhibitors were added to a final concentration of 1 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 25 KU/mL aprotinin. Within 8 h, diluted plasma was chromatographed on an mAb11-AffiGel column equilibrated at 4 °C in PDB (de Silva et al., 1990a). Prior to elution of apoJ with 1 M acetic acid, the column was typically washed with 500 mL of PDB and 50 mL of 3× PDB.

ApoJ/ApoAI Mole Ratio in ApoJ-HDL. To determine the relationship between apoJ and apoAI in affinity-purified apoJ-HDL, mAb11-AffiGel eluates from the plasmas of fasted male donors were dialyzed against 10 mM NH_4HCO_3 containing 0.02% NaN_3 and concentrated with a Centricon 10 000 MWCO ultracentrifugation cup, and protein concentrations were determined with the Pierce BCA reagent. Eluate protein (15 μg) was reduced with 2-mercaptoethanol and electrophoresed on an 11–23% PhorCast gel (Amersham), according to the manufacturer's directions. Protein was stained with 0.2% Coomassie brilliant blue (CBB). Standard curves, made to correct for disproportionate staining of the proteins, were generated with known amounts (amino acid analysis) of apoJ and apoAI in the same gel system. ApoAI was purchased from Biodesign International, and apoJ was purified by high-performance liquid chromatography (de Silva et al., 1990b). Relative apoJ and apoAI amounts were determined by laser desnitometric scanning (LKB Ultrascan) of SDS–polyacrylamide gels. To calculate the mole ratio, the apoJ molecular mass was estimated to be 50 kDa, the approximate mass of the unglycosylated protein since CBB stains protein rather than carbohydrate.

To determine the effect of immunoaffinity chromatography on the apoJ/apoAI mole ratio, 100 mL of diluted plasma was repeatedly chromatographed on mAb11-AffiGel. Prior to elution of apoJ with 100 mL of 1 M acetic acid, the column was washed with 2 L of PDB followed by 450 mL of 3× PDB. The first eluate was immediately dialyzed against 10 mM NH_4HCO_3 containing 0.02% NaN_3. The unbound plasma solution was reapplied to the column, and the entire procedure was repeated to yield the subsequent eluates which were also dialyzed as described above.

Analysis of ApoJ-HDL. ApoJ-HDL were evaluated in plasma and as affinity-purified lipoproteins by one- (1D) and two-dimensional (2D) (Castro & Fielding, 1988) electrophoretic procedures, under nondenaturing conditions; apoJ was visualized by immunostaining. ApoJ-HDL, isolated as described above, were concentrated with an Amicon PM10 UF membrane. For 1D native gradient gel electrophoresis (GGE) (Blanche et al., 1981; Nichols et al., 1986), plasma or apoJ-HDL were fractionated on 4–30% acrylamide gels (Pharmacia LKB) in Tris–borate buffer (90 mM Tris, pH 8.35, containing 80 mM boric acid and 3 mM Na_2EDTA). The gels were electrophoresed at 8 °C for 1800 V-h, soaked for 1 h in blotting buffer (15.6 mM Tris/120 mM glycine) containing 0.5% SDS (Peluso & Rosenberg, 1987), and then transblotted onto nylon membranes (Zetaprobe, Bio-Rad) for 2600 mA-h at 4 °C in blotting buffer. All blots were incubated for 11 h with mouse mAb11 (de Silva et al., 1990a) or rabbit anti-apoJβ (Burkey et al., 1991) or rabbit anti-apoAI (Calbiochem) antibody in BLOTTO (Johnson et al., 1984). The secondary antibody was conjugated to horseradish peroxidase (HRP), except as specified. Vèzina et al. (1988) have shown that the *apparent* molecular weights of lipoproteins determined by GGE under approximately the same conditions are low without bias toward large or small particles.

For evaluation of apoJ-HDL in plasma by 1D agarose electrophoresis, blood (1 mL) from healthy fasted male donors was collected into 2 mM EDTA and immediately transferred to a microfuge tube on ice containing preservatives and protease inhibitors [10 μg/mL α_2-macroglobulin (Kunitake et al., 1990), 0.01% NaN_3, 1 mM benzamidine, and 25 KU/mL aprotinin]. After centrifugation at 4 °C to remove red blood cells, PMSF (final concentration, 1 mM) was added. Plasma was loaded into every other lane of a 1% agarose gel (Ciba). Electrophoresis in 50 mM barbital buffer, pH 8.6, containing 1 mM Na_2EDTA was performed at 10 °C in a Bio-Rad flatbed electrophoresis apparatus operating at 125 V until the marker dye, bromophenol blue, had migrated 5 cm (about 1 h). Proteins were transferred to PVDF paper (Bio-Rad) by press-blotting for 1 h, using five layers of thick filter paper and a 1-kg weight. The blot was incubated overnight in BLOTTO. Lanes containing plasma and standards, bovine serum albumin and α_2-macroglobulin (Calbiochem), were stained with 0.2% CBB. ApoJ was visualized with rabbit anti-apoJβ, diluted 1/1000. ApoAI was visualized with sheep anti-apoAI conjugated to HRP, diluted 1/4000 (the binding site). To increase the sensitivity of the apoAI antibody reaction, enhanced chemiluminescence (Amersham) was used according to the manufacturer's directions. Alternatively, the agarose gels were processed for 2D analysis, as described below.

