Function and Distribution of Apolipoprotein A1 in The Artery Wall Are Markedly Distinct From Those in Plasma

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The poor performance of several recent clinical trials targeting elevation of high-density lipoprotein (HDL) cholesterol and the recent Mendelian genetic studies questioning a causal link between genetic variants controlling HDL cholesterol levels and cardiovascular disease risk argue for a reappraisal of our understanding of HDL. Such a reappraisal demands that we question assumptions about the pathobiology of the lipoprotein, particularly in cases in which direct investigation is lacking. Much of what is known biologically about apolipoprotein A1 (apoA1) in human studies comes from investigations using isolated lipoprotein particles from the circulation (plasma or serum) with the use of buoyant density ultracentrifugation. As a known exchangeable lipoprotein, it is widely recognized that the vast majority of apoA1 within the circulation resides on spherical HDL particles, where it serves as the major structural protein of a complex macromolecular assembly of lipoprotein particles with defined buoyant density (1.063 ≤ d ≤ 1.21). An unproven assumption is that the numerous biological functions observed with HDL or apoA1 recovered from the circulation will mirror what occurs elsewhere in vivo.

**Background**—Prior studies show that apolipoprotein A1 (apoA1) recovered from human atherosclerotic lesions is highly oxidized. Ex vivo oxidation of apoA1 or high-density lipoprotein (HDL) cross-links apoA1 and impairs lipid binding, cholesterol efflux, and lecithin-cholesterol acyltransferase activities of the lipoprotein. Remarkably, no studies to date directly quantify either the function or HDL particle distribution of apoA1 recovered from the human artery wall.

**Methods and Results**—A monoclonal antibody (10G1.5) was developed that equally recognizes lipid-free and HDL-associated apoA1 in both native and oxidized forms. Examination of homogenates of atherosclerotic plaque–laden aorta showed >100-fold enrichment of apoA1 compared with normal aorta (P<0.001). Surprisingly, buoyant density fractionation revealed that only a minority (<3% of total) of apoA1 recovered from either lesions or normal aorta resides within an HDL-like particle (1.063 ≤ d ≤ 1.21). In contrast, the majority (>90%) of apoA1 within aortic tissue (normal and lesions) was recovered within the lipoprotein-depleted fraction (d>1.21). Moreover, both lesion and normal artery wall apoA1 are highly cross-linked (50% to 70% of total), and functional characterization of apoA1 quantitatively recovered from aorta with the use of monoclonal antibody 10G1.5 showed ≈80% lower cholesterol efflux activity and ≈90% lower lecithin-cholesterol acyltransferase activity relative to circulating apoA1.

**Conclusions**—The function and distribution of apoA1 in human aorta are quite distinct from those found in plasma. The lipoprotein is markedly enriched within atherosclerotic plaque, predominantly lipid-poor, not associated with HDL, extensively oxidatively cross-linked, and functionally impaired.

**Key Words:** apolipoproteins ■ arteriosclerosis ■ cardiovascular diseases ■ plaque, atherosclerotic
recovered from human atherosclerotic arterial lesions was selectively targeted for oxidative modification by myeloperoxidase-generated and NO-derived oxidants and that oxidative modification of apoA1 and HDL ex vivo to a comparable extent resulted in loss of cholesterol efflux activity of the lipoprotein. Parallel functional characterization and mass spectrometry studies of circulating HDL isolated by buoyant density ultracentrifugation revealed that higher apoA1 content of oxidative modifications specifically formed by myeloperoxidase- and NO-derived oxidants was associated with impairment in plasma membrane transporter ATP-binding cassette A1 (ABCA1)-dependent cholesterol efflux function of the lipoprotein, lecithin-cholesterol acyltransferase (LCAT) activity, and acquisition of proinflammatory activity. Similar findings have been replicated by other groups, and numerous additional proteomics studies have since mapped site-specific oxidative modifications to apoA1 recovered from the human artery wall. These studies collectively reveal that apoA1 is extensively oxidatively modified within an atherosclerotic-laden artery wall, and similar oxidative modifications to the lipoprotein ex vivo are associated with proatherogenic changes in apoA1 function. However, no studies to date have directly examined the functional properties or the particle distribution of apoA1 recovered from human artery wall. The paucity of direct functional characterization studies is likely a result of the significant challenges that exist in obtaining sufficient quantities of fresh human arterial tissue for such biochemical and biological studies.

Herein we sought to examine both the distribution and the functional properties of apoA1 recovered from the human artery wall. The present studies demonstrate multiple remarkable findings, including direct evidence that the biological function and HDL particle distribution of apoA1 within both normal and atherosclerosis-laden human aortic tissues are markedly distinct from those of circulating apoA1 and HDL. These studies suggest that the historical focus thus far on circulating HDL cholesterol levels may not adequately reflect the actual situation with regard to apoA1 function and HDL particle distribution within the artery wall.

Materials and Methods

Materials
D.O was purchased from Cambridge Isotopes, Inc (Andover, MA). Chelex-100 resin, fatty acid–free bovine serum albumin, and crystalline catalase (from bovine liver; thymol-free) were purchased from Fisher Chemical Company (Pittsburgh, PA). Commercial apoA1 antibodies were from Abcam (Cambridge, MA), Santa Cruz Biotechnologies (South San Francisco, CA), and Genway/Sigma (St Louis, MO). 1,2-Dimyristoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxydazol-4-yl) (NBD-PE) was purchased from Avanti Polar Lipids. All other materials were purchased from Sigma Chemical Company (St Louis, MO) except where indicated.

