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High density lipoproteins bind A β and apolipoprotein C-II amyloid fibrils[§]

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Abstract Disease-associated amyloid deposits contain both fibrillar and nonfibrillar components. The majority of these amyloid components originate or coexist in the bloodstream. To understand the nature of the interaction between the nonfibrillar and fibrillar components, we have developed a centrifugation method to isolate fibril binding proteins from human serum. Amyloid fibrils composed of either A β peptide or apolipoprotein C-II (apoC-II) cosedimented with specific serum proteins. Gel electrophoresis, mass spectrometry peptide fingerprinting, and Western analysis identified the major binding species as proteins found in HDL particles, including apoA-I, apoA-II, apoE, clusterin, and serum amyloid A. Sedimentation analysis showed that purified human HDL and recombinant apoA-I lipid particles bound directly to A β and apoC-II amyloid fibrils. These studies reveal a novel function of HDL that may contribute to the well-established protective effect of this lipoprotein class in heart disease.—Wilson, L. M., C. L. L. Pham, A. J. Jenkins, J. D. Wade, A. F. Hill, M. A. Perugini, and G. J. Howlett. High density lipoproteins bind A β and apolipoprotein C-II amyloid fibrils. *J. Lipid Res.* 2006. 47: 755–760.

Supplementary key words amyloidosis • apolipoprotein A-I • atherosclerosis • human serum • lipid • nonfibrillar component • protein aggregation • protein folding

Amyloid deposits are associated with a large number of debilitating disorders, including Alzheimer's and Parkinson's diseases, transmissible spongiform encephalopathies, type II diabetes, and systemic amyloidoses (1). More than 20 different polypeptides and proteins form amyloid fibrils in vivo; the majority of these are derived from the bloodstream and are deposited extracellularly (2). Amyloid fibrils are universally characterized by their interaction with the dyes thioflavin T and Congo Red and by their cross- β

structure, as revealed by X-ray diffraction. Recent work has focused on the molecular structure of amyloid fibrils, with a particular focus on the organization of the cross- β sheets formed by the A β peptide (3) and the X-ray crystallography of amyloid fibrils composed of small peptides (4, 5).

Less attention has been focused on the nonfibrillar components of amyloid deposits, such as serum amyloid P component (SAP), apolipoprotein E (apoE), clusterin (apoJ), and glycoproteins such as laminin and agrin (6). Although several potential roles for these molecules in the deposition of amyloid plaques have been proposed, conflicting data have confused their precise function. For example, studies with the A β peptide show that apoE and SAP promote A β fibril formation (7, 8), whereas other work shows that apoE and SAP inhibit A β fibril formation (9, 10). Nonfibrillar components may also affect the stability and interaction within amyloid deposits. SAP protects serum amyloid A (SAA) and A β amyloid fibrils from proteolysis by trypsin, chymotrypsin, and pronase (11), whereas apoE and SAP also mediate the self-association and tangling of apoC-II amyloid fibrils (12). Another potential role for nonfibrillar components such as SAP is to affect the recognition of toxic oligomeric amyloid precursors (13) by the innate immune response. Amyloid deposits are often localized to specific tissues, with peptides accumulating in restricted locations (1). The A β peptide, for example, is found in a variety of tissues but is deposited as fibrils only in synapses and the basement membranes of brain blood vessels (14). Such specificity suggests that there are factors, other than the nature of the fibril-forming proteins, that control amyloid deposition.

Because the majority of nonfibrillar components in amyloid deposits are derived from, or at least coexist in, the

Abbreviations: apoC-II, apolipoprotein C-II; SAA, serum amyloid A; SAP, serum amyloid P component.

