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Mass spectral analyses of the two major apolipoproteins of great ape high density lipoproteins

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Abstract

The two major apolipoproteins associated with human and chimpanzee (*Pan troglodytes*) high density lipoproteins (HDL) are apoA-I and dimeric apoA-II. Although humans are closely related to great apes, apolipoprotein data do not exist for bonobos (*Pan paniscus*), western lowland gorillas (*Gorilla gorilla gorilla*) and the Sumatran orangutans (*Pongo abelii*). In the absence of any data, other great apes simply have been assumed to have dimeric apoA-II while other primates and most other mammals have been shown to have monomeric apoA-II. Using mass spectrometry, we have measured the molecular masses of apoA-I and apoA-II associated with the HDL of these great apes. Each was observed to have dimeric apoA-II. Being phylogenetically related, one would anticipate these apolipoproteins having a high percentage of invariant sequences when compared with human apolipoproteins. However, the orangutan, which diverged from the human lineage between 16 and 21 million years ago, had an apoA-II with the lowest monomeric mass, 8031.3 Da and the highest apoA-I value, 28311.7 Da, currently reported for various mammals. Interestingly, the gorilla that diverged from the lineage leading to the human-chimpanzee branch after the orangutan had almost identical mass values to those reported for human apoA-I and apoA-II. But chimpanzee and the bonobo that diverged more recently had identical apoA-II mass values that were slightly larger than reported for the human apolipoprotein. The chimpanzee A-I mass values were very close to those of humans; however, the bonobo had values intermediate to the molecular masses of orangutan and the other great apes. With the already existing genomic data for chimpanzee and the recent entries for the orangutan and gorilla, we were able to demonstrate a close agreement between our mass spectral data and the calculated molecular weights determined from the predicted primary sequences of the respective apolipoproteins. Post-translational modification of these apolipoproteins, involving truncation and oxidation of methionine, are also reported.

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Keywords

Mass spectrometry; truncated apoA-II; dimeric apoA-II. post-translational modifications

1 Introduction

Because of their phylogenetic relationship to humans, the physiological and metabolic processes in the other great apes are of considerable interest. In the area of lipid metabolism, plasma levels of cholesterol and triacylglycerol as well as lipoprotein concentrations have been measured for both wild and captive apes (Srinivasan et al., 1974; Baitchman et al., 2006; Schmidt et al. 2006). As is the case in humans, the distribution of cholesterol associated primarily with either LDL or HDL varied with age. Analytical ultracentrifugal analyses of the distribution of HDL did reveal that great apes differed from humans, having higher percentage of HDL₂ (Ewing et al., 1965; Nelson et al. 1985).

Beginning in the late 1960s, several groups reported the presence of two major apolipoproteins associated with human HDL (Shore and Shore, 1968; Rudman et al., 1970; Kostner and Alaupovic, 1971; Edelstein et al., 1972). The larger apoA-I was sequenced and found to contain 243 amino acids (Brewer et al., 1978) and the smaller apoA-II (Brewer et al., 1972) with 77 amino acids was able to form a homodimer. Sequence analysis of the apoA-II monomer showed that the amino terminus was modified to pyroglutamic acid and that cysteine was at residue 6 (Brewer et al., 1972). The chimpanzee also was reported to have homodimers and to have a cysteine in the N- terminal region at residue 6 (Blaton et al., 1974; Scanu et al., 1974). In contrast sequence analyses of other primate apolipoproteins showed that the apoA-II of macaques (Edelstein et al., 1976) and marmosets (Crook et al., 1990) did not contain cysteine and were monomeric.

Nevertheless, it has been generally assumed that dimeric apoA-II were also present in the other great apes (Herbert et al., 1987; Lund-Katz et al., 1996). Having published several reports on the molecular masses of apolipoproteins associated with the HDL of a variety of mammals (Puppione et al., 2004, 2005, 2006, 2008), we wanted to use these same approaches to determine if indeed other great apes had dimeric apoA-II as well as obtaining molecular mass data on both apoA-I and apoA-II. Mass spectral analyses were carried out on four species of non-human great apes, viz. bonobo, chimpanzee, gorilla and orangutan. As was the case for humans and chimpanzees, we observed dimeric apoA-II in each case. We also report on post- translational modifications of both apoA-I and apoA-II.