Methods

General Procedures
Circulating HDL and plasma-derived apoA1 (purified) were obtained from healthy volunteer donors who gave written informed consent, and the institutional review board of the Cleveland Clinic approved the study protocol. Mouse studies involving monoclonal antibody (mAb) generation were performed under protocols approved by the Institutional Animal Care and Use Committee at the Cleveland Clinic. Lipoproteins, including HDL and HDL-like particles (1.063<d<1.21) from plasma and tissue homogenates, respectively, were isolated by sequential buoyant density ultracentrifugation at low salt concentrations with the use of D2O/sucrose. Protein concentrations were determined by Markwell modified protein assay with bovine serum albumin as standard. Human apoA1 used as control for cholesterol efflux and LCAT activity assays was purified as described. Reconstituted HDL (rHDL) from isolated apoA1 was prepared by the cholate dialysis method with the use of a molar ratio of apoA1:1-palmitol-2-oleyl-sn-glycerol-3-phosphocholine:cholesterol of 1:100:10. HDL particles were further purified by gel filtration chromatography with the use of a Sephacryl S300 column (GE Healthcare, Waukesha, WI) on a Bio-Rad BioFloc FPLC (Bio-Rad, Hercules, CA). Myeloperoxidase (donor: hydrogen peroxide, oxidoreductase, EC 1.11.1.7) was isolated (final A430/A280 ratio of 0.6) as described, and its concentration was determined spectrophotometrically (ε430=170 mmol/L·cm−1) as described. H2O2 concentrations and –OCI concentrations were each determined spectrophotometrically (ε430=39.4 mmol/L·cm−1 and ε238=350 mmol/L·cm−1, respectively) before use. Peroxynitrite (ONOO−) was purchased from Cayman Chemicals (Ann Arbor, MI) and quantified spectrophotometrically before use (ε238=1.36 mmol/L·cm−1). All buffers were passed through a Chelex-100 column and supplemented with 100 μmol/L diethylenetriaminepentaacetic acid (DTPA) to remove any trace levels of redox-active metals. All glassware used was rinsed with 100 μmol/L DTPA, pH 7.4, and then Chelex-100–treated distilled deionized H2O and baked at 500°C before use. SDS-PAGE was performed as described.

Tissue Collection
Fresh surgical specimens of human aortic tissue were obtained as discarded material both at time of organ harvest from transplant donors and during valve/aortic arch (“elephant trunk”) replacement surgery. Tissue was immediately rinsed in ice-cold normal saline until free of visible blood, submerged in argon-sparged 65 mmol/L sodium phosphate buffer (pH 7.4) supplemented with 100 μmol/L DTPA and 100 μmol/L butylated hydroxytoluene, and stored at −80°C in screw-cap specimen containers in which head space was purged with argon. Batyalted hydroxytoluene was omitted from buffer in specimens in which apoA1 was isolated for functional activity assays.

mAb 10G1.5 Generation, Specificity, and Labeling
Hybridoma cell lines were generated by immunizing apoA1−/− mice with purified delipidated human apoA1 isolated from HDL recovered from healthy donors. Among the positive clones, subclones were screened until a mAb with desired binding specificity for equal recognition of all forms of apoA1 (see below) was identified. The sub-clone, mAb 10G1.5, was selected by screening for equal recognition of lipid-free and lipiddated (in rHDL) apoA1 under native conditions, as well as after oxidation by exposure to multiple different systems including myeloperoxidase/H2O2/Cls−, myeloperoxidase/H2O2/N02−, and CuSO4 (oxidized as outlined below). To produce sufficient levels of 10G1.5 for immunoaffinity purification of apoA1 from arterial tissues, hybridoma clones were injected into pristane-treated male BALB/c mice (8 weeks of age). Ascites fluid was collected, precipitated with ammonium sulfate, then bound and eluted from a protein A/G column (Thermo Scientific Pierce, Rockford, IL) to purify mouse mAbs. Isotypes of the mAbs were determined with the use of a mouse mAb isotyping kit (catalog No. 26179, Pierce Rapid Antibody Isotyping Strips plus Kappa and Lambda–Mouse, Thermo Scientific Pierce, Rockford, IL).

