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Isoforms of apolipoprotein C-I associated with individuals with coronary artery disease

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Abstract

Apolipoprotein C-I (apoC-I) is a 6.6kDa serum protein associated with high density lipoproteins (HDL) and triglyceride-rich lipoproteins. In this study, apoC-I was examined in high density lipoprotein subfractions from individuals with and without coronary artery disease (CAD). New isoforms of apoC-I, were detected in the cohort of individuals with CAD using mass spectrometry while the expected apoC-I isoforms were absent. In addition, the apoC-I mass spectra for the CAD cohorts had satellite peaks indicative of the involvement of oxidative processes. Further analysis of the mass spectra of the CAD and non-CAD cohorts suggest that the origin of these new isoforms may be due to genetic mutations that could compromise the function of apoC-I.

Keywords

HDL; Apolipoprotein C-I; Isoforms; MALDI; Atherogenic HDL

Introduction

The focus of this communication is to report the discovery of new isoforms of apolipoprotein C-I (apoC-I) in HDL subfractions from human sera, using a combination of a novel method for density gradient ultracentrifugation (DGU) coupled with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Our analyses revealed the presence of apoC-I isoforms in the mass spectra of the HDL subfractions from individuals with CAD distinct from those without CAD.

Apolipoprotein C-I is a 6.6kDa protein associated with triglyceride-rich lipoproteins and HDL [1]. Composed of 57 amino acids, its basicity and high isoelectric point are attributed to the presence of several lysine residues [2–3]. ApoC-I is involved in the regulation of lipase enzymes [4–5], and has been shown to decrease the clearance of very low density lipoproteins (VLDL) and intermediate density lipoproteins (IDL), from plasma by masking or altering the conformation of the hepatic receptor apoE [6–7]. ApoC-I is known to be a potent stimulator of lecithin-cholesterol acyl transferase (LCAT) with approximately 78% of

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the activity demonstrated by apoA-I [8]; it is also an inhibitor of cholesteryl ester transfer protein (CETP) [9–10].

Isoforms of apoC-containing lipoproteins have previously been identified in biological samples as a result of glycosylation, deglycosylation, and proteolytic activity at the post-translational levels [11–13]. Recently, a functional structural polymorphism of apoC-I, a T45S variant, was identified by mass spectrometry in individuals of American Indian or Mexican ancestry [14]. More recently, two distinct isoforms of apoC-I were identified by mass spectrometry in great apes [15]. These isoforms were designated acidic (apoC-IA), and basic (apoC-IB) based upon their calculated isoelectric points. In addition, a recent proteomics analysis of the mixture of HDL₂ apolipoproteins using MALDI-MS gave clear evidence for protein modifications correlated with CAD [16].

The objective of this communication is to report novel isoforms of apoC-I in the mass spectra of HDL subfractions from individuals with CAD that were not observed in individuals without CAD. These findings reveal a distinct difference between apoC-I spectra in these two populations of individuals, but also give an indication of further post-translational modifications of apoC-I isoforms in the observed oxidation of apoC-I peaks in the MALDI spectra of certain individuals with CAD.

Materials and methods

The work described in this communication was carried out in accordance with *The Code of Ethics of the World Medical Association (Declaration of Helsinki)* for experiments involving humans.

Materials and reagents

The dicesium-cadmium-EDTA complex (Cs₂CdEDTA) was synthesized in our laboratory following a published protocol [17]. The fluorophore 6-((N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)amino)-hexanoyl) sphingosine, NBD (c₆-ceramide), was obtained from Molecular Probes (Eugene, OR). EDTA, sinapinic acid, Dextralip® 50, magnesium chloride hexahydrate, and trifluoroacetic acid (TFA) were from Sigma Aldrich (St. Louis, MO). Reagent-grade methanol, acetonitrile, and dimethyl sulfoxide (DMSO) were obtained from EM Science (Gibbstown, NJ, USA). Strata C18-E solid phase extraction cartridges and syringe adapter caps were purchased from Phenomenex (Torrance, CA). De-ionized water used in all experiments was from a Milli-Q water purification system (Millipore, Bedford, MA).