For 2D electrophoretic analysis of apoJ-HDL in plasma, agarose electrophoresis followed by GCE was performed. One lane of the agarose gel was stained at 55 °C for lipid with 0.03% Fat Red 7B (Sigma) in 80% methanol plus 0.25% Triton X-100. Other lanes were excised, and each was annealed to a 4–30% precast polyacrylamide gel (Pharmacia LKB) with 0.75% agarose in Tris–borate buffer. The gradient gels were electrophoresed in Tris–borate buffer at 4 °C for 2040 V-h. Equilibrium was not achieved in order to retain small (<70-kDa) particles containing apoAI on the gel and to facilitate transblotting. Gels were soaked for 2 h in blotting buffer containing 0.5% SDS, rinsed for 5 min in blotting buffer, and transblotted at 200-mA constant current onto two sheets of nylon membrane (Zetaprobe, Bio-Rad) for 54 h at 4 °C. A strip of PVDF paper was placed between the lane containing native standards (Pharmacia) and the nylon membrane, stained with Ponceau S, and a standard curve (R = 0.996) was constructed to estimate the molecular weight. Subsequently, blots were incubated simultaneously with rabbit anti-apoAI, diluted 1/5000, and with mouse anti-apoJ (mAb11), diluted 1/5000. ApoAI was visualized with goat anti-rabbit–HRP conjugate (Bio-Rad), diluted 1/5000. To visualize apoJ, blots were incubated with 1.5 μCi of sheep anti-mouse ^{125}I-IgG (New England Nuclear) and exposed to Kodak X-OMAT AR film for 3 days at −70 °C.

ApoJ–ApoAI Association. Freshly drawn human plasma (25 mL), obtained and diluted in the usual manner, was depleted of apoJ by repeated extractions with mAb11-AffiGel, as outlined above. Aliquots (3 mL) of HPLC-purified apoJ (de Silva et al., 1990b) in 50% CH_3CN/0.1% TFA were dried in a Savant vacuum concentrator and resolubilized in 26.5 mL of apoJ-depleted plasma, incubated for 5 h at 37 °C with gentle agitation, and applied to mAb11-AffiGel columns (1 × 5 cm). Columns were washed with 500 mL of PDB and 100 mL of PDB plus 500 mM NaCl before elution of apoJ-

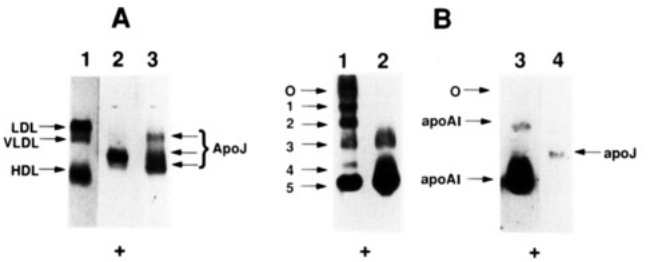

FIGURE 1: ApoJ-HDL are α_2-HDL which do not share electrophoretic properties with the majority of HDL. Plasma (1 μL, A; 2 μL, B) was obtained from a normolipemic fasted male volunteer and subjected to electrophoresis in 1% agarose, as described under Experimental Procedures. (A) Lane 1 was stained for lipid with Fat Red; lanes 2 and 3, for apoJ with mAb11 followed by anti-mouse IgG conjugated to HRP. Lanes 1 and 2 show fresh plasma; lane 3, plasma stored for 14 days at 4 °C. (B) Reference standards marked in lane 1 are as follows: 1 = fibrinogen; 2 = LDL + transferrrin + C3 + C4; 3 = α_2-macroglobulins + haptoglobins; 4 = α_1-antitrypsin; 5 = albumin. Lane 2 contained α_2-macroglobulin (1 μg) and bovine serum albumin (2 μg). Lanes 1 and 2 were stained with CBB, lane 3 with anti-apoAI–HRP conjugate, and lane 4 with rabbit anti-apoJβ followed by anti-rabbit IgG–HRP.

HDL with 40 mL of 1 M acetic acid. Eluates were dialyzed into 10 mM NH_4HCO_3 containing 1 mM Na_2EDTA and then into H_2O containing 0.01% NaN_3. Protein concentrations were determined by the Bradford method (Bio-Rad). Equivalent volumes of each eluate were vacuum-concentrated, electrophoresed under denaturing conditions on an 11–23% PhorCast gel (Amersham), stained with CBB, and scanned as described above.

Lipids and Apolipoproteins. ApoB was determined by a monoclonal antibody-based competitive ELISA (Young et al., 1986). An identical ELISA, except using an apoAI-specific mAb, was developed to quantitate apoAI. All lipid measurements were performed in a NHLBI-CDC standardized laboratory. HDL-cholesterol (HDL-C) was measured after precipitation of LDL and VLDL with heparin/Mn^{2+} (Albers et al., 1978). Cholesterol and triglycerides were determined by enzymatic methods on an Hitachi 737 (Steiner et al., 1981), using serum calibrators provided by the CDC. LDL-cholesterol (LDL-C) was calculated from total cholesterol, HDL-C, and triglyceride concentrations (Friedewald et al., 1972).