Specificity of mAb 10G1.5 was tested with the use of apoA1 or rHDL either in native form or after incubation at 37°C in 60 mmol/L Na[PO4] buffer (pH 7.4) with multiple different oxidation systems. The different myeloperoxidase systems consisted of 19 mmol/L myeloperoxidase, 100 μmol/L DTPA, 40 μmol/L H2O2, and either 100 mmol/L NaCl or 1 mmol/L KBr or 1 mmol/L NaN3 as indicated. Horseradish peroxidase (19 mmol/L) was used with 40 μmol/L H2O2. ApoA1 and rHDL were exposed to myeloperoxidase and horseradish peroxidase
for 90 minutes at 37°C, and the reactions were stopped by addition of 2 mmol/L methionine and 300 mmol/L catalase. All other oxidation reactions were performed for 24 hours at 37°C. Final concentrations of oxidants used were as follows: H₂O₂, 40 µmol/L; ONOO⁻, 40 µmol/L; ONOO⁻/HO.Cl, 40 µmol/L each; CuSO₄, 10 µmol/L; CuSO₄/H₂O₂, 10 and 40 µmol/L, respectively; FeCl₃, 10 µmol/L; and FeCl₃/H₂O₂, 10 and 40 µmol/L, respectively. ApoA1 or HDL (prepared from apoA1 or their various oxidized versions) was coated at 0.5 µg/mL into enzyme immunoassay plates and probed with 10 ng/mL anti-total apoA1 mAb 10G1.5 at room temperature for 1 hour. For Western blot analyses, mAb 10G1.5 was IRDye labeled (LI-COR Biosciences, Lincoln NE) with the use of the LI-COR IRDye 800CW high-molecular weight dye at a protein ratio at 4:1 and visualized by infrared imaging. The IRDye 800CW dye bears an N-hydroxysuccinimide ester reactive group that couples to free amino groups on the antibody, forming a stable conjugate with antibody. Coupling was performed according to the manufacturer’s instructions.

**Human ApoA1 Quantification**

Human apoA1 was quantified by a Food and Drug Administration–approved apoA1 immunoassay on the Abbott ARCHITECT ci8200 Integrated Analyzer System (Abbott Labs, Abbott Park, IL). All other apoA1 was quantitated by quantitative immunoblot analysis with the use of mAb 10G1.5 as the detector antibody, as determined against a standard curve of known purified apoA1 standard. Immunoactive bands were quantitated with the use of Image Studio software (version 2. LI-COR) or Image J (version 1.46; http://rsbweb.nih.gov/). All nascent HDL particles, as well as isolated human HDL2 and HDL3, were further purified by gel filtration chromatography with the use of a Sephacryl S300 column (GE Healthcare, Waukesha, WI) on a Bio-Rad Biologics DuoFlo FPLC.

**Aortic Tissue Homogenization**

Atherosclerotic lesions from aortic tissues were from subjects (n=20) with an average age of 83±3 years. Normal human aortic tissues were obtained from transplant donors (n=5) from the Cleveland Clinic and had an average age of 23±7 years. All tissue homogenization and lipoprotein fractionation procedures were performed within a cold room to ensure maintaining tissue and sample temperatures <4°C. Frozen tissue blocks (submerged in 65 mmol/L sodium phosphate buffer, pH 7.4, under argon, within screw-cap containers) were thawed by placing the containers in an ice/water bath. Immediately before complete thaw, ice-cold Ca²⁺- and Mg²⁺-free Chelex-100-treated PBS supplemented with 100 µmol/L DTPA, pH 7.4, was added to rinse the tissue 5 times to remove any residual blood from tissue. The aorta segment was cleaned of adventitial fat and again rinsed 3 times with ice-cold PBS supplemented with 100 µmol/L DTPA. Wet weight of the aorta was determined, and the tissue was cut into 3 times with ice-cold PBS supplemented with 100 µmol/L DTPA. The tissue was tied with silk threads to prevent tissue from floating during homogenization.

**Immunoaffinity Isolation of ApoA1 From Aortic Tissue Homogenate**

Immunoaffinity resin was generated by covalently coupling mAb 10G1.5 to AminoLink Plus (Pierce Chemical, Rockford, IL) resin at a density of 1.5 mg antibody per milliliter of resin in an amine-free buffer (PBS, pH 7.4) according to the manufacturer’s instruction. Reactive non–antibody-bound sites on the resin were blocked with the addition of excess ethanolamine. The affinity gel was drained, and antibody concentration in the flow-through was determined to calculate cross-linking efficiency, which was >90%. The gel was then rinsed extensively with 1 mol/L TRIS, pH 7.4, and 1 mol/L NaCl and then equilibrated in 1× PBS, pH 7.4, before use or storage (0.002% sodium azide was added if stored). Individual 1-time use affinity columns (1 mL, drained resin) were prepared with immobilized 10G1.5, and artery wall apoA1 was purified from individual samples of aortic homogenates under conditions that quantitatively recovered apoA1, as confirmed with the use of Western blot analyses of column fractions.

**HDL Imaging**

HDL was dual-labeled and incubated with mouse peritoneal macrophages to individually monitor the fate of phospholipid versus protein components of the particle as follows. Briefly, the protein component of isolated human HDL was first labeled with Alexa Fluor 633 cyanine dye (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. HDL lipid was next labeled by first forming a phospholipid film of NBD-PE by evaporation of a 1:4 methanol/chloroform solution overnight under vacuum and then rehydrating the film with Alexa Fluor 633–labeled HDL in prefiltered PBS and 4 cycles of alternating rounds of sonication at 0°C for 1 minute, followed by an 1-minute interval on ice. Dual-labeled HDL was centrifuge filtered and washed numerous times with PBS before incubation with macrophages. Thioglycolate-elicited peritoneal macrophages from C57Blk/6J mice were collected and cultured as described. Dual-labeled HDL (125 µg protein per milliliter) was incubated with cells at 37°C for 1 hour, and then images were captured on a Zeiss LSM 510 Meta confocal microscope.

