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The Protein Cargo of HDL: Implications for Vascular Wall Biology and Therapeutics

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HDL is proposed to inhibit atherosclerosis by a number of different pathways, including promotion of reverse cholesterol transport and inhibition of inflammation. However, both mouse and human studies suggest that quantifying HDL-cholesterol levels provide limited information about HDL's cardioprotective effects. This article briefly reviews our current thinking about the functional and cardioprotective effects of HDL and the role of the HDL proteome in these processes.

Clinical and epidemiological studies consistently demonstrate a strong, inverse association of HDL cholesterol (HDL-C) level with CAD risk (1,2). Moreover, hypercholesterolemic mice with genetically engineered deficiencies in proteins implicated in HDL metabolism have strong atherosclerotic phenotypes (3), providing compelling evidence that HDL plays an important role in atherogenesis. These observations have triggered intense interest in targeting HDL levels for therapeutic intervention.

However, several recent lines of evidence weaken the hypotheses that HDL-cholesterol (HDL-C) levels relate directly to CAD status and that elevating HDL-C is necessarily therapeutic. For example, several genetic variations that affect HDL-C levels do not strongly associate with altered CAD risk (4). Also, certain drugs that elevate HDL-C levels, such as fibric acid derivatives, have shown no clear benefit in clinical populations, and treating CAD subjects with CETP inhibitor, torcetrapib, that increased HDL-C by ~75% but raised the incidence of CAD events (5). Moreover, genetically engineered deficiencies in proteins involved in specific aspects of murine HDL metabolism greatly increased both HDL-C levels and atherosclerosis (6).

These observations raise the possibility that HDL-C levels provide limited information about HDL's cardioprotective effects. Moreover, they suggest that quantifying HDL-C may be a poor way to explore HDL function. This article briefly reviews our current thinking about HDL's cardioprotective effects. It then discusses our studies of HDL's protein cargo and their implications for HDL biology and therapeutics.

HDL mediates cholesterol efflux from macrophages

Cholesterol-rich VLDL and LDL, the major apoB-containing lipoproteins, deliver cholesterol to macrophage foam cells, the cellular hallmark of atherosclerosis (7). HDL's cardioprotective effect is attributed in part to its ability to mobilize excess cholesterol from

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artery wall macrophages. Two pathways involve membrane ATP-binding cassette transporters (ABCA1 and ABCG1), which are highly induced when macrophages over-accumulate cholesterol (3,8). ABCA1 mediates the transport of cholesterol and phospholipids from cells to lipid-poor apolipoproteins. ABCG1 mediates the transport of cell cholesterol to lipidated HDL particles.

The ABCA1 and ABCG1 pathways prevent macrophages from hoarding cholesterol

Mouse and human studies have shown that defects in the apolipoprotein/ABCA1 pathway are important determinants of CAD (3,8). Ablating the *Abca1* gene in macrophages increases atherosclerotic lesions in atherosclerosis-susceptible mouse models (9,10), and overexpressing human ABCA1 in transgenic mice retards atherogenesis (11,12). The macrophage cholesterol export activity of ABCG1 predicts that this transporter should also be atheroprotective. However, studies of mice deficient in ABCG1 have yielded conflicting results. In contrast, when mice lack both ABCA1 and ABCG1, atherosclerosis is dramatically accelerated (13). Thus, the two cholesterol transporters may need to function together to block the disease.

HDL has potent anti-inflammatory effects in vivo

It has been proposed that HDL's cardioprotective effects depend in part on its ability to inhibit inflammation and that HDL in humans with established CAD is dysfunctional (14,15). Indeed, animal studies convincingly demonstrate that changes in proteins involved in HDL metabolism, such as SR-B1, can promote atherosclerosis even when plasma levels of HDL cholesterol levels are elevated (6).

Systemic inflammation has been proposed to convert HDL to a dysfunctional form that loses these anti-inflammatory and anti-atherogenic effects (14,15). Also, antioxidant and anti-inflammatory properties have been attributed to proteins such as paraoxonase that are cotransported with HDL in plasma. Importantly, mice deficient in paraoxonase are susceptible to atherosclerosis (16). Loss of anti-inflammatory proteins, perhaps in concert with gain of pro-inflammatory proteins, may thus be another key component in the dysfunctionality of HDL.

Mass spectrometry is a powerful proteomics tool

Proteomics—a global approach to understanding protein expression, regulation, and function—transcends analysis of individual components. One of its major tools, mass spectrometry, measures molecular mass and therefore can also detect and characterize posttranslational modifications of proteins (17).