RESULTS

ApoJ Lipoproteins Migrate as α_2-HDL. When plasma is fractionated rapidly on a discontinuous salt gradient by ultracentrifugation, a small portion of apoJ is present in HDL_2 with the majority in VHDL (de Silva et al., 1990a). To define this apparent heterogeneity further, we determined the electrophoretic properties of plasma apoJ, under nondenaturing conditions. Initially, plasma was subjected to 1D agarose electrophoresis (Figure 1A). As reference, the major lipoprotein classes, LDL with β mobility, VLDL with preβ mobility, and HDL with α mobility, were visualized with the lipid stain Fat Red (lane 1). Lane 2, immunostained for apoJ, documents that all of the apoJ that was detected by mAb11 was located in a relatively broad band between VLDL and HDL. Thus, apoJ was not associated with the predominant lipoprotein classes. The region between electrophoretically separated VLDL and HDL contains both preβ- (Kunitake et al., 1985; Ishida et al., 1987), and α_2-HDL (Ishida et al., 1990).

To determine the position of apoJ migration with greater precision, apoJ was compared with known plasma proteins (Laurell, 1972), including apoAI (Figure 1B). ApoJ was

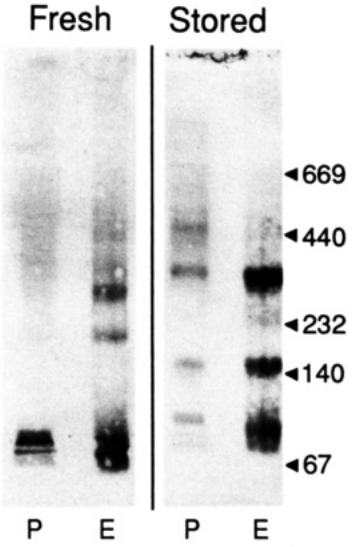

FIGURE 2: Immunoaffinity-isolated apoJ-HDL resemble apoJ-HDL in plasma. ApoJ-HDL were isolated, as described under Experimental Procedures, from fresh plasma or from plasma that had been stored for 17 days at 4 °C. Plasma (P, 4.3 μL) or apoJ-HDL (E, 0.3 μg) was loaded into alternate lanes of a 4–30% acrylamide gel and subjected to electrophoresis. ApoJ was identified by immunostaining using rabbit anti-apoJβ followed by anti-rabbit IgG–HRP.

determined to have α_2 mobility, on the basis of its migration in the region of the standard α_2-macroglobulin (lanes 1 and 2). The electrophoretic mobility of apoJ (lane 4) was different from that of a minor species of apoAI-HDL which migrated to the preβ position (lane 3). ApoAI in preβ-HDL represents 2–15% (Castro & Fielding, 1988; Kunitake et al., 1985; Ishida et al., 1987; Smith et al., 1984; Neary & Gowland, 1987) of the total apoAI in plasma obtained from fasted normolipemic donors. Minor subclasses of mouse apoAI-containing HDL migrate as two discrete bands with preβ and α_2 mobility, respectively, between VLDL and the majority of HDL (Ishida et al., 1990). The α_2-migrating apoAI may also be present in human plasma, but at low levels not immunostained under these conditions.

ApoJ-HDL Are Unstable. ApoJ-containing α_2-HDL were found to be unstable. The reorganization of apoJ-containing species during plasma storage was initially detected by agarose electrophoresis (Figure 1A, lane 3). In contrast to the single predominant α_2-migrating species of apoJ in fresh plasma, faster and slower migrating species of apoJ were also present in stored plasma. The species with faster electrophoretic mobility remained within the α_2 region, whereas that with the slower mobility migrated with properties comparable to VLDL in the preβ position.

The distribution of apoJ in fresh and stored plasma was compared in greater detail by nondenaturing gradient gel electrophoresis (GGE) followed by immunostaining (Figure 2, lanes labeled P). Blanche et al. (1981) demonstrated the advantages of nondenaturing GGE in resolving complex mixtures of plasma lipoproteins with good resolution and minimum artifacts. A polyclonal antibody preparation (rabbit anti-apoJβ) was used to identify apoJ to eliminate the possibility that results were biased by the selectivity of a monoclonal antibody. In fresh plasma, predominant apoJ-containing species were 70–90 kDa, with other species distributed between 140 and 1000 kDa. In stored plasma, the 70–90-kDa species were significantly reduced with concomitant increases in the 150-, 320-, and >400-kDa forms of apoJ. This method underestimates the sizes of the lipoprotein particles (Vézina et al., 1988). On the basis of reflective

scanning analysis of gel photos obtained in repeated experiments, the distribution of apoJ was determined for fresh vs stored plasma. Arbitrary size ranges of 70–100, 100–230, 230–440, 440–670, and >670 kDa were selected, and the average percent of apoJ in fresh/stored plasma was 47/14, 0/14, 27/34, 24/33, and 2/4, respectively.

The changes in the apparent size of apoJ-HDL with plasma storage were not due to degradation of the apoJ protein. On the basis of immunostain analysis of denatured plasma samples electrophoresed through polyacrylamide, no low molecular weight forms of apoJ were revealed (data not shown). The reorganization was not retarded by the sulfhydryl-specific inhibitor of lecithin:cholesterol acyltransferase (LCAT) 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Neary et al., 1991), the antioxidant butylated hydroxytoluene, or freezing (–20 °C) or thawing, but was accelerated by increased temperature (data not shown).