**Proteomic Analyses**

To confirm that the protein recovered after immunoaffinity isolation (with the use of mAb 10G1.5) from normal aortic tissue homogenate was apoA1, the major SDS-PAGE gel bands at molecular weights 25 and 50 kDa were excised. The samples were first treated with dithiothreitol/iodoacetamide (Sigma, St Louis, MO) to carbamidomethylate any cysteine in the protein(s), and then proteins were digested with the use of Mass Spec grade trypsin (Promega, Madison, WI) at 37°C overnight. Tryptic peptides were loaded onto an IntegrA Frit sample trap (ProteaPep C18, 300 Å, 150 µm x 2.5 mm, New Objective, Woburn, MA) at 1 µL/min with 5% acetonitrile and 0.1% formic acid to desalt the samples. The peptides were subsequently eluted through a column (75 µm x15 cm) packed in-house with XperTek 218TP, C18, 300 Å pore size, 150 µm particle size (Cobert Associates, St Louis, MO) at 200 nL/min with the use of a Proxeon Easy-nLC II system (Thermo Scientific, Waltham, MA).
with a gradient of 5% to 65% acetonitrile, 0.1% formic acid over 120 minutes into a LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific, Waltham, MA). Peak lists were generated with the use of Proteome Discoverer 1.1 (Thermo Fischer Scientific, Waltham, MA). The resulting Unified Search Files (*.srf) were searched against the Uniprot FASTA of all apolipoproteins and also against a human protein database downloaded from the European Bioinformatic Institute (release: 2013_02). Modifications used for searches included carbamidomethylated cysteine (fixed), oxidized methionine and tryptophan (variable), 3-chlorotyrosine, and 3-nitrotyrosine (variable). Only strictly tryptic peptides with a maximum of 2 missed cleavage sites were allowed in the database searches. Monoisotopic precursor ions were searched with a tolerance of 100 ppm with 0.8 Da for the fragment ions on the data obtained from the hybrid LTQ-Orbitrap Velos mass spectrometer. Unidentified fragment ions in all fragmentation spectra were manually validated with the use of Protein Prospector (University of California, San Francisco).

**Statistical Analysis**

Nonparametric statistical methods were used to determine statistical differences attributable to sampling numbers. The Wilcoxon rank sum test was used for 2-group comparisons, and the Kruskal-Wallis test was used for multiple-group comparisons (>2 groups). In cases in which the Kruskal-Wallis test was performed for multiple-group comparisons and found to be significant (P<0.05), multiple-comparison procedures such as the Dunn test were used for pairwise comparison between groups and controls. The Wilcoxon rank sum test was also used for pairwise comparison. Where indicated, the 1-sample robust Hotelling T² test was used to determine statistical significance when enzymatic activity between the control group and experimental group was compared.

**Results**

**mAb 10G1.5 Recognizes ApoA1 Equally Well in its Lipid-Free or HDL-Associated, Native, and Oxidized Forms**

We initially sought to accurately quantify and immunospecificity isolate apoA1 from artery wall tissue homogenate (from both normal and atherosclerotic lesions). We reasoned that this would require a sufficiently tight binding antibody that demonstrated minimal recognition bias between lipidated versus nonlipidated forms of the lipoprotein, as well as oxidized versus nonoxidatively modified forms. Examination of every commercially available antibody we could find (both monoclonal and polyclonal) showed significant bias in recognizing one form or another (typically recognition of oxidized forms preferentially, and with inadequate affinity). Figure 1A illustrates the biases observed with 3 characteristic commercial antibodies (2 polyclonal and 1 mouse mAb). Despite equal mass of protein loaded into adjacent lanes from native versus oxidized apoA1, and HDL versus oxidized HDL, the commercial antibodies show varied intensity of staining (eg, commercial antibody 1 shows oxidized HDL>>oxidized apoA1>>apoA1 or HDL; commercial antibody 2 shows oxidized apoA1>>oxidized HDL>>apoA1 or HDL; and commercial antibody 3 shows oxidized apoA1 or oxidized HDL>>apoA1 or HDL). We therefore initially sought to develop a suitable antibody that met our strict apoA1 recognition criteria. Purified delipidated human apoA1 (isolated from plasma HDL) was injected into several apoA1−/− mice. After screening >5000 hybridoma clones for their ability to recognize apoA1 forms equally well, a small number (4) met our screening program requirements. One mAb, 10G1.5, was selected on the basis of specific activity of recognition by ELISA, immunoblot analysis, its ability to immunoprecipitate apoA1, and the growth characteristics of the hybridoma clone. Figure 1B illustrates that mAb 10G1.5 recognizes native apoA1 and apoA1 reconstituted into HDL particles equally well. Furthermore, mAb 10G1.5 recognizes apoA1 in native versus oxidized forms equivalently, with the use of a wide variety of oxidation schemes (Figure 1B). We further examined the ability of mAb 10G1.5 to quantify different concentrations of purified apoA1 (lipid-poor) versus equivalent amounts of...
The majority of apoA1 isolated from lesions is highly cross-linked and not HDL associated. The particle distribution of apoA1 within human atherosclerotic lesions has not been reported. We therefore homogenized human aortic atherosclerotic lesions (n=10 different subjects) and used sequential buoyant density ultracentrifugation to initially remove the VLDL/LDL-like fraction (d<1.063) and then recover both the HDL-like fraction (1.063≤d≤1.21) and the LPD fraction (density >1.21), as described in Methods. Samples were first examined on gradient (5% to 15%) SDS-PAGE separations with the use of Sypro Ruby Red protein staining, which shows minimal protein-to-protein differences in staining; equally stains lipoproteins, glycoproteins, and other difficult-to-stain proteins; and does not interfere with subsequent mass spectrometry analyses. Visual inspection showed a complex protein mixture, with an unknown band migrating at ≈27 kDa, the molecular weight of apoA1 (Figure 2A). Western analysis with anti-total apoA1 antibody (mAb 10G1.5) of a membrane containing transferred proteins from a parallel-run duplicate gel readily detected within lesion homogenates a band at the molecular weight of the apoA1 monomer (Figure 2B). Remarkably, the vast majority of apoA1 within the aortic lesion was observed to be present not within the HDL-like fraction but rather within the LPD fraction (Figure 2B). After substantial increase in exposure of the immunoblot, apoA1 was detected within the HDL-like particle fraction (Figure 2C). Also notable within the immunoblots were prominent slower migrating forms of immunoreactive apoA1-containing protein bands at molecular weights of ≈50, ≈75, and ≈100 kDa present in particular within the starting material (homogenate) and the LPD fraction (d>1.21) but noticeably diminished in the HDL-like particle fraction (1.063≤d≤1.21). The sizes of these slower migrating apoA1-immunoreactive bands are consistent with the sizes of oxidatively cross-linked dimeric and multimeric apoA1 forms.