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bloodstream, we initiated a search for serum proteins that bind amyloid fibrils. Amyloid fibrils composed of A β peptide, α -synuclein, and apoC-II were chosen for this study. A β amyloid fibrils are formed from the A β peptide, which is cleaved from the amyloid precursor protein and deposited in plaques associated with neuronal dysfunction in Alzheimer's disease (15). α -Synuclein is the major component of Lewy bodies, the fibrillar intraneuronal inclusions associated with Parkinson's disease. Soluble α -synuclein has class A amphipathic helices and binds lipid in a similar manner to apolipoproteins (16). Human apoC-II is a component of very low density lipoproteins, in which it plays an essential role in activating lipoprotein lipase during lipid metabolism. ApoC-II readily aggregates in lipid-free conditions to form homogeneous amyloid fibrils (17). ApoC-II aggregates are present in human atherosclerotic plaques, and apoC-II fibrils initiate macrophage inflammatory responses (18). Our results identify a subset of serum proteins that bind A β and apoC-II amyloid fibrils.

MATERIALS AND METHODS

Materials

A β peptide was synthesized and α -synuclein and apoC-II were expressed and purified as described previously (17, 19, 20). Amyloid fibrils were prepared by incubation in phosphate buffer [100 mM sodium phosphate, 0.1% (w/v) sodium azide, pH 7.4] using final concentrations of 0.4 mg/ml (A β), 3 mg/ml (α -synuclein), and 0.4 mg/ml (apoC-II) at 37°C with shaking for 5 days for A β and α -synuclein (1,400 rpm Thermomixer; Eppendorf, Hamburg, Germany) and at room temperature without shaking for 5 days for apoC-II. The presence of amyloid fibrils was confirmed using the dye thioflavin T, as described previously (17). Initial studies were conducted with one fasted serum sample from a healthy volunteer. Serum samples with increased SAA levels were provided by consenting patients from a previous study conducted at St. Vincent's Hospital, Melbourne (21). SAA levels were determined by immunonephelometry (BN-II nephelometer; Dade-Behring, Marburg, Germany). Human HDL, rabbit anti-human apoA-I antibodies, goat anti-human apoA-II antibodies, and goat anti-rabbit peroxidase-conjugated secondary antibodies were purchased from Calbiochem (La Jolla, CA). Human apoA-I was purchased from Sigma (St. Louis, MO). Goat anti-human apoE antibodies, goat anti-human clusterin antibodies (apoJ), and rabbit anti-goat horseradish peroxidase secondary antibodies were purchased from Chemicon (Temecula, CA). Reconstituted HDL (22) was kindly provided by CSL Bioplasma (Melbourne, Australia).

Binding of serum proteins to amyloid fibrils

Human serum diluted 10-fold in phosphate buffer (350 μ l) or purified lipoprotein (300 μ l, 0.1 mg/ml) was mixed, by brief vortexing, with amyloid fibrils (100 μ l) or phosphate buffer (100 μ l) at room temperature and immediately layered on top of 20% sucrose (500 μ l) in phosphate buffer in a 1 ml polycarbonate ultracentrifuge tube. The samples were then centrifuged in a TL100 benchtop ultracentrifuge (Beckman Coulter, Fullerton, CA) at 100,000 rpm (355,000 *g*) for 10 min at 20°C. The supernatants of both serum-alone controls and serum amyloid fibril mixtures were removed, and the pellets were gently washed twice with 500 μ l of phosphate buffer and resuspended in gel-loading buf-

fer (60 μ l). The samples (30 μ l) were analyzed using 16% Tris-Tricine SDS-PAGE gels or Tris-glycine 4–20% gradient SDS-PAGE gels (Gradipore) and stained with Coomassie Brilliant Blue R250 or colloidal silver (Plus One silver stain; Amersham, Piscataway, NJ), as indicated.

Mass spectrometry

Gel slices corresponding to specific protein bands were rinsed (25 mM ammonium bicarbonate) and incubated in 10 mM dithiothreitol for 1 h at 56°C, then alkylated with 55 mM iodoacetamide for 45 min in the dark. Gel pieces were dried and incubated in trypsin (Promega, Madison, WI; 20 μ l of 12.5 ng/ μ l in 25 mM ammonium bicarbonate) at 37°C overnight. The supernatant was collected and mixed in a 5:2 ratio with α -cyano-4-hydroxycinnamic acid in 50% acetonitrile and 1% trifluoroacetic acid for mass spectrometry analysis. The instrument used was a Voyager-DE STR Perkin-Elmer Applied Biosystems (Foster City, CA) time-of-flight mass spectrometer equipped with a 337 nm N₂ laser matrix-assisted laser desorption ionization source. Parent ions were measured in reflector and positive modes. Mass spectrometry fingerprint spectra were analyzed using MS-FIT (<http://prospector.ucsf.edu/>).