Affinity-Purified and Native ApoJ-Lipoproteins Are Similar. To ascertain whether immunoaffinity-purified apoJ-HDL are representative of apoJ-HDL in plasma, their size distributions were compared by GGE. Rabbit anti-apoJβ was used to visualize apoJ. As is shown in Figure 2, there was reasonable similarity between the apoJ in isolated apoJ-HDL (lanes labeled E) and in plasma (lanes labeled P). ApoJ-HDL isolated from freshly drawn blood were approximately 50% 70–90-kDa species, with the remainder present as components of 230 and 280 kDa and higher. ApoJ-HDL isolated from stored plasma contained less of the 70–90-kDa species, about 30%, and significantly increased amounts of 150- and 320-kDa complexes, comparable to the heterogeneity of apoJ-HDL present in the stored plasma. The distribution of apoJ in apoJ-HDL obtained from fresh/stored plasma between 70–100-, 100–230-, 230–440-, 440–670-, and >670-kDa species was 56/34, 8/17, 30/41, 7/5, and 0/3, respectively (average percent). The differences between isolated apoJ-HDL and apoJ-HDL in plasma (see previous discussion of lanes labeled P) are possibly exaggerated due to increased concentrations of apoJ in isolated apoJ-HDL vs plasma and to the immunochemical method used to identify apoJ. The size heterogeneity of the apoJ-HDL present in both stored plasma and isolated from stored plasma, shown in Figure 2, resembles that of the apoJ-HDL reported previously (de Silva et al., 1990a). We know now that conversion of apoJ-HDL from the "fresh" to the "stored" type of size heterogeneity is evident after only 48 h at 4 °C.

ApoJ-HDL Contain ApoAI at a Constant ApoJ/ApoAI Mole Ratio. Plasma apoJ and apoAI have, to a great extent, distinct electrophoretic properties (Figure 1). Moreover, only 1–4% of total plasma apoAI is associated with affinity-purified apoJ-HDL (de Silva et al., 1990a; Jenne et al., 1991). Given that plasma apoAI levels are typically 10 times those of apoJ and that there is no correlation between plasma apoJ and apoAI levels (Jenkins et al., 1990), the physiological relevance of the association between apoAI and apoJ is an issue. We addressed the relevance of the apoJ–apoAI association by evaluating their relationship in apoJ-HDL in mAb11-AffiGel eluates obtained by repeated extractions of the same plasma, by a single extraction of plasma from one donor over several days (individual variation), and by single extractions of plasmas from multiple donors (between-individual variation).

We hypothesized that a relevant apoJ–apoAI interaction would result in a constant apoJ/apoAI mole ratio in apoJ-HDL preparations isolated from plasma by sequential immunoaffinity extractions of apoJ. To test this prediction, plasma from one individual was chromatographed on mAb11-AffiGel, and the unbound fraction and the eluate were collected. This process was repeated 3 times, each time rechromatographing the unbound fraction and collecting the eluate. Proteins in the sequential eluates were analyzed by SDS–PAGE: Figure 3A shows the results when equal amounts of protein were loaded on the gel; Figure 3B, when equal volumes were loaded. Typically, most (70–85%) of the apoJ was removed in the first chromatographic step, and each successive step removed less and less apoJ (Figure 3B). The mole ratios of apoJ/apoAI in the mAb11-AffiGel eluates were determined by densitometric scans of CBB-stained gels shown in Figure 3A. When apoJ/apoAI ratios in all four eluates were compared, the mole ratio was 5/1 (5.0 ± 2.5, n = 4), indicating that depletion of apoJ from plasma caused a proportional decrease in apoAI. This result is also important since it documents that the apoJ-HDL isolated by a single immunoaffinity extraction of plasma are representative of the total apoJ-HDL present.

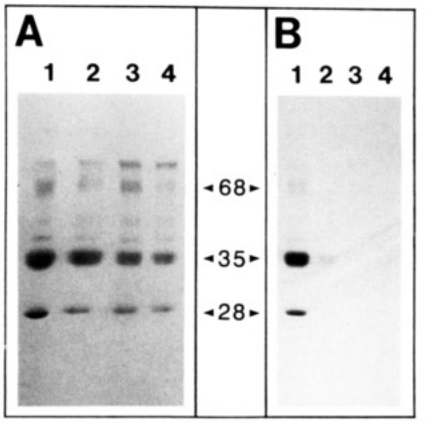

FIGURE 3: Ratio of apoJ/apoAI in immunoaffinity-purified apoJ-HDL remains constant through sequential plasma depletion steps. Plasma was obtained from a normolipemic fasted male volunteer, and repeatedly chromatographed on mAb11-AffiGel, as outlined under Experimental Procedures. At each step, protein bound to the column was eluted with 1 M acetic acid and evaluated by SDS–PAGE. Proteins were visualized with CBB staining. Panel A shows the result when equivalent amounts (15 μg) of eluted protein were loaded and panel B when equivalent volumes (400 μL) of eluate were loaded. The apoJ/apoAI mole ratio was calculated by densitometric scanning (panel A), taking into account the fact that apoAI stains 3× more intensely than apoJ and using molecular mass values of 28 kDa for apoAI (Baker et al., 1974) and 50 kDa for apoJ (de Silva et al., 1990c).