Serum collection

Serum samples were collected in the fasting state into 9.5ml Vacutainer tubes treated with polymer gel and silica activator (366510, Beckton-Dickinson Systems, Franklin Lakes, NJ). Serum was separated from erythrocytes by centrifugation at 3200rpm for 20min at 5°C. The supernatant (serum) was aspirated and stored at –80°C prior to analyses. Seven normolipidemic subjects with diagnosed CAD and 8 non-CAD controls determined by angiography or medical record were used in this study. Serum samples were selected from our laboratory serum library, which consists of donated serum from Scott & White Hospital (Temple, TX) patients as part of a study of atherogenic HDL. Informed consent was obtained from all donors.

Separation of lipoprotein particles by preparative density gradient ultracentrifugation

Prior to ultracentrifugation, serum was first treated with dextran sulfate and magnesium chloride to precipitate out apoB-containing lipoproteins. A 10g/L solution of dextran sulfate

and 0.5M magnesium chloride was added to a sample of serum at a volume of 10% of the serum volume. This mixture was vortexed and allowed to sit at room temperature for 10min. The mixture was then centrifuged by tabletop centrifuge at 12,000rpm for 5min. The supernatant was then prepared for ultracentrifugation. Into a 1.5ml Eppendorf tube, 200ul of the serum supernatant was subsequently stained (for imaging) with 10ul of a solution of NBD in DMSO (1mg/ml), mixed with 1100ul 0.300M Cs₂CdEDTA, and vortexed at 1400rpm for 1min. This homogenous mixture was then incubated at room temperature for 30min. A 1000ul aliquot was transferred to a 1.5ml thick-walled polycarbonate open-top ultracentrifuge tube (Beckman Instruments, Fullerton, CA). This solution was spun for 6h at 120,000rpm and 5°C in a Beckman Optima™ TLX-120 Ultracentrifuge with a 30° fixed angle TLA 120.2 rotor.

Fluorescence imaging and generation of lipoprotein density profile

A custom-built fluorescence imaging system was used to measure the distribution of the lipoprotein particles in the ultracentrifuge tube following ultracentrifugation. The light source used was a Fiber-Lite MH-100 Illuminator, (MH100A, Edmund Industrial Optics). A digital color microscope camera (S99808, Optronics, Goleta, CA) was used. The camera and light source were placed orthogonally to each other on an optical bench and a slit (1cm x 4cm) was placed 8cm away from the tube holder and suspended by a post-holder to collimate the excitation beam. A gain of 1.0000 and an exposure time of 41.1mS were chosen using the accompanying MicroFire camera software. A blue-violet excitation filter (BG-12, Schott, Edmund Industrial Optics) with a bandwidth centered at 407nm and a yellow emission filter (OG-515, OEM, Edmund Industrial Optics) with a bandwidth centered at 570nm was chosen to match the NBD (C₆-ceramide) excitation and emission. From the image capture of the UC tube, a density profile was generated as described by a published method [17]. Using Origin 7.0 software, the image was first converted into grayscale intensity values as a function of pixels in two dimensions. Later the grayscale intensity from a small strip of 10 pixels oriented in the center of the UC tube was averaged. This average grayscale intensity was then plotted as a function of tube coordinate (0–34mm) to give the final lipoprotein density profiles. This method also provides for the separation of lipoprotein subclasses and their subsequent recovery from serum. Excision of HDL fractions was achieved through the determination of the position of these fractions in an ultracentrifugation tube based upon their respective tube coordinates. The tube coordinate scale ranged from 0–34mm which corresponds to the distance from the top to the bottom of the ultracentrifugation tube respectively. The cut points corresponded to the density ranges of 1.063–1.125g/mL for HDL₂, and 1.125–1.210g/mL for HDL₃. These defined density ranges were used to determine the cut positions for all serum samples in this study.