Western analysis

Samples were transferred from SDS-PAGE gels to nitrocellulose membranes by electroblotting. The membranes were incubated in blocking buffer (5% skim milk 10 mM Tris, 150 mM sodium chloride, and 0.01% Tween 20, pH 7.4) at 4°C overnight, followed by 1 h at room temperature in a 1:1,000 dilution of primary antibody in blocking buffer, three washes in 10 mM Tris, 150 mM sodium chloride, and 0.01% Tween 20, pH 7.4, and then 1 h at room temperature in secondary antibody, diluted 1:10,000. Bound antibody was visualized using insoluble tetramethylbenzidine.

Analytical ultracentrifugation

Sedimentation velocity analysis was performed using a Beckman model XL-A analytical ultracentrifuge, as described previously (12). ApoC-II amyloid fibrils and HDL samples were centrifuged at 7,000 rpm (4,000 *g*), with radial absorbance data acquired at 280 nm. Data were fitted to a least-squares $g^*(s)$ distribution model, $ls-g^*(s)$, using the program SEDFIT (23).

Electron microscopy

Samples were applied to freshly glow-discharged carbon-coated copper grids, negatively stained with 2% (w/v) potassium phosphotungstate, pH 6.8, and examined using a FEI Tecnai 12 transmission electron microscope equipped with a Soft Imaging System MegaView III charge-coupled device camera. Micrographs were recorded at nominal magnifications of 150,000 \times .

Phospholipid assay

Total phospholipid levels of pellet fractions were measured using the phospholipid C assay for the determination of phospholipids in serum (Wako, Osaka, Japan). Total pellet fractions (60 μ l, resuspended in phosphate buffer) of apoC-II amyloid fibrils spun alone, apoC-II amyloid fibrils spun in the presence of serum, and serum spun alone were analyzed.

RESULTS

Mixtures of human serum and preformed α -synuclein, A β peptide, or apoC-II amyloid fibrils were centrifuged

through a 20% sucrose solution, and the pellet fraction was analyzed by SDS-PAGE. Serum proteins that do not interact with amyloid fibrils remain in the supernatant fraction (data not shown). **Figure 1A** shows the results obtained, together with control samples containing serum centrifuged alone. Under the conditions used, there was no specific binding of serum proteins to α -synuclein fibrils. In contrast, $A\beta$ and apoC-II fibrils bound and sedimented with a selective and common set of serum proteins. There was a small subset of proteins from serum that sedimented through the 20% sucrose in the absence of amyloid fibrils; these protein bands were not analyzed further. Similar results were obtained using human plasma (data not shown). The results shown in Fig. 1A were sensitive to the amount of serum present in the initial mixture. Serum

dilutions of 1:10 and 1:50 yielded different amounts of protein in the pellet fraction (Fig. 1B).

The identities of the serum proteins that interact with $A\beta$ and apoC-II amyloid fibrils were investigated using mass spectrometry (**Table 1**) (see also supplementary data). Bands labeled d–g in Fig. 1 were identified as apoA-I, apoE, apoA-IV, and clusterin, respectively. These identifications were based on the measured masses of between 6 and 21 tryptic peptides derived from the individual bands. The identifications were made using MS-FIT and searching the SwissProt database; in each case, there was one predominant match to a human serum protein that was consistent with the known mobility of the denatured protein using SDS-PAGE. The proteins identified are all components of serum HDL. Western analysis