We further hypothesized that the individual and between-individual values of the apoJ/apoAI mole ratio would be constant. The apoJ/apoAI mole ratio of affinity-purified apoJ-HDL, obtained from a single individual on 4 successive days, was consistent at 6.4 ± 1.0. The between-individual apoJ/apoAI mole ratio of apoJ-HDL, determined for five male volunteers, was 4.9 ± 0.6 (Table I). The apoJ/apoAI mole ratio in apoJ-HDL did not depend on other plasma parameters measured, specifically total cholesterol and triglyceride, HDL-C or LDL-C, or on the plasma concentrations of apoB, apoAI, or apoJ (Table II). The concentrations of apoAI and apoJ in the plasmas of the five donors for the multidonor experiment varied by 1.4-fold, from 109 to 152 mg/dL and from 8.3 to 12 mg/dL, respectively. The LDL-C and HDL-C values varied by 1.4- and 1.7-fold, respectively, similar to the extent of variation in apoAI and apoJ.

The results, taken together, indicate a reproducible relationship between apoJ and apoAI in apoJ-HDL. To determine if this ratio is influenced by plasma storage, under conditions

Table I: Interdonor Variation of ApoJ/ApoAI Mole Ratio[a]

storage time (days) for subject	apoJ/apoAI molar ratio			
	0	2	4	7
1	5.8	ND	ND	7.7
2	4.9	6.1	7.7	8.6
3	4.7	ND	ND	9.5
4	4.8	ND	ND	6.2
5	4.2	ND	5.4	6.6
mean	4.9	ND	6.5	7.7
standard deviation	0.6			1.4

[a] ApoJ-HDL (15 μg) were obtained from five fasted, normolipemic male volunteers and evaluated by SDS–PAGE. The apoJ/apoAI mole ratio was calculated on the basis of CBB staining by comparison to standard curves. Plasma was stored at 4 °C.

Table II: Plasma Parameters, ApoJ/ApoAI Experiment[a]

subject	plasma parameters (mg/dL)						
	TC	TG	HDL-C	LDL-C	apoB	apoAI	apoJ
1	144	50	42	92	79	110	12.0
2	184	44	70	105	89	152	9.2
3	174	64	50	111	71	109	11.9
4	205	159	41	132	100	109	8.3
5	179	60	61	106	78	146	11.1
6	187	86	66	104	75	155	10.0

[a] Plasma was obtained from fasted, normolipemic male volunteers, and the plasma lipids and apolipoproteins were quantitated, as described under Experimental Procedures. TC, total cholesterol; TG, triglyceride, C, cholesterol. The between-individual variation in the apoJ/apoAI mole ratio was determined, using subjects 1–5; the individual variation in the ratio was determined for subject 6.

where the apoJ species undergo significant changes in electrophoretic mobility (Figure 1) and apparent molecular weight (Figure 2), apoJ-HDL were obtained from the stored plasmas of the five donors evaluated for the multidonor experiment, and the apoJ/apoAI mole ratios were determined (Table I). There was a significant increase in the apoJ/apoAI mole ratio in the apoJ-HDL isolated from the plasma from each subject after 7 days of plasma storage at 4 °C. The increase was continuous with storage time over the 7-day period examined, and varied between donors. For these individuals, the mean apoJ/apoAI mole ratio had increased from 4.9 ± 0.6 to 7.7 ± 1.4 in 7 days. The cause of this increase appeared to be an increase in the amount of apoJ extracted, not a decrease in the amount of apoAI, in immunoaffinity-purified apoJ-HDL. This conclusion is based on the results of two subsequent experiments, using different donors and performed in triplicate, in which the amount of apoJ extracted from fresh vs stored plasma by single-pass mAb11-AffiGel chromatography increased by an average of 29% and 43%, respectively, after plasma storage. In contrast, the amount of apoAI was relatively unchanged, an average decrease of 5% and an increase of 6%, respectively, in the two experiments.

ApoJ Interacts with ApoAI. The individual and between-individual reproducibility of the apoJ/apoAI mole ratio implies an interaction between the two apolipoproteins. To test for interaction between apoJ and apoAI, HPLC-purified apoJ was added to apoJ-depleted plasma (diluted 1/3) prepared by immunoaffinity chromatography. Since only a small amount of the total apoAI is removed with apoJ, the apoJ-depleted plasma retained considerable apoAI, 100–140 mg/dL (S. H. Jenkins, unpublished data). Following apoJ addition, apoJ was reextracted by immunoaffinity chromatography, and the apoJ/apoAI mole ratio was determined (Table III). The recovery of apoJ in the reextraction was 80–90% in three experiments. The amount of apoJ added to 26.5 mL of apoJ-depleted plasma was increased from 160 to 470 μg. The highest concentration of added apoJ gave a final concentration equivalent to 7.5 mg/dL, close to the normal value of ~10 mg/dL (Jenkins et al., 1990). ApoAI was reisolated with apoJ in each case, with the amount of apoAI proportional (R = 0.90) to the amount of apoJ added. The correlation between apoJ and apoAI was equally good (R = 0.93) when the amount of apoAI extracted by mAb11-AffiGel was evaluated against the amount of apoJ extracted. The apoJ/apoAI mole ratio decreased with increasing apoJ, from 30.4 ± 3.1 at 160 μg to 11.2 ± 0.6 at 470 μg of apoJ. At the highest amount of added apoJ, the apoJ/apoAI mole ratio in affinity-purified apoJ-HDL was about twice that of apoJ-HDL isolated from native plasma. No known apolipoprotein, other than apoAI, was detected in apoJ-HDL by SDS–PAGE.

