The HDL proteome: a marker–and perhaps mediator–of coronary artery disease

Jay W. Heinecke[1]

Department of Medicine, University of Washington, Seattle, WA 98195

Abstract   One important cardioprotective function of HDL is to remove cholesterol from lipid-laden macrophages in the artery wall. HDL also exerts anti-inflammatory effects that might inhibit atherogenesis. However, HDL has been proposed to be dysfunctional in humans with established coronary artery disease (CAD), though the underlying mechanisms are unclear. Therefore, we used mass spectrometry to investigate the roles of HDL proteins in inflammation and cardiovascular disease. Shotgun proteomic analysis identified multiple complement regulatory proteins, protease inhibitors, and acute-phase response proteins in HDL, strongly implicating the lipoprotein in inflammation and the innate immune system. Moreover, mass spectrometry and biochemical analyses demonstrated that HDL$_3$ from subjects with clinically significant CAD was selectively enriched in apolipoprotein E, suggesting that it carries a distinctive protein cargo in humans with atherosclerosis. HDL from CAD subjects also contained markedly elevated levels of chlorotyrosine and nitrotyrosine, two characteristic products of myeloperoxidase, indicating that oxidative damage might generate dysfunctional HDL. Aggressive lipid therapy with a statin and niacin remodeled the HDL proteome to resemble that of apparently healthy subjects.▮ Collectively, our observations indicate that quantifying the HDL proteome by mass spectrometry should help identify novel anti-inflammatory and cardioprotective actions of HDL and provide insights into lipid therapy.—Heinecke, J. W. **The HDL proteome: a marker–and perhaps mediator–of coronary artery disease.** *J. Lipid Res.* 2009. **50:** S167–S171.

HDL and LDL are the major carriers of cholesterol in human blood (1–3). Epidemiological, genetic, and clinical studies demonstrate that elevated levels of LDL or low levels of HDL are important risk factors for coronary artery disease (CAD). In striking contrast, high levels of HDL are cardioprotective. LDL promotes heart disease by delivering cholesterol to macrophages, a key early event in atherogenesis (1–3). HDL protects against atherosclerosis by removing cholesterol from artery wall macrophages by a process termed reverse cholesterol transport.

HDL exhibits other biological activities that may contribute to its anti-atherogenic properties, such as the ability to inhibit inflammation (4). It has been proposed that its cardioprotective effects depend on the types of particles generated in vivo and that HDL in humans with established CAD is dysfunctional (4). Indeed, animal studies convincingly demonstrate that changes in proteins involved in HDL metabolism can promote atherosclerosis, even when plasma levels of HDL-cholesterol are elevated (5, 6). Moreover, in vitro studies demonstrate that oxidative damage impairs the ability of apolipoprotein A-I (apoA-I), the major HDL protein, to remove cholesterol from macrophages (7).

One possible contributor to oxidative damage to HDL is myeloperoxidase (7). This heme protein, which is expressed by macrophages in human atherosclerotic lesions (8), generates an array of reactive oxygen and nitrogen species in vitro. Moreover, quantification by isotope dilution gas chromatography-mass spectrometry of oxidized amino acids derived from the lipoprotein has implicated myeloperoxidase in HDL oxidation in vivo (7). For example, circulating HDL isolated from humans with established CAD contains markedly elevated levels of chlorotyrosine and nitrotyrosine, two characteristic products of myeloperoxidase, suggesting that oxidative damage by the enzyme helps generate dysfunctional HDL (7, 8).

During inflammation, HDL also acquires proteins that may either protect or harm the artery wall (9, 10). For example, it becomes enriched in amyloid A, which can induce it to bind to proteoglycans in the artery wall, where it can be converted into an atherogenic form (11). Chronic inflammation, as monitored by blood proteins such as C-reactive protein, is associated with an increased risk of CAD (11). Thus, oxidative and nonoxidative pathways associated with inflammation could render HDL dysfunctional in vivo.

MASS SPECTROMETRY IS A POWERFUL TOOL FOR INVESTIGATING THE PROTEIN COMPOSITION OF HDL

HDL is a circulating complex of lipids and proteins that was originally defined on the basis of its density on ultra-centrifugation (12). This approach tends to equate buoyant density with functional significance.

By contrast, Alaupovic (13) proposed more than 30 years ago that HDL is a family of distinct particles that contain apoA-I but also vary in protein composition. This concept has been confirmed by the isolation and characterization of subspecies of HDL particles (14–17). For example, immuno-affinity isolation has identified two broad families of particles, one containing only apoA-I and the other containing both apoA-I and apoA-II (15).