Quantification of the distribution of protein and apoA1 forms recovered within homogenates from multiple distinct human atherosclerotic plaque–laden aorta (n=10) is shown in Figure 3. The majority (81.0±5.6%) of the total protein in the lesion homogenate was found within the LPD fraction, whereas the HDL-like fraction contained only 1.7±0.2% (Figure 3A). Quantitative analysis of the anti-total apoA1–specific immunoblots indicated that nearly all of the apoA1 isolated from lesions was lipid-poor and found within the LPD fraction (d>1.21), where 0.7±0.4 mg apoA1 per gram wet weight of lesion material was recovered (Figure 3B), corresponding to 92.4±4.1% of total apoA1 in the artery wall (Figure 3C). Surprisingly, only a nominal amount (<3%) of apoA1 within the artery wall (lesions) was recovered in the HDL-like particle fraction (Figure 3B and 3C).

Figure 2. Apolipoprotein A1 (apoA1) from human atherosclerotic lesions is not located on high-density lipoprotein (HDL)-like particles and is heavily cross-linked. Proteins in atherosclerotic lesion homogenate or after buoyant density ultracentrifugation fractionation into HDL-like particles and lipoprotein-depleted (LPD) fractions from the indicated density ranges were separated on 5% to 15% reducing SDS-PAGE gels. A, Sypro Ruby-stained gel of the indicated protein samples (10 µg) from homogenate and the indicated density ranges obtained from different atherosclerotic lesion tissue samples (n=5). B, Western blot membrane of a duplicate run gel as in A with 2.4, 0.4, and 2.4 µg of homogenate, HDL-like, and LPD fraction proteins, respectively, probed with anti-total apoA1 monoclonal antibody (mAb) 10G1.5. C, Overexposure of Western blot in B to show apoA1 in HDL-like fraction. Monomeric and dimeric apoA1 immunoreactive bands and molecular weight markers are indicated.
Atherosclerotic Plaque-Laden Aorta

**Figure 3.** Particle distribution of apolipoprotein A1 (apoA1) obtained from human atherosclerotic artery wall. A, Percentages of total protein in starting homogenate and in buoyant density ultracentrifugation fractions for low-density lipoprotein (LDL)-like, high-density lipoprotein (HDL)-like, and lipoprotein-depleted (LPD) fractions determined by bicinchoninic acid protein assay are indicated. B, ApoA1 (mg) recovered per gram of lesion tissue (wet weight) in the HDL-like and LPD fractions. C, Percent apoA1 total apoA1 from atherosclerotic lesion homogenate present in the HDL-like and LPD fractions. D, Cross-linked apoA1/total apoA1 within each fraction as a percentage was determined by quantitative Western blot analysis of apoA1 immunoreactive bands in Figure 2B and additional blots (not shown). Values were determined from samples (n=10); error bars represent SD. Mean values are indicated by a heavy horizontal line. Kruskal-Wallis test was used in **A** and **D** and found to be significant (P=0.0001 and P=0.0003, respectively). Dunn test was used to adjust for multiple comparisons, and Wilcoxon rank sum test was used in pairwise comparisons. Actual P values are listed when P<0.05.

Actually modestly underestimated because we know that under the conditions used, 15% to 20% of the total apoA1 remains unrecovered in the “pellet” from the initial tissue homogenate. This modest loss appeared acceptable because control studies with repeated homogenization of the pellet and fractionation of the recovered material revealed, within both crude homogenate and subsequent buoyant density isolated fractions, banding patterns and results that were virtually identical to the original homogenate and fractions on the basis of both protein staining and Western blot analyses (data not shown).