Table III: ApoJ Interacts with Plasma ApoAI[a]

apoJ added (μg)	peak area		apoJ/apoAI mole ratio
	apoJ	apoAI	
0	0	0	
160	15.2	1.7	30.4 ± 3.1
310	55.9	5.1	16.6 ± 1.5
470	86.9	13.2	11.2 ± 0.6

[a] Purified apoJ was added to 26.5 mL of apoJ-depleted plasma (1/3 dilution), and apoJ-HDL were recovered by chromatography on mAb11-AffiGel. Proteins in apoJ-HDL were separated by SDS–PAGE and quantitated as the peak area by scanning the CBB-stained gel. Area was determined by peak weight and is shown as milligrams. The apoJ/apoAI mole ratio was calculated (n = 3) as described under Experimental Procedures.

Majority of ApoJ-HDL Do Not Contain ApoAI. Since 2D electrophoresis has provided considerable information about the heterogeneity of apoAI-containing lipoproteins (Castro & Fielding, 1988; Ishida et al., 1987), we utilized this technique to provide insight into the distribution of apoAI in plasma apoJ-HDL (Figure 4). Freshly drawn (panels A–C) and stored (panels D–F) plasma samples were subjected to electrophoresis, sequentially in agarose and polyacrylamide (4–30% gradient), and apoJ and apoAI were visualized by immunostaining. Most of the apoAI (panels A and D) migrated in the α position. Most of the apoJ (panels B and E) was detected in the region just ahead of VLDL and in alignment with a small fraction of the apoAI. The amount of apoAI in the region of overlap with apoJ is visually underrepresented by the immunochemical method of detection. Vèzina et al. (1988) suggested that the interaction of apoAI with other proteins, particularly in complexes of ≥350 kDa, reduces its accessibility to antibodies.

In fresh plasma, the apparent molecular masses of the apoJ species were concentrated in the 70–90-kDa range; other species were evident at 150, 200, 250, and 280 kDa and higher (panel B). The use of ^{125}I-IgG secondary antibody coupled with long development of the radioautograph to visualize apoJ exaggerates the apparent contribution of the low-abundance, large species relative to the pattern visualized by the HRP-conjugated secondary antibody (Figure 2). The minor amount of apoAI that migrated between VLDL and the majority of HDL appeared as four distinct species: 350, 400, and 500 kDa and a small <67-kDa species. This pattern agrees qualitatively with that reported by Francone et al. (1989); the 350–500-kDa and the <67-kDa species correspond to $LpAI_{pre\beta 2}$ and $LpAI_{pre\beta}$, respectively. Since equilibrium was not achieved during electrophoresis in the second dimension, the molecular mass values are approximate. Comparison of panel A with D (apoAI) and panel B with E (apoJ) indicates that plasma storage caused only minor changes in the electrophoretic

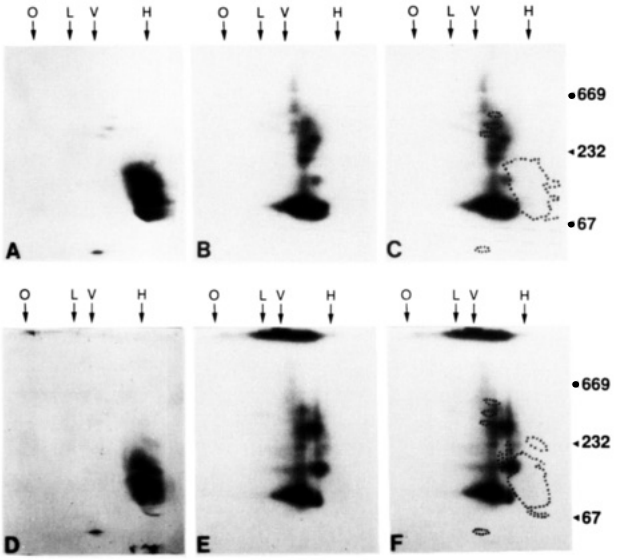

FIGURE 4: ApoJ and apoAI comigrate in α_2-HDL. Plasma (4 μL), fresh or stored for 5 days at 4 °C, was subjected to 2D electrophoresis, as outlined under Experimental Procedures. ApoJ and apoAI were visualized simultaneously by immunostaining, using mAb11 followed by sheep anti-mouse ^{125}I-IgG and anti-apoAI followed by goat anti-rabbit IgG–HRP, respectively. Panels A–C show the results with fresh plasma; panels D–F, with stored plasma. Panels A and D show apoAI, panels B and E show apoJ, and panels C and F indicate the overlap of apoJ and apoAI. The designations O, L, V, and H represent the origin and the positions of migration of LDL, VLDL, and HDL, respectively, during agarose electrophoresis.

properties of the apoAI-HDL whereas there were shifts in apoJ-HDL in the β and α directions, as is also evident in Figure 1A.