HDL subspecies are thought to be distinct metabolic entities with numerous functions, including lipid transport and promotion of cholesterol efflux from macrophages and other peripheral cells. However, HDL also has antioxidant and antiparasitic activities (4, 16). The proteins it contains with potential antioxidant properties include paraoxonase-1, lecithin-cholesterol acyltransferase, and lipoprotein-phospholipase A$_2$, and alterations to those proteins have been proposed to impair its cardioprotective function (4).

We hypothesized that quantifying HDL's protein composition might provide insights into its antiatherogenic and anti-inflammatory properties. We therefore used shotgun proteomics, tandem mass spectrometry (MS/MS) analysis of a complex mixture of proteins (18, 19), to study the HDL proteome. In this approach, HDL is digested with trypsin, and the resulting peptides are separate by liquid chromatography and then analyzed with MS and MS/MS (20).

In the first step, the mass of intact precursor peptides is determined by MS. In the second, precursor ions are isolated from all other peptide ions and fragmented by collision-induced dissociation, which generates a set of C-terminal and N-terminal peptide ions. The masses of these product ions (i.e., the MS/MS spectrum) are searched against a protein database to identify the parent protein that contains the specific peptides that would generate the observed MS/MS spectrum (19). The MS/MS spectra can also be used to deduce peptide sequence and to identify site-specific modifications.

Using gel electrophoresis with MS/MS analysis, other investigators have identified 12 proteins associated with HDL$_3$ (the dense fraction of HDL) and 14 proteins with total HDL (21). We identified all of those proteins, as well as many others (see below), suggesting that liquid chromatography MS/MS methods are more sensitive than gel-based proteomic methods for detecting HDL-associated proteins.

HDL CARRIES PROTEIN FAMILIES THAT REGULATE COMPLEMENT ACTIVATION AND PROTEOLYSIS

Using shotgun proteomics, we identified 48 proteins in HDL isolated by ultracentrifugation from healthy controls and/or CAD subjects (20). They included 22 of 23 known HDL proteins with well-characterized roles in lipid metabolism, which validates our experimental approach. Importantly, we found 13 proteins not previously known to reside in HDL.

We used annotations by the Gene Ontology Consortium to connect the complex array of proteins we identified in HDL to biological processes (**Fig. 1**). Surprisingly, acute-phase response proteins (23 of 48), whose plasma concentrations are altered markedly by acute inflammation (20), outnumbered proteins implicated in lipid metabolism. Several proteins not previously known to reside in HDL, including complement factors C4A/C4B and C9 as well as the complement regulatory protein vitronectin, were identified. Vitronectin is an extracellular matrix protein, which raises the possibility that certain HDL components can be derived from noncellular sources or cells distinct from those that synthesize apoA-I in the liver and intestine. The detection of multiple proteins with roles in complement activation, together with pioneering studies of HDL protein complexes that kill protozoa (16), is consistent with the suggestion that HDL serves as a platform for the assembly of proteins involved in the innate immune response.

Given that proteolysis of structural proteins in atherosclerotic lesions is thought to play a critical role in plaque rupture, the major cause of myocardial infarction and sudden death in subjects with CAD (22), it is noteworthy that we found a family of proteins in HDL that contain serine proteinase inhibitor domains. Serine protease inhibitors, termed serpins, are key regulators of numerous biological pathways involved in inflammation, coagulation, angiogenesis, and matrix degradation. Moreover, we also detected the thiol proteinase inhibitor kininogen-1 and haptoglobin-related protein, which contains a crippled catalytic triad residue that may allow it to act as a decoy substrate to prevent proteolysis. These observations suggest that HDL plays a previously unsuspected role in preventing plaque rupture, perhaps by protecting vascular lesions from promiscuous proteolysis.

Our detection of multiple complement regulatory proteins in HDL (20) is also noteworthy. Complement activation helps mediate tissue damage in animal models of acute myocardial infarction (23). HDL also blocks the assembly of the terminal complement attack complex on cultured endothelial cells (24). Inhibition of complement deposition by HDL may limit injury to cardiac cells and prevent activation of the coagulant response in endothelium and platelets, two critical events in acute thrombosis.