ApoA1 is Markedly Enriched in Lesions, and Normal Aortic Tissue ApoA1 Similarly Is Lipid-Poor and Highly Cross-Linked

Our initial studies focused on apoA1 within atherosclerotic lesions. However, given the surprising finding that virtually all apoA1 within aortic lesions was not on an HDL particle, and fully two thirds of all apoA1 within lesions was cross-linked, we decided to examine apoA1 within normal aortic tissue for comparison. Normal aortic tissue was obtained at time of organ harvest from transplant donors. For illustrative purposes, images of a typical normal aortic specimen and a typical atherosclerotic plaque–laden aortic specimen are shown in Figure 4A. Homogenates of normal aortic tissue (n=5) were prepared and fractionated by buoyant density ultracentrifugation as described in Methods. Fractionation of protein from normal artery and lesion homogenates on 5% to 15% gradient reducing SDS-PAGE gels stained with Sypro Ruby Red for protein revealed that although there are similarities in the protein banding pattern, the pattern is noticeably different in normal versus lesion homogenates (Figure 4B). Notably, immunoblot probing with apoA1-specific anti-total apoA1 (mAb 10G1.5) of parallel SDS-PAGE gels transferred to membranes showed that compared with lesion-derived homogenates, there is very little immunoreactive apoA1 in homogenates prepared from normal aortic tissue (Figure 4C). Because extremely low levels of apoA1 were observed within normal artery wall tissue, and especially the HDL-like fraction, an increased protein amount was loaded onto SDS-PAGE gels to permit visualization by Western blot and comparison of HDL-like and LPD fractions from the normal artery wall homogenate (Figure 4D). The majority of protein in the normal artery wall homogenate was found to be in the LPD fraction (Figure 1 in the online-only Data Supplement). As observed for aortic lesion apoA1, a significant portion of apoA1 was lipid-poor and recovered within the LPD fraction. Furthermore, there was a high degree of immunoreactive apoA1 forms in the normal artery wall that migrate at higher molecular weights, consistent with that of oxidatively cross-linked apoA1 dimer and higher multimeric forms, particularly within the LPD fraction (Figure 4D).

Quantification of apoA1-specific immunoblots (with volumes of starting homogenate and density cut fractions recovered taken into account) revealed that the total apoA1 recovered per gram of wet weight aortic tissue from normal artery wall was 120-fold less than that recovered from lesion-laden aorta (Figure 4E; note log scale for y axis). Remarkably, the distribution of apoA1 within normal artery resembled those found in the same fractions from atherosclerotic lesions, with only 3% of apoA1 being HDL associated and 92% of the total apoA1 present in the normal artery wall residing in the LPD fraction (Figure 4F). Similar to our observation within lesions, nearly half (43.4±13.9%) of the total apoA1 within the normal artery wall was cross-linked and was observed to reside not on the HDL-like particle (3.6±2.6%) but in the LPD fraction (61.4±23.0%; Figure 4G).

Because the levels of apoA1 in the normal artery wall–derived homogenates were so low, we wanted to verify that the bands detected on Western blots with the use of mAb 10G1.5 were in fact apoA1. We therefore immunopurified apoA1 from normal aortic tissue homogenates (n=5) using 10G1.5 (as described in Methods), and immunoaffinity isolated proteins were separated on a gradient nonreducing (5% to 15%) SDS-PAGE gel and stained for protein. The major protein bands observed corresponded to doublet bands.
migrating at ≈25 and ≈50 kDa (Figure II in the online-only Data Supplement). These were individually excised and digested with trypsin for mass spectrometry analyses, as described in Methods. Tandem mass spectrometry analyses of tryptic peptides for each excised band revealed apoA1 as the dominant protein within each, with 40% to 62% peptide coverage in each of the gel-excised bands (Tables I through IV in the online-only Data Supplement).

**Plasma ApoA1 Is Predominantly HDL Associated and Has Decreased ApoA1 Cross-Links in the HDL-like Fraction Compared With the LPD Fraction**

Given the surprising HDL particle distribution and cross-link prevalence of apoA1 found in the artery wall (Figures 2–4) and the known preponderance of apoA1 within the HDL fraction in plasma, we examined the distribution of apoA1 in plasma using the same anti-total apoA1 mAb (10G1.5). Plasma from normal healthy consenting donors was fractionated by sequential buoyant density ultracentrifugation as described in Methods. The indicated amounts of protein from the starting material plasma, HDL-rich fraction, and LPD fractions were run on gradient (5% to 15%) reducing SDS-PAGE gels and stained with Coomassie Blue (Figure 5A) or transferred for immunoblot analyses with anti-apoA1 mAb 10G1.5 to show apoA1 and cross-linked apoA1. E, ApoA1 (mg) recovered per gram of normal aortic tissue (wet weight) in the HDL-like and LPD fractions. F, ApoA1 to total apoA1 as a percentage from normal aortic tissue homogenate present in the HDL-like and LPD fractions. G, Cross-linked apoA1/total apoA1 within each fraction as a percentage was determined by quantitative Western blot analysis of apoA1 immunoreactive bands in C and D. Monomeric and dimeric apoA1 are indicated. Values were determined from samples of n=5 (G) and n=3 in (D) and additional blots (not shown); error bars represent SD. Mean values are indicated by a heavy horizontal line. Kruskal-Wallis test was used in G and found to be significant (P=0.006). Wilcoxon rank sum test was used in pairwise comparisons to determine statistical differences. Actual P values are listed when P<0.05.