In panels C (fresh plasma) and F (stored plasma), the apoAI profile has been traced onto the apoJ profile to facilitate evaluation of the apoJ–apoAI relationship. In fresh plasma, there was overlap of apoJ and apoAI which occurred primarily in particles of 350–400 kDa. However, the majority of apoJ did not comigrate with apoAI. When plasma had been stored prior to analysis, there was increased comigration of apoJ and apoAI in the 150-kDa range, with reduced overlap in the 350–400-kDa range.

DISCUSSION

Plasma lipoproteins that contain apoJ, bimodal in size and relatively poor in lipid, exist within the HDL class (de Silva et al., 1990a; James et al., 1991; Jenne et al., 1991). Our results reported here establish that all of the apoJ-HDL in freshly isolated plasma have α_2 electrophoretic mobility in agarose. This is true not only if the plasma is obtained from a fasted subject, as is demonstrated here, but also if it is obtained from a subject whose plasma triglycerides are increased by 3-fold by an oral fat load given 4 h prior to phlebotomy (S. H. Jenkins, W. D. Stuart, and J. A. K. Harmony, unpublished data). It is interesting that purified apoJ has also been reported to have α_2 electrophoretic mobility (Murphy et al., 1988), which suggests that the electrophoretic properties of apoJ-HDL are, in large part, governed by the protein. Using analytical methods that do not denature lipoproteins, specifically agarose electrophoresis coupled with gradient gel electrophoresis, we found that the apoJ-HDL in freshly isolated plasma are approximately equally distributed into a class of 70–90 kDa and one of >200 kDa. There is little difference between the size distribution of apoJ-HDL in plasma and in mAb11 eluate, indicating that the conditions used here to elute apoJ from the mAb11-AffiGel column and to concentrate it for analysis do not significantly disrupt the structures of lipoproteins containing apoJ.

The instability of apoJ-HDL in plasma contrasts with the stability of apoJ-HDL isolated by immunoaffinity chromatography. It is important to note this instability since other investigators are also evaluating the structures of apoJ lipoproteins in various physiological fluids. During plasma storage, the mean size of apoJ-HDL increases, and different subspecies have increased and decreased electrophoretic mobility, respectively. In contrast, isolated apoJ-HDL remain stable during weeks of storage at 4 °C (data not shown). Plasma factors are therefore likely to be responsible for the instability. As determined by SDS–PAGE coupled with electroimmunoblot analysis, apoJ degradation does not contribute to the instability of apoJ-HDL. The mechanism of the reorganization is unknown, but may involve protein aggregation and/or lipid redistribution. The tendency of apoJ to aggregate has been noted previously by Griswold et al. (1986), Tsuruta et al. (1990), and Sylvester et al. (1991b). The process appears to result in increased affinity of the anti-apoJ–apoJ interaction (these results and S. H. Jenkins, preliminary data). As a consequence, more apoJ can be removed from stored vs fresh plasma by a single immunoaffinity extraction. The magnitude of increased extraction depends on storage conditions and time and on the individual from whom the plasma was obtained. In contrast, the amount of apoAI associated with immunoaffinity-purified apoJ is not affected by plasma storage. It is therefore likely that the apoJ which is not associated with apoAI undergoes reorganization with storage, implying that apoAI can stabilize apoJ (see below).

Several investigators (Kunitake et al., 1990; Ishida et al., 1990; Neary et al., 1987, 1991) have reported that apoAI-containing preβ-HDL are also unstable in plasma, although others (Kunitake et al., 1985; Ishida et al., 1987) have found them to be stable. In our experience, the extent of reorganization of apoJ-HDL, determined by 1D and 2D electrophoretic analysis, is significantly greater than that of preβ-migrating apoAI. DTNB has no effect on the reorganization of apoJ-HDL in plasma, suggesting that LCAT does not participate in this process. However, this cannot be concluded unequivocally since DTNB has, depending on the report, either no (Ishida et al., 1990) or an inhibitory effect (Ishida et al., 1990; Neary et al., 1991) on changes in apoAI-containing preβ-HDL. ApoJ-HDL in plasma are not stabilized by reduced temperature, which has also been reported to be the situation for apoAI-containing preβ-HDL (Neary et al., 1987). The documented instability of apoJ-HDL necessitates the use of fresh biological fluids when assessing the structure of apoJ-containing lipoproteins until a suitable inhibitor of their reorganization is identified.

The amount of apoAI associated with apoJ-HDL in freshly isolated plasma is constant. The mole ratio of apoJ/apoAI measured for five young healthy male subjects was approximately 5/1. The apoJ-HDL that contain both apoJ and apoAI also have α_2 electrophoretic mobility but have molecular masses in the 350–400-kDa range (approximate). If the stoichiometry per particle is 1/1, apoJ/apoAI, the apoJ/apoAI-HDL comprise <20% of the total apoJ-HDL. Purified apoJ can reassociate with apoAI in plasma, producing apoJ–apoAI complexes in which the apoJ/apoAI mole ratio approaches that which existed originally in the plasma. The fact that the mole ratio depends on the amount of apoJ added suggests that the contribution of apoJ/apoAI-HDL to the pool of apoJ-HDL is governed by the amount of apoJ present.