HDL IS A FAMILY OF PARTICLES WITH DISTINCT PROTEIN CARGOES

Our studies of the HDL proteome (20), together with those of immunoaffinity-isolated HDL particles (14–16), strongly support the view that HDL is a mixture of particles that carry distinct protein cargoes. Many of these proteins are involved in lipid transport and metabolism. However, others may contribute to inflammation, complement acti-
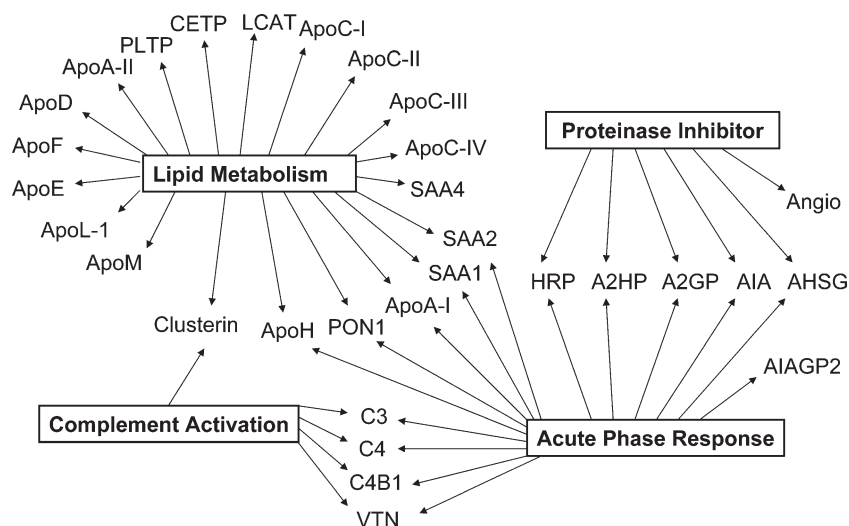
Fig. 1. Global view of biological processes and molecular functions of HDL proteins. Gene Ontology process annotations were used to associate HDL proteins with biological functions (20, 35). This approach demonstrated significant overrepresentation of proteins in several categories, including lipid metabolism ($P = 2 \times 10^{-27}$), the acute-phase response ($P = 1 \times 10^{-18}$), protease inhibitor activity ($P = 2 \times 10^{-6}$), and complement activation ($P = 5 \times 10^{-5}$). APOH, β-2-glycoprotein I; AGT, angiotensinogen; AHSG, α-2-HS-glycoprotein; AMP, bikunin; CETP, cholesteryl ester transfer protein; FBA, fibrinogen; HPR, haptoglobin-related protein; HPX, hemopexin; ITIH4, inter-α-trypsin inhibitor heavy chain H4; KNG1, kininogen-1; LCAT, lecithin-cholesterol acyltransferase; ORM2, α-1-acid glycoprotein 2; RBP4, retinol binding protein; SERA1, α-1-antitrypsin; SERF2, α-2-antiplasmin; SERF1, serpin peptidase inhibitor (clade F, member 1); TF, transferrin; TTR, transthyretin; VTN, vitronectin. Reproduced with permission from (20).

vation, and proteolysis. Some of the particles could be cardioprotective by inhibiting inflammation, removing toxic proteins and lipids from the artery wall, and delivering protective agents to arterial cells. Others could have deleterious effects, such as promoting cholesterol accumulation by arterial cells and inhibiting other cardioprotective pathways.

HDL OF HUMANS WITH ESTABLISHED CAD HAS A DISTINCT PROTEOMIC SIGNATURE

Our observations support the proposal that alterations in HDL's protein cargo might boost the formation of inflammatory particles (4, 9), raising the possibility that quantifying those proteins could provide insights into atherogenesis. To explore this idea, we used shotgun proteomics to compare the protein composition of HDL$_3$ isolated from control and CAD subjects (20). Importantly, the CAD subjects were newly diagnosed (though at least 3 months past any acute CAD event) and not on medications that alter lipid levels (see below). Using statistical approaches based on spectral counting [the number of MS/MS spectra unique to a protein (25, 26)] and random permutation analysis (20, 27), we found five proteins that were apparently enriched in HDL isolated from the CAD subjects. These proteins related to lipid metabolism (apoE, apoC-IV, and apoA-IV), oxidative stress (paraxonase-1), and the immune system (complement factor C3).

ApoA-IV, located in the apoA-I/apoC-III/apoA-IV gene cluster, inhibits atherosclerosis in mouse models of hypercholesterolemia (28). ApoE, apoC-IV, and C3 are expressed by macrophages (1, 29). C3 is needed to assemble the mem-

brane attack complex of the complement system, suggesting a link between HDL, macrophages, and innate immunity. Moreover, apoE and apoC-IV are part of a gene cluster that is upregulated in LXR-stimulated macrophages (30). A number of factors related to atherosclerosis might regulate the levels of these proteins in HDL, including their production by the liver, their expression in macrophages, and remodeling of HDL particles.

Our proteomic analyses suggested that apoE was enriched in HDL$_3$ isolated from CAD subjects (20). To confirm this observation, we measured apoE levels immunochemically in HDL$_3$ isolated from a second set of 64 subjects enrolled in a different study: 32 with established CAD and 32 age- and sex-matched controls. To control for differences in lipid metabolism, we matched the subjects for HDL-cholesterol and triglyceride levels (20). Levels of apoE were significantly higher in HDL$_3$ isolated from the CAD subjects. In striking contrast, the two groups had similar levels of apoA-I and apoA-II.