Recovery of HDL fractions

Following ultracentrifugation, the tubes were frozen in liquid nitrogen by lowering into a custom 10-slot holder, causing the liquid in the tubes to freeze from the bottom to the top. To cut the respective fractions a micrometer/tube holder assembly was used to dial in the correct cut points. This micrometer/tube holder assembly contains a micrometer head, which functions to advance the position of the UC tube relative to the location of the notch for the saw blade. A Dremel® scroll saw (Racine, WI) was fitted with 0.25mm blades for the cutting of the tubes.

Delipidation and desalting of lipoprotein fractions

HDL fractions were desalted and delipidated using a Phenomenex C18 cartridge (Torrance, CA). Briefly, the HDL fractions were mixed with an equal volume of 0.1% TFA in water. This sample mixture was loaded on a cartridge that had been conditioned with 3ml 0.1% (v/v) TFA in acetonitrile, followed by 3ml of 0.1%(v/v) TFA in water, the cartridge was then

rinsed with 3ml 0.1%(v/v) TFA in water to remove any salts and water soluble contaminants from the ultracentrifugation spin. Proteins were eluted in 100ul aliquots of 0.1% (v/v) TFA in acetonitrile and the first elution which contained all serum proteins, was evaporated to dryness, and reconstituted in 100ul 0.1%(v/v) TFA in water for MALDI analysis.

MALDI-TOF mass spectrometry

MALDI spectra of HDL fraction proteins were recorded using an Applied Biosystems Voyager-DE STR (Foster City, CA, USA) in linear mode at an acceleration voltage of 25kV using a 93% transmission acceleration grid. Delay time was set at 575ns and 100 shots per spectrum were collected. Mass ranges were acquired between 5,000 and 35,000 m/z . The system was calibrated externally with bovine insulin (m/z 5733.6), bovine serum albumin (m/z 66,341), and *Escherichia coli* thioredoxin (m/z 34,622). In a 1:1 ratio, 1ul of each sample was added to 1ul of 10mg/ml sinapinic acid and spotted onto a 100 spot stainless steel plate.

Results

The overall strategy of the study was to recover HDL₂ and HDL₃ from serum and determine whether there are differences in the mass spectra of the apolipoproteins for the CAD and non-CAD cohorts. Both cohorts are normolipidemic participating in an ongoing clinical study of atherogenic HDL. The two HDL subclasses were separated and recovered by ultracentrifugation and the apolipoprotein fractions analyzed by MALDI mass spectrometry. Representative results obtained for these two cohorts are summarized in this section.

Lipoprotein particle density profile

The uptake of a fluorescent probe by the lipoprotein particles allows for the visualization and identification of the distribution of lipoproteins in a lipoprotein density profile [Fig. 1]. All apoB containing lipoproteins (LDL, VLDL, and TRL) were removed by dextran sulfate precipitation prior to DGU. The first “peak” in Fig. 1 is an imaging artifact that corresponds to the reflection of fluorescence radiation emanating from the lipoprotein layers to the meniscus in the UC tube. The HDL₂ and HDL₃ particle distributions span the density range from 1.063–1.210g/mL. The distribution above this range consists of proteins, mainly albumin, that have incorporated the fluorescent label and sediment to the bottom of the tube. This method is used to not only characterize the density of the lipoprotein particles but also to provide a means of recovering specific subclasses for further characterization as described in the methods section. For the profile shown in Fig. 1, the cut points used to recover the HDL₂ and HDL₃ fractions are indicated by the blue, vertical lines which correspond to 1.063–1.125g/mL and 1.125–1.210g/mL respectively, for HDL₂ and HDL₃. The density profiles for the two cohorts showed considerable variability in the relative intensities of the two HDL subclasses.