2 Methods

2.1 Source of great ape plasmas

The San Diego Wild Animal Park, a repository for frozen mammalian plasmas, provided samples from the following animals: bonobo (*Pan paniscus*) (male and 2 females); chimpanzee (*Pan troglodytes*) (3 females); gorilla (*Gorilla gorilla gorilla*) (2 females and 1 male); Sumatran orangutan (*Pongo abelii*) (male and female). The frozen plasmas (approximately 1.5 mL from each ape) were transported on dry ice to the Los Angeles campus of the University of California. Protocols and analyses, described below, were carried out on each individual sample. The samples were not pooled. The resulting values were averaged and appear in the Results section.

2.2 Ultracentrifugal isolation of HDL fractions

Solution densities were adjusted as described previously (Schumaker and Puppione, 1986). The lipoproteins were separated using a TLA-100 rotor centrifuged in a Beckman Optima-TLX ultracentrifuge at 80,000 rev./min (240,000 g at r_{av}) at 20 °C. Initially, tubes containing 0.18 mL of plasma adjusted to a density of 1.063 g/mL were centrifuged for 4 h. After removing the top 0.060 mL, the infranatants were adjusted to a density of 1.210 g/mL. Following 4 h of centrifugation, the HDL were recovered in the top 0.040 mL. All salt solutions used for ultracentrifugation contained 0.04% Na₂ EDTA and 0.05% NaN₃.

2.3 Determination of apolipoprotein mass by size exclusion chromatography-mass spectrometry (SEC-MS)

The apolipoproteins were separated by SEC prior to analysis in the mass spectrometer (Whitelegge et al., 1998). HDL fractions were first dialyzed against a NaCl solution (density = 1.0063 g/mL). The dialyzed fractions then were acidified by mixing 0.01 mL with 0.09 mL of 90% formic acid immediately prior to SEC-MS. For samples run under reduced conditions, 0.005 mL of 1.0 M DTT were added to the sample and allowed to incubate for 10 min before the addition of 0.085 mL of 90% formic acid. SEC-MS was performed in CHCl₃/MeOH/1% aqueous formic acid (4/4/1; v/v/v) using a Super SW 2000 column (4.6 × 300 mm, Tosoh Bioscience, Montgomeryville, PA, USA) at 0.250 mL/min and 40°C. Immediately prior to entering the mass spectrometer, the column effluent was monitored with a UV detector set at 280 nm. Mass spectrometry (ESI-MS) was performed using a triple quadrupole instrument (API III, Applied Biosystems) (Whitelegge et al., 1999). Data were processed using MacSpec 3.3, Hypermass and BioMultiview 1.3.1 software (Applied Biosystems). The resulting molecular mass values were compared with calculated molecular weights derived from genomic entries in various databases that included the National Center for Biological Information (www.ncbi.nlm.nih.gov), the Genome Bioinformatics website of the University of California at Santa Cruz (<http://genome.ucsc.edu/>) and the Ensembl website (<http://www.ensembl.org/index.html>). Calculated molecular weights were obtained using ProtParam at the proteomic server of the Swiss Institute of Bioinformatics (<http://ca.expasy.org/>).

3 Results

3.1 Great ape apoA-I molecular mass values

The average values for the molecular masses of chimpanzee and gorilla apoA-I were very close. Based on the mass spectral measurements on the plasma apo HDL, the value for three chimpanzees apoA-I was 28079.33 ± 1.258 Da and the apoA-I of three gorillas had an average value of 28078.67 ± 0.833 Da. Representative spectra are compared in Fig. 1A. Comparable measurements on three bonobos gave a value of 28108.80 ± 0.361 Da. Spectra for the bonobo and the orangutan are compared in Fig. 1B. The orangutan spectrum shown contains two major peaks having a molecular mass value of 28134.0 Da and 28166.0 Da. One other orangutan sample was also analyzed and only a single major peak at 28166.7 Da was observed (data not shown). The intensity of the signals and the difference in mass between the two peaks (32.0 Da), seen in Fig. 1B suggest that there are two variations for the orangutan apoA-I sequence probably due to a change in one or two amino acid residues resulting from point mutations. The orangutan with mass values shown in Fig. 1B presumably would then be an apoA-I heterozygote