**ApoA1 Isolated From Atherosclerotic Lesions Is Dysfunctional**

In a final series of experiments, apoA1 was isolated from additional atherosclerotic plaque–laden aortic tissues (n=10
Human plasma Western blot

Sample # 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
protein (ng): 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4

HDL fraction 1.063-1.21
LDL fraction >1.21
LPD fraction >1.21

Western blot

Probe: anti-total apoA1 mAb 10G1.5

Figure 5. Apolipoprotein A1 (apoA1) in plasma is on high-density lipoprotein (HDL) particles and behaves differently than artery wall–derived apoA1. Plasma from normal healthy human volunteers (n=5) was fractionated by buoyant density gradient centrifugation. A, Coomassie Blue–stained 15% to 5% SDS-PAGE gel of plasma, high-density lipoprotein (HDL), and lipoprotein-depleted (LPD) fraction proteins. B, Western blot analysis of proteins on membrane from a duplicate fractionated protein gel probed with anti-total apoA1 monoclonal antibody (mAb) 10G1.5 shows apoA1 predominantly in the HDL fraction and not cross-linked. C, Percentage of total protein recovered in starting material and in very-low-density lipoprotein (VLDL)-low-density lipoprotein (LDL), HDL-like, and LPD fractions. Quantitative analyses of B and overexposed B reveal percentage of apoA1 to total apoA1 present in the plasma HDL and LPD fractions in D and cross-linked apoA1 in plasma apoA1 and were determined with the use of the 1-sample robust Hotelling T² test.

Discussion

The present studies reveal multiple remarkable findings about apoA1 within the artery wall. First, the vast majority of apoA1 within both normal and atherosclerotic human arterial tissue, in contrast to within the circulation, is lipid-poor (ie, in the LPD fraction, d>1.21) and does not reside on an HDL-like (1.063≤d≤1.21) particle. Second, the content of apoA1 in atherosclerotic lesions is >100-fold higher than that observed within normal artery wall. Third, the majority of apoA1 within arterial tissues (both normal and atherosclerotic) is oxidatively cross-linked. Fourth, apoA1 within arterial tissues is dysfunctional, with ≥80% reduction in cholesterol acceptor activity and ≥90% reduction in capacity to activate LCAT. Fifth, the majority of oxidatively cross-linked apoA1 within the circulation is not HDL particle associated but rather resides within the LPD fraction (d>1.21). Collectively, the present studies thus argue that examination of total apoA1 and HDL function within the circulation may not adequately represent what is occurring within the artery wall, especially with respect to cholesterol acceptor and LCAT activities. Moreover, the overall strategy applied in the present studies may prove useful in the examination of other posttranslational modifications to apoA1, such as glycation, and site-specific oxidative modifications as antibodies become available. Finally, the present studies suggest that the common practice of isolating circulating HDL for study of its biological properties and discarding the lipid-poor (non-HDL associated) apoA1 may in fact be throwing out the very fraction (lipid-poor) that more closely

Figure 6. Functional characterization of lesion apolipoprotein A1 (apoA1). Macrophage cholesterol efflux activity and lecithin-cholesterol acyltransferase (LCAT) activity were measured in apoA1 immunopurified from human atherosclerotic-laden plaque (n=10 different subjects), as described in Methods. ApoA1 isolated from plasma high-density lipoprotein recovered from healthy donors (n=3) and reconstituted high-density lipoprotein formed from these apoA1 particles served as controls for total efflux activity and LCAT activity, respectively. Bars represent triplicate determinations; error bars represent SD. P values represent comparison between subject samples vs control apoA1 and were determined with the use of the 1-sample robust Hotelling T² test.
reflects the environment within the artery wall. Of note, the extent of oxidatively cross-linked apoA1 within the lipid-poor (LPD) fraction of plasma was 3-fold enriched relative to the HDL-like fraction (Figure 5E). On the basis of the cumulative results herein, one might speculate that dysfunctional forms of HDL monitored within the circulation will most likely reside not on the HDL particle itself but as lipid-poor forms in the LPD fraction (d>1.21). It is notable that the apoA1 found within the artery wall, which was predominantly within the LPD fraction in both atherosclerotic lesions and normal artery wall tissue, is remarkably highly cross-linked (50% to 70%). This value is even higher than previously observed in Western blots (though cross-linking was not quantified per se) in studies examining apoA1 oxidation levels in the artery wall based on buoyant density recovery of HDL-like particles. Through use of stable isotope dilution mass spectrometry–based approaches, these studies suggested that an upper boundary of up to 1 of every 2 HDL-like particles recovered from the artery wall carried an oxidative modification from either myeloperoxidase- or NO-derived oxidants.8–10,13 Other studies have also reported that oxidative cross-linking noted here may help to provide an answer, as well as mass spectrometry studies in the past.8–10,13 Oxidatively modified proteins tend to be less soluble and relatively protease resistant and might thus be “retained” within the subendothelial space, particularly within the hydrophobic environment of the atherosclerotic plaque. The phenomenon of lipoprotein retention within the subendothelial compartment of the artery wall has been suggested previously. Originally proposed for apoB lipoproteins, retention or trapping of LDL particles in the initial stages of atherosclerosis is suggested to cultivate formation of modified LDL, which may incite biological and inflammatory responses that initiate or advance the atherosclerotic process.30 Progressive apoB lipoprotein retention through the actions of secretory acid sphingomyelinase and lipoprotein lipase are thought to lead to accelerated lesion progression.31,32 Although we have no data to implicate lipase activation at present in depleting lipids from HDL within the artery wall leading to lipid-depleted forms, the presence of similar phospholipids on HDL suggests that a similar retention scheme for apoA1 lipoprotein retention is feasible and could thus contribute to the observed accumulation of apoA1 in artery wall lesions over time, in addition to enhanced lipid and sterol uptake into macrophages producing foam cells. Other studies have also shown that increased endothelial cell permeability in atherosclerotic lesions could lead to increased LDL and HDL migration into the diseased vessel wall and accelerate the retention process.33–35