Our demonstration of elevated levels of apoE in HDL$_3$ isolated from two independent groups of subjects with established CAD raises the possibility that alteration in HDL's protein content serves as a marker, and perhaps a mediator, of CAD.

INTENSIVE LIPID THERAPY REMODELS THE HDL PROTEOME

We used MS to test the hypothesis that aggressive lipid-lowering therapy alters the HDL proteome in humans with established CAD (31). To quantify changes in abundance,

we used two complementary methods for quantifying proteins: spectral counting and extracted ion chromatograms. Spectral counting, the number of detected MS/MS spectra for a given protein, is based on the observation that tryptic peptides derived from proteins that are more abundant in a sample have a higher probability of being identified by MS/MS (25, 26). For extracted ion chromatograms, a peptide's ion current and charge state extracted from full scan mass spectra are used to construct a chromatogram (32). Relative abundance is monitored as the peptide's ion current.

This approach offers important technical advantages. First, both methods are label-free (i.e., do not require peptides or proteins to be labeled with mass tags or isotopes) and therefore are well suited to analyzing HDL under clinical conditions. Second, spectral counting is readily implemented with the data analysis systems used for MS/MS (25, 26). Third, extracted ion chromatograms estimate protein ratios more accurately than does spectral counting (32). Finally, it is possible to compare the extracted ion chromatogram ratios of multiple peptides detected from the same protein, which should increase confidence in the results.

HDL$_3$ was isolated from six newly diagnosed CAD subjects prior to and 1 year after combination therapy with niacin (a potent HDL-cholesterol elevating drug) and atorvastatin (which markedly lowers LDL-cholesterol levels). After digesting HDL proteins with trypsin, we analyzed the resulting peptide mixture by liquid chromatography-Fourier transform-mass spectrometry (31). Spectral counting was used to initially identify proteins that appeared to be differentially expressed in HDL$_3$ before and during therapy. To confirm these observations, we quantified extracted ion chromatograms derived from multiple peptides for each candidate protein.

This approach initially identified three HDL$_3$ proteins whose relative abundance appeared to change significantly as a result of treatment: apoE, apoF, and phospholipid transfer protein (PLTP) (31). The combination lipid therapy lowered levels of apoE; by contrast, it boosted levels of apoF and PLTP. Spectral counting also detected trends with borderline significance toward lower apoC-II and higher apoJ levels in the treated subjects. It is important to note that proteins with low spectral counts (e.g., apoF, PLTP, and apoJ) exhibit greater relative variability (27).

We took advantage of the greater precision of extracted ion chromatography to confirm our observations (32), finding that therapy with atorvastatin and niacin significantly lowered apoE and increased apoJ, apoF, and PLTP levels in HDL$_3$ isolated from CAD subjects (31). Although niacin increases levels of HDL-cholesterol and apoA-I in plasma, we failed to observe a significant change in the apoA-I content of HDL$_3$. This observation suggests that niacin increases the number of HDL particles but not the amount of apoA-I per particle. Alternatively, it may increase apoA-I levels in HDL species distinct from HDL$_3$.

To confirm that our MS techniques can quantify changes in the HDL proteome, we used two complementary approaches (31). First, we observed a strong linear correlation between apoE levels as assessed by extracted ion

chromatograms and nephelometry in our initial studies. Second, we demonstrated biochemically that combination therapy with niacin and statin reduced levels of apoE in HDL$_3$ in an independent group of 18 subjects. The validity of our approach is further supported by the observation of decreased apoE levels in HDL during statin treatment of hypertriglyceridemic subjects (33) or hypertriglyceridemic subjects with type 2 diabetes mellitus (34).

It is noteworthy that we found elevated levels of apoE in HDL$_3$ isolated from subjects with established CAD (20) and that aggressive lipid therapy lowered apoE levels in HDL$_3$ (31). We also found that aggressive lipid therapy raised levels of apoJ, apoF, and PLTP, all of which were present at lower levels in CAD subjects than control subjects (31). Thus, combination therapy remodels the HDL$_3$ proteome to make it resemble that of apparently healthy subjects.

THE HDL PROTEOME: FUTURE DIRECTIONS

Our observations implicate HDL in inflammation and host defense mechanisms. They further suggest that its proteome could serve as a marker, and perhaps mediator, of cardiovascular disease in humans. Monitoring the HDL proteome may provide insights into the efficacy of lipid therapy and should help identify novel cardioprotective actions of HDL.

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