3.2 Post-translational modifications of great ape apoA-I

Closer examination of the chimpanzee and bonobo spectra indicates that a small percentage of apoA-I had undergone post-translational modifications. The loss of the C- terminal glutamine (Δ mass of 128 Da) is indicated by the peaks observed at 27951.0, 27953.0, and 27956.0 Da

in three chimpanzee (average 27953.3 Da) and at 27985.0 Da and 27983.0 Da in two of the three bonobos (average 27984.0 Da).

3.3 Great ape apoA-II Molecular mass values

Measurements of the apoA-II gave average values of 17471.0 ± 1.00 Da for three bonobos and 17469.7 ± 1.15 Da for three chimpanzees. The average molecular mass for the apoA-II of three different gorillas was 17380.7 ± 0.58 Da. Unexpectedly, in the case of the two orangutans, apoA-II had a much lower molecular mass 16060.5 ± 0.71 Da (Fig. 2). Upon reduction, molecular masses also were determined for monomeric forms of apoA-II, with values of 8735.3 Da for the bonobo, 8690.3 Da for the gorilla and 8032.0 Da for the orangutan (data not shown). These values demonstrate that as in chimpanzees and humans, apoA-II circulates as a homodimer in the other great apes as well.

3.4 Post-translational modifications of great ape apoA-II

In addition to the peaks associated with the homodimer, heterodimers, having lower molecular mass values of 17342.0 Da were detected in both the bonobo and chimpanzee spectra (data not shown). Similarly, the gorilla had a heterodimer, having a value of 17258.0 Da. These lower values, as was the case for apoA-I, are due to the loss of the C-terminal glutamine from one of the monomers forming the dimer. The chimpanzee spectrum had an additional peak at 17214.0 Da, due to the loss of this same amino acid from both chimpanzee monomers. In both orangutan spectra, a minor peak was observed at 16076.0 Da that would correspond to a methionine being converted to methionine sulfoxide (Fig. 2).

4 Discussion

Previous studies of human apo HDL using mass spectrometry have demonstrated that the molecular mass measurements could be compared with the calculated molecular weights of apoA-I (28078.6 Da) and apoA-II (17379.8 Da) (Pankhurst et al., 2003). In this way, both the validation of the reported amino acid sequences, previously determined by protein analyses, as well as the detection of post-translational modifications of these apolipoproteins could be made. At the time our measurements were done, only partial amino acid sequences for these apolipoproteins existed for the chimpanzee (Scanu et al., 1974); however, the entries in the genomic databases for the chimpanzee did provide us with complete sequences enabling such comparisons to be made for at least one of these great apes. The gorilla and the orangutan genomic entries appeared later. At first, we were wondering about the gorilla data because the molecular masses of both apoA-I and apoA-II were almost identical to reported human values (Pankhurst et al., 2003). Also, the very low mass for orangutan apoA-II suggested that the protein might have undergone extensive enzymatic degradation. Nevertheless, later entries in the databases eliminated our concerns as discussed below. Unfortunately, there are no genomic entries for the bonobo.

Like humans, the genes for chimpanzee ([NC_006478.2](#)) and orangutan (ENSPPYG00000003904) apoA-I are located on chromosome 11. In both cases, the genes yield a 267 amino acid protein, consisting of an 18 amino acid signal sequence, a 6 amino acid propeptide and a 243 amino acid mature protein. Our molecular mass measurements for both the chimpanzee (28079.3 Da) and the gorilla (28078.7 Da) were very close to calculated molecular weight of human apoA-I. In agreement with these measurements, genomic data also have revealed that the primary sequences of the chimpanzee (NC_006478.2) and the gorilla (ENSGGOG00000008006) are identical to that of human apoA-I. The signal sequences and the propeptides of each of the great apes obtained from genomic data are also identical to the human sequences. However, the orangutan 243 amino acid sequence has five variations at residues 6, 53, 138, 167 and 197, with a threonine instead of a serine, a methionine instead of