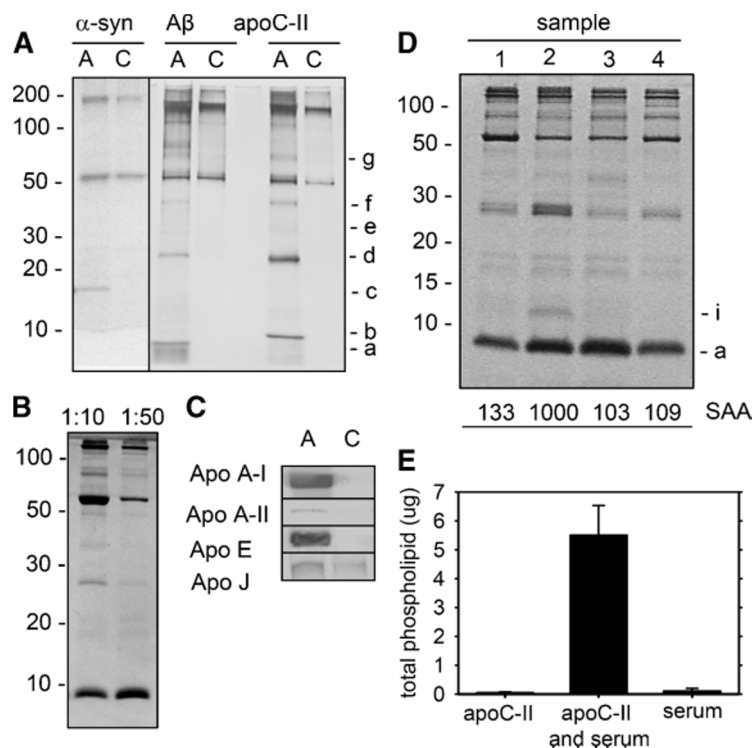


Fig. 1. Analysis of the binding of serum proteins to amyloid fibrils. All samples were centrifuged through 20% sucrose for 10 min at 355,000 *g*. Samples centrifuged in the presence of amyloid fibrils are marked A, whereas control serum samples centrifuged alone are also shown (C). A: Pellet fractions from a serum sample centrifuged in the presence of amyloid fibrils composed of α -synuclein (α -syn), $A\beta$, and apolipoprotein C-II (apoC-II) were analyzed by 4–20% Tris-glycine gradient SDS-PAGE and silver staining. Lowercase letters identify specific bands. Bands a, b, and c indicate the position of $A\beta$, apoC-II, and α -synuclein, respectively. Bands d–g were analyzed further (Table 1). The positions of molecular mass makers in kDa are shown. B: Pellet fraction serum (diluted 1:10 and 1:50) centrifuged in the presence of amyloid fibrils composed of apoC-II and analyzed by 4–20% Tris-glycine gradient SDS-PAGE and silver staining. C: Western analysis of amyloid binding proteins from serum. Pellet fractions were analyzed using peroxidase conjugate antibodies to apoA-I, apoA-II, apoE, and apoJ (clusterin), as indicated. The bands correspond to known mobilities of apoA-I, apoA-II, apoE, and apoJ. D: Analysis of apoC-II amyloid binding proteins in different serum samples (1–4). Each different serum sample was mixed with apoC-II amyloid fibrils and centrifuged, and the pellet fraction was analyzed using 16% Tris-Tricine SDS-PAGE with Coomassie Brilliant Blue staining. The band labeled a corresponds to apoC-II, and the band labeled i was analyzed further (Table 1). Serum amyloid A (SAA) levels are shown for each sample in mg/l. E: Total phospholipid content of the pellet fractions of apoC-II amyloid binding samples from human serum. Pellet fractions of apoC-II amyloid alone, apoC-II and serum, and serum alone were centrifuged and analyzed for total phospholipid. Error bars indicate SEM (*n* = 3).

TABLE 1. Identification of amyloid binding proteins

Band ^a	Molecular Mass ^b	Percentage Coverage	Peptides Matched ^c	Molecular Weight Search Score ^d	Protein
	<i>kDa</i>	<i>%</i>			
d	28	40	12/18	4.3 E+06	ApoA-I
e	34	51	13/16	3.7 E+03	ApoE
f	48	71	21/24	5.7 E+08	ApoA-IV
g	75	31	8/15	1.2 E+05	Clusterin (apoJ)
i	12	54	6/8	3.2 E+03	Serum amyloid A

ApoA-I, apolipoprotein A-I.