MALDI analysis of HDL fractions

Non-CAD cohort—Fig. 2 shows the MALDI spectra for the HDL apolipoproteins recovered from HDL₂ and HDL₃ fractions from a subject without CAD. Fig. 2A shows the spectrum in the mass range from m/z 5,000 to 35,000 for the HDL₂ region. As shown in the lower portion of Fig. 2A, the dominant peaks in the spectrum are from apoA-I and apoC-I with significant contributions from a cluster of apoC-II and apoC-III ions in the region around m/z 9000. ApoC-I is the dominant peak in the mass spectrum despite its factor of twenty lower serum concentration relative to the dominant apoA-I. This enhancement is due to the high proton affinity of lysine-rich apoC-I in the gas phase desorption plume of the MALDI laser. Utilizing this feature, coupled with the low mass spectral background in the apoC-I region, we elected to focus on this molecule for our initial studies. Fig. 2B shows the

corresponding mass spectra for the HDL₃ subclass fraction. The mass spectra are similar to that for HDL₂ with some differences in the apoC-II and apoC-III regions but apoC-I is the dominant peak, as observed for HDL₂. Figs. 2C–D are an expanded version of the apoC-I region. Two peaks were observed that were designated as apoC-I' and apoC-I based upon their molecular weights. Bondarenko et al. previously identified and established that apo C-I' is an isoform that lacks Thr-Pro residues at the N terminus [18]. For the seven angiographically confirmed non-CAD cohort of patients, all spectra displayed apoC-I peaks whose masses are in accordance with the literature values. Table 1 shows the average masses and standard deviation of apoC-I' and apoC-I for the control cohort compared to the calculated masses of these proteins. (The inherent accuracy in this mass region was determined to be ± 1.2 u using an insulin standard).

CAD cohort—Figs. 3–4 are the MALDI spectra of the apoC-I region for two subjects from the CAD cohort. Figs. 3A–B are the apoC-I spectra for the HDL₂ and HDL₃ subclasses respectively. Both the full and truncated forms were detected however *their masses were higher by approximately 90 Da* compared to the controls. All seven of the CAD individuals were found to have this new isoform, but none of the controls. This new isoform appears to be particularly susceptible to the oxidative stress associated with CAD. An example is shown in Fig. 4. Fig. 4A is the spectrum derived from the HDL₂ fraction showing satellite peaks 16 u higher for both the full form identified as apoC-I₁ and truncated form (apo C-I₁'). For the HDL₃ fraction however, only the truncated form has an oxidation satellite [Fig. 4B].

The apoC-I isoform spectra for the remaining CAD cohorts showed considerable variability in the degree of oxidation as well as molecular weight. This variability is reflected in the larger standard deviation of the measured average mass compared to the controls as shown in Table 1.

A special case—One of the individuals included in the study is a 56 year old subject who was initially selected as a control based primarily on having a sustained symptom-free healthy profile. This patient also has a family history of premature CAD. This case is included because it may have relevance to understanding the origin of the heavier isoforms of apoC-I. The subject's apoC-I mass spectral profile matches that of the CAD cohort shown in Figs. 3A–B. In addition, the subject's HDL₂ fraction was found to induce smooth muscle apoptosis (unpublished results), a potential atherogenic property linked to apoC-I [19].

Discussion

It is clear, even from this limited amount of data, that this new isoform of apoC-I is linked to CAD, at least for this cohort of normolipidemic CAD subjects. The approximate 90 Da increase for both the full apoC-I₁ and its truncated form (apoC-I₁') indicates that a mutation of the apoC-I gene is most likely involved and that the truncation has occurred after transcription. If the mutation takes place at a site that is involved in the LCAT/CETP activity of apoC-I within the HDL system, its role in lipoprotein metabolism could be compromised contributing to an atherogenic form of HDL.

The pattern of oxidation of the new isoforms is particularly intriguing. Referring to Fig. 4, the apoC-I₁ isoform is not oxidized in the HDL₃ fraction but is oxidized in the truncated form (apoC-I₂') suggesting that it is more amenable to oxidation. When HDL₃ to HDL₂ remodeling occurs however, apoC-I₁ becomes more vulnerable to oxidation to form apoC-I₂. For some of the CAD subjects, apoC-I₁ and apoC-I₁' show oxidized forms in both the HDL₂ and HDL₃ subclasses.