Shotgun proteomics is a powerful mass spectrometric approach that can detect and quantify hundreds or even thousands of proteins in one sample (18). It uses liquid chromatography in concert with electrospray ionization and tandem mass spectrometric analysis (LC-ESI-MS/MS) to identify peptides in tryptic digests. To identify the proteins in the original mixture, the MS/MS spectrum collected for each peptide is searched against a database of all the theoretical spectra for a digest of the relevant genome.

Using shotgun proteomics, we analyzed the protein composition of HDL isolated by ultracentrifugation from blood of apparently healthy subjects and subjects with established CAD (19). This approach identified 48 proteins, including virtually every HDL protein previously implicated in lipid metabolism. This important control showed that we could identify the proteins known to be present in HDL. The only apolipoprotein (apo) we did not

identify was apoA-V, the least abundant apo in HDL—its concentration is only $\sim 1/1,000^{\text{th}}$ that of apoA-I, and the dynamic range of shotgun proteomics is generally ~ 100 .

HDL is rich in acute-phase response proteins and carries protein families that regulate complement activation and proteolysis

Our proteomic analyses of HDL produced several unanticipated observations (19). First, we identified more acute phase response proteins in HDL than proteins implicated in lipid metabolism. This finding supports the idea that HDL is involved in inflammation biology. Second, we found two protein families—protease inhibitors and regulators of complement activation—that were not previously known to reside in HDL. Protease inhibitors are of interest because acute thrombosis over a ruptured plaque—rather than progressive arterial narrowing—is the cause of most clinical events in humans with heart disease (20). Moreover, animal models suggest that proteolysis is a key event in promoting unstable plaque. Thus, the presence of protease inhibitors in HDL is consistent with the idea that proteolysis might be another anti-atherogenic mechanism. Complement activation is of interest because studies it may play a role in atherogenesis (21). Thus, abnormal HDL might activate the complement system in humans, promoting another key event in ischemic myocardium.

These observations support the notion that HDL is involved in the innate and acquired immune responses. Indeed, it is well established that a specific subspecies of HDL can kill parasitic trypanosomes in vitro, and that this process requires a complex of three HDL proteins: apoA-I, haptoglobin-related protein, and apoL (22). This may be relevant to human biology because a person became infected with a trypanosome species that normally is found only in cows (23). Importantly, this individual was deficient in apoL, one of the components of the tri-molecular killing complex, again suggesting that HDL might be an important component of the immune system.

Why do we have lipoproteins?

These observations raise a fundamental question: why do we have lipoproteins? One intriguing hypothesis boils it down to sex: that lipoproteins help us live long enough to reproduce. Over most of human evolution, two key factors—adequate nutrition and surviving infection—have been critical for passing genes from one generation to the next.

Current thinking on lipoproteins is driven by the “nutrient hypothesis,” which suggests that lipoproteins’ key biological function is to deliver nutrients. For example, apoB is centrally important in transporting triglycerides from the gut and the liver to adipose tissue for storage and to muscle for energy.

Many lines of evidence support the nutrient hypothesis for the apoB-containing lipoproteins, chylomicrons, VLDL, and LDL. For HDL, we favor an alternative hypothesis: that evolution gave HDL a key role in both eliminating excess cholesterol from extrahepatic tissues and perhaps of equal importance, a role in the immune system in fighting infection (Fig. 2). It is well established that HDL interacts with macrophages—key players in both the innate and adaptive immune systems. One intriguing idea is that HDL exists in part to help macrophages remove apoptotic cells at sites of tissue damage and infection. Under these conditions, the inflammatory state of macrophages must be modulated so the cells can deal with both infectious organisms and the burden of cholesterol in the dead cells they ingest.

Our notion is that macrophages in this setting donate proteins and lipids to HDL, ridding themselves of excess cholesterol. However, we suspect that HDL also donates lipids and

proteins to macrophages and that this dialog is important for regulating the cells' inflammatory state. A similar dialog may be important for controlling the proliferation of monocytes and their entry into tissue, as was elegantly demonstrated recently (24).

HDL of CAD subjects carries a distinct protein cargo

Using shotgun proteomics, we compared the protein composition of HDL isolated from control subjects and subjects with newly diagnosed CAD who were not yet on lipid-lowering therapy (19). We used spectral counting to demonstrate that HDL₃—the dense fraction of HDL—isolated from plasma of the CAD subjects had significantly higher concentrations of apoE, complement C3, apoC-IV, paraoxonase-1, and apoA-IV than HDL₃ from the controls. We confirmed these observations biochemically by measuring apoE levels in HDL₃ isolated from a different set of 64 subjects: 32 with established CAD and 32 age-matched controls. Levels of apoE were significantly higher in HDL₃ isolated from the CAD subjects. In striking contrast, the two groups had similar levels of apoA-I and apoA-II.