^a Bands shown in Fig. 1.

^b Apparent molecular mass relative to the mobility of SDS-PAGE molecular mass standards.

^c Peptides identified by peptide mass fingerprinting using MS-FIT (<http://prospector.ucsf.edu/>) as a fraction of total peptides observed.

^d Molecular Weight Search (MOWSE) scores were calculated using MS-FIT (<http://prospector.ucsf.edu/>).

confirmed the identity of apoA-I and apoE as amyloid binding proteins (Fig. 1C). In addition, the binding of apoA-II, another HDL protein component, which was not initially identified by mass peptide fingerprinting, was also demonstrated by Western analysis. Clusterin was also identified in the apoC-II amyloid serum pellet fraction by Western analysis and to a lesser extent in the serum-alone pellet fraction.

During inflammation, there is a large increase in SAA levels and a corresponding increase in the levels of SAA in HDL (24). We analyzed four different serum samples with SAA levels of 103, 109, 133, and 1,000 mg/l (Fig. 1D). SDS-PAGE analysis of the pellet fraction for the sample with the highest SAA level revealed an additional band (i) at a mobility corresponding to SAA (lane 2). Mass spectrometry confirmed this band as SAA (Table 1). Control serum-alone spins did not show the presence of SAA in any of the pellet fractions.

Figure 1E shows the phospholipid content of the pellet fractions. Samples of apoC-II amyloid fibrils alone or serum alone contained negligible phospholipid levels. In contrast, phospholipid levels were much higher in the pellet fractions for apoC-II amyloid fibrils centrifuged in the presence of serum. Comparisons with serum samples before centrifugation (data not shown) suggest that ~3% of serum phospholipid sediments with apoC-II amyloid fibrils.

The sucrose sedimentation method was used to investigate the direct interaction of HDL with apoC-II amyloid fibrils. Sedimentation experiments with purified human HDL demonstrate that HDL binds to apoC-II amyloid fibrils, as shown by protein bands at 28 and 18 kDa corresponding to apoA-I and dimeric apoA-II (Fig. 2A). These protein bands were not observed in the HDL-alone control sample. Under the conditions used (50 µg/ml HDL, 100 µg/ml apoC-II fibrils), a significant amount of HDL remained in the supernatant. The interaction of reconstituted HDL, composed of apoA-I combined with soy phospholipid, with apoC-II amyloid fibrils was also investigated. The results shown in Fig. 2A demonstrate the presence of a protein band in the pellet fraction corre-

sponding to the mobility of apoA-I that was not present in the sample containing reconstituted HDL alone. Additional experiments showed that lipid-free purified human apoA-I did not bind amyloid fibrils under these conditions (Fig. 2A). Electron micrographs of the apoC-II amyloid fibril control pellet (Fig. 2B) and the pellet fraction for apoC-II fibrils sedimented in the presence of HDL (Fig. 2C) show particles both free in solution and bound to the fibrils when HDL is present. The average size of the particles associated with the fibrils was 10.8 nm, consistent with the size of intact HDL particles.

We investigated whether the binding of purified HDL to apoC-II fibrils produced a change in the rate of fibril sedimentation (Fig. 3). HDL causes a concentration-dependent increase in the average sedimentation rate of