The plasma compartment may, in fact, be the site at which the apoJ–apoAI interaction normally occurs. We (Burkey et al., 1992) have found that human liver HepG2 cells, which secrete both apoJ and apoAI at equivalent rates (Burkey et al., 1991), secrete apoJ-HDL that do not contain apoAI, consistent with the hypothesis that apoJ-HDL acquire apoAI extracellularly.

What are the implications of the apoJ–apoAI interaction? Since the physiologic function(s) of apoJ remain(s) to be elucidated, the impact of its association with apoAI can only be speculative at this time. The capacity of purified apoJ to interact with apoAI in plasma, and its failure to interact with other known apolipoproteins such as apoAII, apoE, or the apoC's, is indicative of a direct physical interaction between apoJ and apoAI. The results of Jenne et al. (1991) support this conclusion. Since the amount of plasma apoAI normally far exceeds that of apoJ, it is possible that not all of the apoAI in plasma is "eligible" to interact with apoJ. Lipoproteins that contain both apoJ and apoAI are likely to have different lipid compositions, representing different stages of metabolism, and to have different catabolic fates from those that contain apoJ only. ApoAI has been reported to bind to bovine seminal plasma proteins (Manjunath et al., 1989) and to a 120-kDa protein isolated from term placenta (Keso et al., 1987), as well as to a protein purified from adipose cells (Barbaras et al., 1990). These various protein associations imply roles for apoAI that are distinct from its role in cholesterol homeostasis or, alternatively, specialized functions for apoAI within the theme of cholesterol homeostasis.

HDL appear to occupy a key position in the interorgan transport of lipids [reviewed in Reichl and Miller (1989)]. They serve as a reservoir of apolipoprotein CII (apoCII) which, when transferred from HDL to chylomicrons and very low density lipoproteins (VLDL), facilitates the intravascular hydrolysis of triglycerides catalyzed by lipoprotein lipase. Subclasses of small HDL promote the efflux of cholesterol from extrahepatic cells, and apoAI facilitates the sequestration of this cholesterol as cholesteryl ester by activating plasma LCAT. The coupled processes of cholesterol efflux and esterification serve to maintain cholesterol balance in extrahepatic tissues. The cholesteryl esters are subsequently delivered to the liver for further metabolism or excretion. The entire pathway has been referred to as reverse cholesterol transport.

ApoAI-containing HDL with preβ electrophoretic mobility may be major players in accepting cholesterol in reverse cholesterol transport. In addition to preβ HDL, these species have been termed "free" apoAI (Børresen & Berg, 1980) and $LpAI_{pre\beta}$ (Francone et al., 1989). Although there is some disagreement on the exact size, concentration, composition, and stability of preβ apoAI-HDL, it is generally agreed that—like about 50% of the apoJ-HDL—they are small (<100 kDa) dense particles which do not contain apoAII. As predicted for species involved in reverse cholesterol transport, these small HDL are found in elevated levels in patients with hyperlipidemias that predispose to atherosclerosis and CHD (Ishida et al., 1987; Neary & Gowland, 1987; Daerr et al., 1986), appear in the interstitial fluid of aortic plaques (Smith et al., 1984; Heideman & Hoff, 1982), and have been shown to be a primary acceptor of radiolabeled cholesterol from cultured fibroblasts (Castro & Fielding, 1988). In addition, Neary et al. (1991) recently reported that preβ HDL levels are negatively correlated with LCAT activity, suggesting that these particles are the preferred LCAT substrates.

ApoJ-HDL may similarly accept cholesterol from extrahepatic tissues and then combine with apoAI to facilitate cholesterol esterification by LCAT. Alternatively, since the apoJ gene is expressed in a wide variety of tissues, secreted apoJ may mediate cholesterol efflux directly. Such a scenario would place apoJ in a pivotal position in the process of reverse cholesterol transport. Two lines of evidence support the link between apoJ and apoAI in this hypothetical mechanism of cholesterol efflux. ApoAI (Zannis et al., 1985) can be expressed in a number of the same extrahepatic tissues as apoJ (de Silva et al., 1990c; Murphy et al., 1989), and, in some situations, e.g., neuronal injury–regeneration, expression of both appears to be similarly induced (Léger et al., 1987; Bettuzzi et al., 1989; Buttyan et al., 1989; Boyles et al., 1989).

In spite of a bewildering number of functions attributed to apoJ and its homologs [summarized in Sylvester et al. (1991a)], a primary role of apoJ appears to be the transport of small hydrophobic molecules, including cholesterol and triglyceride. This role is supported by the predicted secondary structure of apoJ, containing amphipathic domains necessary for lipid binding (de Silva et al., 1990c), and by the association of lipids with the affinity-purified protein (de Silva et al., 1990a; Jenne et al., 1991). ApoJ is secreted and circulates in body fluids (de Silva et al., 1990a; O'Bryan et al., 1990). The hydrophobic molecules associated with apoJ may be needed for a variety of biological processes, including energy metabolism, membrane construction and remodeling, communication, or lubrication. ApoJ expression is increased significantly in cells/tissues that are undergoing apoptosis and degeneration (Buttyan et al., 1989; Monpetit et al., 1986; Sawczuk et al., 1989; Duguid et al., 1989; May et al., 1990; McNeill et al., 1991), both of which are situations where cell membrane constituents are reutilized. Resolution of the exact role of apoJ in such lipid transport phenomena will require a careful analysis of the components associated with apoJ that are isolated directly from the cells that secrete it.

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