Although multiple studies have noted extensive oxidative modification of apoA1 recovered from the artery wall,9–11,13–16,36 there has been disagreement about which residues are the main sites of oxidation. Our prior proteomic mapping studies used polyclonal antibodies (chicken anti-apoA1 or anti-HDL) to immunoprecipitate apoA1 from arterial tissue homogenates.11,15 In contrast, proteomic mapping studies from alternative groups typically have used buoyant density isolation to recover the HDL-like particle fraction within lesion homogenates.11,15 On the basis of the present studies using the mAb 10G1.5, which was developed specifically to allow both recovery and equal quantification of apoA1 in lipid-free versus lipiddated and native versus oxidized forms, it is now clear
that analysis of recovered HDL-like particles from arterial tissues only examines a very small fraction (<3%) of the total apoA1 within the artery wall. Whether the small amount of apoA1 (and its associated proteome) recovered in an HDL-like particle from the artery wall provides a “snapshot” of the apoA1 on its way to particle “disintegration” and formation of the lipid-poor apoA1 that is the predominant form remains to be determined. The present studies suggest that results that focus on HDL-like particles recovered from the artery wall need to be interpreted within the context of recognizing their minor quantitative contribution to total apoA1 in the artery wall. An interesting question, although not examined in the present studies, is whether changes in the HDL-associated proteome within the circulation observed in subjects with cardiovascular disease or within subjects at heightened risk for cardiovascular disease have any relevance to the marked changes in the environment of apoA1 observed within the artery wall.

The complexity surrounding the role of the HDL particle in the pathogenesis of cardiovascular disease has been highlighted recently because of several high-profile clinical trial failures targeting raising of HDL cholesterol and recent Mendelian randomization studies on HDL cholesterol levels. The present studies suggest that traditional HDL–measured parameters within the circulation, such as HDL cholesterol and apoA1 mass, may not adequately reflect the biology of apoA1 occurring within the artery wall. Recent studies suggest that functional measures of cholesterol efflux activity within apolipoprotein B–depleted serum may serve as a superior surrogate for HDL function. However, this too has recently been questioned because the HDL particle was found to account for only a minority of the cholesterol acceptor activity in the cholesterol efflux activity assays performed. Furthermore, despite the reported inverse association between cholesterol efflux activity and prevalent cardiovascular disease, in a separate large clinical study of similar patients (ie, sequential subjects undergoing elective diagnostic coronary angiography), enhanced cholesterol efflux activity was observed to be associated paradoxically with increased prospective cardiovascular event risk. It is remarkable that what we now recognize as HDL was first described nearly a century ago. Yet, studies focusing on HDL still continue to surprise us and reveal how little we know about its complex biology. The present and recent studies suggest that measurement in the circulation of HDL cholesterol, apoA1, or even cholesterol efflux activity may not adequately reflect what is happening within the artery wall. Rather, development of dysfunctional HDL assays that detect structurally specific modified forms of apoA1 formed in the artery wall but that diffuse back out into the circulation may be what is needed to provide insights into the processes occurring within the artery wall.

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References


Much of what is known about apolipoprotein A1 (apoA1) and high-density lipoprotein (HDL) comes from investigations using isolated HDL particles from the circulation with the use of buoyant density ultracentrifugation and not from HDL or apoA1 directly recovered from the human artery wall. A longstanding assumption is that measurement of circulating HDL cholesterol, apoA1, or even cholesterol efflux activity provides insights into biological processes relevant within the artery wall. ApoA1 recovered from human atherosclerotic lesions, however, is known to be highly oxidized, and ex vivo oxidation of apoA1 or HDL to an extent comparable to that observed in vivo both cross-links apoA1 and inhibits cholesterol efflux and lecithin-cholesterol acyltransferase activities of the lipoprotein. Herein we directly examined the function and distribution of apoA1 recovered from normal and atherosclerotic plaque-laden human aorta. Remarkably, the distribution and function of apoA1 in the artery wall were markedly distinct from those observed within the circulation. First, apoA1 was 100-fold enriched within atherosclerotic plaque compared with normal arterial tissue. Furthermore, arterial wall apoA1 was predominantly lipid-poor, not associated with HDL, extensively oxidatively cross-linked, and functionally impaired with respect to both cholesterol efflux and lecithin-cholesterol acyltransferase activities. Collectively, these findings argue that circulating levels of HDL cholesterol, HDL particles, or apoA1 inadequately reflect apoA1 biology within the artery wall. Instead, our findings suggest that examination of circulating lipid-poor apoA1 forms or detection of structurally specific oxidatively modified forms of apoA1 may better reflect processes important within the vessel wall and for rendering HDL dysfunctional.