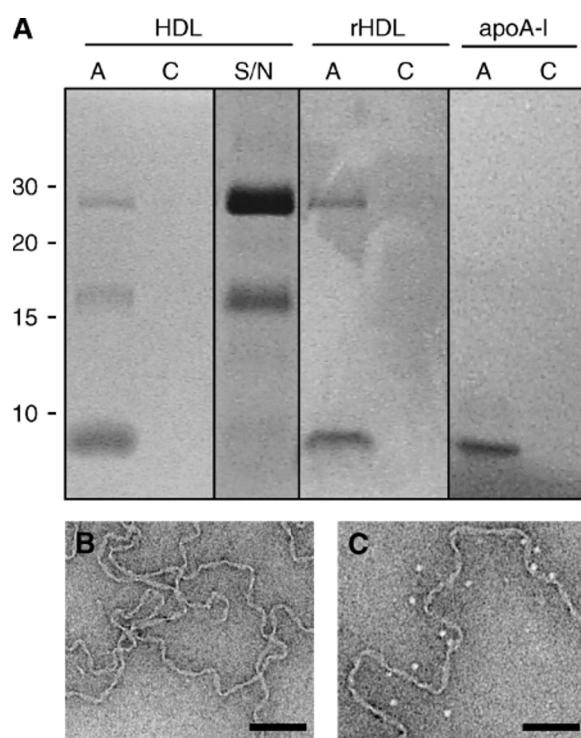


Fig. 2. Interaction of apoC-II amyloid fibrils with HDL and reconstituted apoA-I lipid particles (rHDL). A: Samples were centrifuged through 20% sucrose for 10 min at 355,000 *g*, analyzed by 16% Tris-Tricine SDS-PAGE, and stained with Coomassie Brilliant Blue. The positions of molecular mass makers in kDa are shown. Lanes labeled HDL: Pellet fractions of apoC-II amyloid fibrils centrifuged in the presence of human HDL (50 µg/ml) (A) and the control human HDL sample (50 µg/ml) (C) sedimented alone. The supernatant fraction for the human HDL and apoC-II sample is shown (S/N). Lanes labeled rHDL: Pellet fractions of rHDL (50 µg/ml) centrifuged in the presence of apoC-II amyloid fibrils (A) and the control (C) rHDL sedimented alone. Lanes labeled apoA-I: Pellet fractions of human apoA-I (40 µg/ml) centrifuged in the presence of apoC-II amyloid fibrils (A) and the control (C) apoA-I sedimented alone. B: Transmission electron micrograph of apoC-II amyloid fibrils (0.4 mg/ml; 100 mM sodium phosphate, pH 7.4, 0.1% NaN₃) centrifuged alone. C: Transmission electron micrograph of apoC-II amyloid fibrils centrifuged in the presence of human HDL (50 µg/ml). Bars = 100 nm.

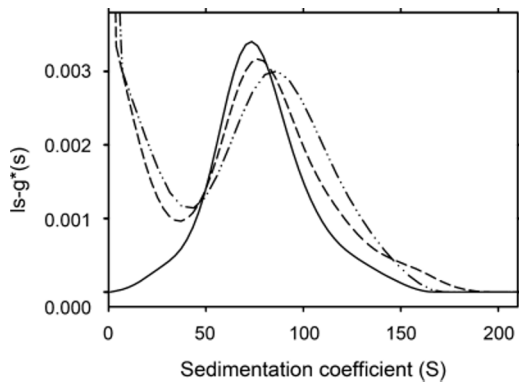


Fig. 3. Sedimentation velocity analysis of apoC-II amyloid fibrils. ApoC-II amyloid fibrils and HDL were centrifuged at 4,000 *g* in an analytical ultracentrifuge. Continuous sedimentation coefficient distributions [$ls\cdot g^*(s)$] are shown for apoC-II amyloid fibrils (solid line) and apoC-II amyloid fibrils in the presence of HDL at concentrations of 0.18 mg/ml (dashed line) and 0.32 mg/ml (dashed-dotted line).

apoC-II fibrils, as observed previously for apoE and SAP and attributed to fibril-fibril interactions and tangling (12).