Supporting the notion that these new isoforms of apoC-I may be linked to genetic mutation is the results of the “special case”. Although the subject was asymptomatic for CAD, [the signature of this new apparently atherogenic apoC-I₁ isoform was present in the HDL fractions. This feature, if driven by genetic mutation, could underlie the known family history of premature CAD for this individual.

A new isoform of apoC-I, approximately 90 Da heavier in molecular weight than the literature value, has been identified in a cohort of normolipidemic CAD subjects. This new isoform was not observed in a matching set of non-CAD controls and is potentially a new genetically-based risk factor for CAD.

Research highlights

- Isoforms of apoC-I detected in HDL subfractions from individuals with coronary artery disease (CAD) that were not detected in the non-CAD cohort.
- *m/z* shifting of apoC-I isoform peaks observed at approximately +90Da in all individuals with CAD.
- Further modifications observed in the apoC-I spectra of some individuals with CAD which showed doublet apoC-I peaks with mass differences indicative of oxidation.
- Findings contribute to the understanding of the unique functional properties of HDL.

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References

1. Shulmann RS, Herbert PN, Wehrly K, Frederickson DS. The complete amino acid sequence of C-I (apoLP-Ser), an apolipoprotein from human very low density lipoproteins. *J Biol Chem.* 1975; 250:182–190. [PubMed: 166984]
2. Jong MC, Hofker MH, Havekes LM. Role of ApoCs in lipoprotein metabolism: functional differences between ApoC1, ApoC2, and ApoC3. *Arterioscler Thromb Vasc Biol.* 1999; 19:472–484. [PubMed: 10073946]
3. Shacter NS. Apolipoproteins C-I and C-III as important modulators of lipoprotein metabolism. *Curr Opin Lipidol.* 2001; 12:297–304. [PubMed: 11353333]
4. Conde-Knape K, Bensadoun A, Sobel JH, Cohn JS, Shachter NS. Overexpression of apoC-I in apoE-null mice: severe hypertriglyceridemia due to inhibition of hepatic lipase. *J Lipid Res.* 2002; 43:2136–2145. [PubMed: 12454276]
5. Berbee JF, van der Hoogt CC, Sundararaman D, Havekes LM, Rensen PC. Severe hypertriglyceridemia in human APOC1 transgenic mice is caused by apoC-I induced inhibition of LPL. *J Lipid Res.* 2005; 46:297–306. [PubMed: 15576844]
6. Kowal RC, Herz J, Weisgraber KH, Mahley RW, Brown MS, Goldstein JL. Opposing effects of apolipoproteins E and C on lipoprotein binding to low density lipoprotein receptor-related protein. *J Biol Chem.* 1990; 265:10771–10779. [PubMed: 2355022]
7. Weisgraber KH, Mahley RW, Kowal RC, Herz J, Goldstein JL, Brown MS. Apolipoprotein C-I modulates the interaction of apolipoprotein E with β -VLDL to low density lipoprotein receptor-related protein. *J Biol Chem.* 1990; 265:22453–22459. [PubMed: 2266137]