Importantly, Sacks and colleagues reported similar results in a large, prospective study called the CARE trial (25). The two strongest predictors of cardiac events in people with established CAD were elevated levels of apoC-III in apoB-containing lipoproteins and of apoE in HDL. Moreover, levels of these proteins were stronger predictors of CAD risk than levels of HDL-C or LDL-C. These observations suggest that HDL's composition may reveal quite a bit about cardiovascular disease risk and, perhaps, about disease pathogenesis, and that this information may be independent of HDL-C levels.

Rader, Rothblat, and colleagues recently showed that human serum HDL's ability to promote sterol efflux from cultured macrophages can vary markedly, despite similar levels of HDL-C and apoA-I (26). Thus, HDL-C level is not the only determinant of macrophage sterol efflux in this system. Those workers also demonstrated that the efflux capacity of serum HDL was a strong predictor of CAD status (27). Moreover, that association was independent of HDL-C and apoA-I levels. Taken together, these observations suggest that serum HDL's capacity to promote sterol efflux from macrophages in vitro reflects its functionality, raising the possibility that the assay provides insights into HDL biology and CAD risk that are distinct from the level of HDL-C.

Clinical implications and HDL's utility in predicting CAD risk

We propose that HDL is at the interface of many of the processes that are critical in atherogenesis, including lipid metabolism, inflammation, and macrophage biology (Fig. 3). This idea raises the possibility that HDL may play a wider role in inflammation biology than has previously been appreciated. In this scenario, HDL is cardioprotective by a number of different pathways, including promotion of reverse cholesterol transport and inhibition of inflammation.

A major unresolved issue is whether dysfunctional HDL is relevant to human biology. There is strong evidence for this hypothesis in mouse models of atherosclerosis, but much less is known in humans. However, the results of the first CETP inhibitor trial are consistent with the idea that certain forms of HDL may not be cardioprotective and may even be atherogenic (28). However, the inhibitor used in these trials clearly had off-target effects. Studies of other CETP inhibitors that lack these side effects are ongoing, and it will be of great interest to determine whether they lower CAD risk.

Finally, both genetic studies in mice and proteomic and functional analyses of human HDL suggest that quantifying HDL-C may be a poor way to explore HDL's functions and that HDL-C levels provide limited information about the lipoprotein's cardioprotective effects.

The demonstration that HDL₃ of humans with established CAD is enriched in apoE and that apoE levels in HDL predict future clinical events in humans with established CAD raises the possibility that quantifying levels of proteins in HDL can improve the assessment of CAD risk (19,25). Moreover, these proteins may play important roles in disease pathogenesis, perhaps by modulating HDL's anti-inflammatory and sterol-modulating properties. It clearly would be of great interest to develop new biochemical and functional HDL assays that could broaden our understanding of the factors that are important in the pathogenesis—and perhaps treatment—of atherosclerotic vascular disease.

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Abbreviations

apo	apolipoprotein
CAD	coronary artery disease
HDL	high density lipoprotein
LC-ESI-MS/MS	liquid chromatography-electrospray ionization tandem MS analysis
LDL	low density lipoprotein
MS	mass spectrometry

Figure 1. Global view of biological and molecular functions of HDL's protein cargo

We used Gene Ontology annotations to associate HDL proteins identified in our shotgun proteomic studies (REF) with biological functions. This approach demonstrated significant overrepresentation of proteins in 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 ($P=5\times 10^{-5}$). Abbreviations: APOH, β -2-glycoprotein I; AGT, angiotensinogen; AHSG, α -2-HS-glycoprotein; AMP, bikunin; CETP, cholesteryl ester transfer protein; FGA, fibrinogen; HPR, haptoglobin-related protein; HPX, hemopexin; ITIH4, inter-alpha-trypsin inhibitor heavy chain H4; KNG1, kininogen-1; LCAT, lecithin-cholesterol acyltransferase; ORM2, α -1-acid glycoprotein 2; PLTP, phospholipid transfer protein; 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 Ref. ¹⁹.

Figure 2. Proposed role of HDL in tissue repair and the immune system
TG, triglyceride; CE, cholesteryl ester; FC, free cholesterol.

Figure 3. HDL resides at the interface between lipid metabolism, inflammation, and macrophage biology