DISCUSSION


This study examines the direct interaction of serum proteins with preformed amyloid fibrils. The results shown in Fig. 1 demonstrate that HDL is a major species in human serum that binds amyloid fibrils composed of A β and apoC-II. In contrast, serum proteins did not bind α -synuclein fibrils. α -Synuclein amyloid fibrils are deposited intracellularly, which may account for the difference in binding properties. Although the results shown in Fig. 1 indicate that HDL apolipoproteins are the principal low molecular weight serum proteins that bind A β and apoC-II amyloid fibrils, it is important to note that several unresolved high molecular weight species also bind. We cannot preclude the possibility that apoB, a high molecular weight protein marker of LDL and VLDL, is one of these proteins. An unexpected finding of this study was the lack of evidence for an interaction between SAP and either A β or apoC-II fibrils. SAP is a nonfibrillar component of amyloid deposits and is present in human serum at a concentration of ~ 40 μ g/ml (25). Western analysis also failed to detect the binding of SAP to the fibrils, although SAP was detected in serum (data not shown). Possible explanations for the lack of an interaction are that SAP is complexed to other components in serum or that HDL competes more efficiently for fibril binding.

The protein composition of HDL changes during inflammation (24), and the results shown in Fig. 1D indicate that these changes are reflected in the amyloid binding protein profiles. The identification of SAA as an amyloid binding protein suggests that the protein composition of HDL during inflammation could affect the metabolism of the amyloid fibrils. Moreover, these results suggest that the composition of HDL in serum determines the amyloid

binding protein profile. This implies that HDL binds as a single entity rather than as the binding of individual components. Phospholipid assays demonstrate the presence of lipid in the pellet fractions for apoC-II amyloid serum spins, supporting the conclusion that whole HDL particles are associated with amyloid fibrils, not just the protein components. We cannot discount the possibility that there is some disruption of the HDL particles on association with the amyloid fibrils. However, the presence of such an extensive list of HDL protein components in the pellet fraction and the electron micrographs of pellet fractions showing particles corresponding to the expected size of HDL (10 nm in diameter) suggest that relatively intact HDL particles sediment with amyloid fibrils. The question arises of what determines the recognition of amyloid fibrils by HDL. Our results show that reconstituted apoA-I lipid particles, but not lipid-free apoA-I, bind amyloid fibrils, indicating either that lipid bound apoA-I or lipid is sufficient to mediate this binding.

Previous studies have demonstrated interactions between HDL components and soluble amyloidogenic proteins. Soluble A β peptide interacts with HDL via apoA-I, apoA-II, apoE, and apoJ (26), whereas soluble transthyretin, another amyloidogenic protein, interacts with HDL via apoA-I (27). Immobilized A β peptide binds clusterin (apoJ) from plasma and cerebrospinal fluid (28), and soluble A β in human serum associates with HDL and VLDL lipoprotein particles (29). Purified apoA-I interacts with the A β peptide and inhibits fibril formation (30). The ability of HDL components to interact with monomeric and fibrillar proteins may control the kinetics of formation and equilibrium between the soluble and fibrillar disease forms of amyloidogenic proteins.

HDL has been widely studied as a negative risk factor for heart disease. Most attention has focused on the proposed role of HDL in reverse cholesterol transport (31). Support for this role is provided by studies with transgenic animals, in which a loss of the HDL receptor responsible for reverse cholesterol transport, scavenger receptor class B type I, leads to increased atherosclerosis (32). However, despite extensive studies, it has not been possible to unequivocally demonstrate a link between reverse cholesterol transport and the development of heart disease. Recent studies demonstrate that HDL therapy using HDL and reconstituted HDL can limit the progression of atherosclerosis in both animal and human trials (33–35). This study identifies a novel function for HDL: the specific binding of HDL to amyloid fibrils composed of apoC-II and A β . Recently, it was shown that amyloid fibrils composed of A β or apoC-II interact with the CD36 receptor of macrophages (18, 36) and promote cell signaling pathways, an early event in the conversion of macrophages to foam cells and the development of atherosclerosis. Lipoproteins are widely observed in atherosclerotic plaques, and apoA-I, apoB, and apoE are part of the response-to-retention hypothesis, which proposes that retained plasma lipoproteins promote foam cell formation (37). The binding of HDL to amyloid fibrils may inhibit macrophage activation via the CD36 receptor, leading to protection from

foam cell formation and heart disease. This ability of HDL to bind to amyloid fibrils may also influence the rate of formation, stability, and tangling of amyloid fibrils. Therefore, these effects may contribute to the well-established protective role of HDL in the development of heart disease. 

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