8. Soutar AK, Garner CW, Baker HN, Sparrow JT, Jackson RL, Gotto AM Jr, Smith LC. Effect of the human plasma apolipoproteins and phosphatidylcholine acyl donor on the activity of lecithin:acyltransferase. *Biochemistry*. 1975; 98:845–855.
9. Gautier T, Masson D, Pais de Barros JP, Athias A, Gambert P, Aunis D, Metz-Boutigue MH, Lagrost L. Human apolipoprotein C-I accounts for the ability of plasma high density lipoprotein to inhibit the cholesteryl ester transfer protein activity. *J Biol Chem*. 2000; 275:37504–37509. [PubMed: 10978346]
10. Dumont L, Gautier T, Pais de Barros J-P, Laplanche H, Blache D, Ducoroy P, Fruchart J, Fruchart J-C, Gambert P, Masson D, Lagrost L. Molecular mechanism of the blockade of plasma cholesteryl ester transfer protein by its physiological inhibitor apolipoprotein CI. *J Biol Chem*. 2005; 280:38108–38116. [PubMed: 16159884]
11. Hussain M, Zannis V. Intracellular modification of human apolipoprotein AII (apoAII) and sites of apoAII mRNA synthesis: comparison of apoAII with apoCII and apoCII isoproteins. *Biochemistry*. 1990; 29:209–217. [PubMed: 2108716]
12. Zannis V, McPherson J, Goldberger G, Karathanasis S, Breslow J. Synthesis, intracellular processing, and signal peptide of human apolipoprotein E. *J Biol Chem*. 1984; 259:5495–5499. [PubMed: 6325438]
13. Scanu A, Byrne R, Edelstein C. Proteolytic events affecting plasma apolipoproteins at the co- and post-translational levels and after maturation. *J Lipid Res*. 1984; 25:1593–1601. [PubMed: 6442340]
14. Wroblewski MS, Wilson-Grady JT, Martinez MB, Kasthuri RJ, McMillan KR, Flood-Urdangarin C, Nelsestuen GL. A functional polymorphism of apolipoprotein C1 detected by mass spectrometry. *J Lipid Res*. 2006; 273:4707–4715.
15. Puppione DL, Ryan CM, Bassilian S, Souda P, Xiao X, Ryder OA, Whitelegge JP. Detection of two distinct forms of apoC-I in great apes. *Comp Biochem Physiol*. 2010; 5:73–79.
16. Vaisar T, Mayer P, Nilsson E, Knopp ER, Prazen B. HDL in humans with cardiovascular disease exhibits a proteomic signature. *Clinica Chimica Acta*. 2010; 411:972–979.
17. Johnson JD, Bell NJ, Donahue EL, Macfarlane RD. Metal ion complexes of EDTA as solutes for density gradient ultracentrifugation: influence of metal ions. *Anal Chem*. 2005; 77:7054–7061. [PubMed: 16255609]
18. Bondarenko PV, Farwig ZN, McNeal CJ, Macfarlane RD. MALDI- and ESI-MS of the HDL apolipoproteins; new isoforms of apo A-I, A-II. *Int J Mass Spectrom Ion Process*. 2002; 219:671–680.
19. Kolmakova A, Kwiterovich P, Virgil D, Alaupovic P, Knight-Gibson C, Martin SF, Chatterjee S. Apolipoprotein C-I induces apoptosis in human aortic smooth muscle cells via recruiting neutral sphingomyelinase. *Arterioscler Throm Vasc Biol*. 2004; 24:264–269.

Fig. 1.

A representative lipoprotein density profile from an individual with CAD following dextran sulfate precipitation to remove all non-HDL lipoproteins is shown above. The density ranges used to define HDL₂ and HDL₃ by this method are shown by the blue, vertical lines. The y-axis corresponds to the fluorescence intensity of the lipophilic fluorophore NBD (c₆-ceramide).

Fig. 2. MALDI-TOF mass spectra of HDL₂ and HDL₃ fractions from an individual without CAD. (A) shows the HDL₂ fraction, (B) the HDL₃ fraction, (C) the expanded HDL₂ fraction, and (D) the expanded HDL₃ fraction.

Fig. 3. MALDI-TOF mass spectra of (A) expanded HDL₂ and (B) expanded HDL₃ human serum fractions from an individual with CAD.

Fig. 4. MALDI-TOF mass spectra of apoC-I variants in the (A) expanded HDL₂ and (B) expanded HDL₃ human serum fractions from an individual with CAD showing further modifications.

Table 1Summary of m/z values for apoC-I peaks

	Control Cohort Apo C-I'	Control Cohort Apo C-I	CAD Cohort Apo C-I ₁ '	CAD Cohort Apo C-I ₁
Average Mass (Da)	6432.95 ± 8.23	6632.52 ± 1.98	6520.03 ± 16.04	6721.58 ± 8.10
Calculated Mass	6432.5	6630.6	6432.5	6630.6
Difference (Da)	0.45 ± 8.23	1.92 ± 1.98	87.53 ± 16.04	90.98 ± 8.10

Data was collected and averaged from 16 patients.