a valine, a histidine instead of a glutamine, threonine instead of a serine and a serine instead of a threonine, respectively (Table 1). As a result, orangutan apoA-I has a calculated molecular weight of 28133.7 Da that is in close agreement with one of the observed peaks with measured value of molecular mass 28134.0 Da. An examination of the entries in the Ensembl database for 15 different mammals indicates that this currently is the largest reported value for mammalian apoA-I. Our data for bonobo apoA-I were intermediate to the values of the orangutan and the other great apes, *viz.* 28108.8 Da. An observed post-translational modification of apoA-I due to loss of a C-terminal glutamine ($\Delta -128$ Da) was observed in the bonobo and chimpanzee spectra, consistent with the observed average mass values of 27984.0 Da and 27953.3 Da, respectively.

The gene for apoA-II is located on chromosome 1 in chimpanzees (ENSPTRG00000001580) and orangutans (**FJ997614**), as is the case in humans. The respective genes encode an 18 amino acid signal peptide, a 5 amino acid propeptide and a 77 amino acid mature protein. Genomic data for human apoA-II (ENSP00000356969) reveal that the signal sequence and the propeptide are identical to those of the other great apes and that in each case a cysteine is present at residue 6. The mature apoA-II of the chimpanzee and the orangutan do vary from the human sequence as indicated in Table 2. Chimpanzee apoA-II has an aspartate and an asparagine at residues 8 and 9, respectively and a methionine at residue 68. The calculated molecular weight, after correcting for the conversion of the N-terminal glutamine to pyroglutamic acid, is 8735.9 Da. The resulting homodimer would then have a molecular weight of 17469.8 Da. Our measured molecular masses for the dimeric apoA-II of the chimpanzee (17469.7 Da) and the bonobo (17471.0 Da) were very close to this calculated value, suggesting that the sequences of these two great apes are probably the same. Our analyses of the orangutan apo HDL revealed that the apoA-II is currently the smallest to be reported. The three exons encoding this apolipoprotein initially were located in a 15 kb genomic entry (**ABGA01295244.1**). In the third exon, the codon that would have encoded a glycine as the 71st residue instead has been replaced by a stop codon. Using the UCSC genome browser, these three exons also can be located between coordinates 90083319 and 90084578 on chromosome 1. The orangutan apoA-II also varies from the human sequence with a proline, a glutamine, an asparagine and a lysine at residues 10, 37, 65 and 68, respectively (Table 2). The resulting 70 amino acid sequence has a corrected calculated molecular mass of 8031.3 Da. This would result in the dimer having a molecular weight of 16060.6 Da, in agreement with our measured molecular mass value. It should be noted that like the other great apes, most mammals studied also have a 77 amino acid apoA-II (Puppione et al., 2004). Rats and mice apoA-II on the other hand are slightly larger with 79 amino acids (Puppione et al., 2004, 2006). The sequence of gorilla apoA-II was obtained from a 5.4 kb entry (**CABD01119658.1**) in the whole genome shotgun database. The resulting protein sequence, found to be identical to human apoA-II, yielded a corrected calculated molecular weight for the dimer of 17379.8 Da. Our average molecular mass value was 17380.7 Da.

As was observed for apoA-I, there is also a truncated form of human apoA-II due to a loss of a C-terminal glutamine (Deterding et al., 2002 ; Pankhurst et al., 2003). This results in the formation of two dimers having lower molecular masses, *viz.* a heterodimer and a homodimer. Both of these were detected in the apoA-II spectra of the chimpanzee. Lower molecular weight heterodimers were also present in the bonobo and gorilla spectra. Similar truncation also has been reported for monomeric mouse apoA-II, involving the loss of a C-terminal lysine (Puppione et al., 2006). The apoA-II of great apes have a methionine residue at residue 26 and the chimpanzee apoA-II has an additional one at residue 68. Of the four groups of great apes studied here, only the orangutan apoA-II spectrum, with an additional peak at 16076.0 Da showed evidence of oxidation. The methionine at residue 68 in human apoA-II also is susceptible to oxidation and has been associated with the development of atherosclerosis (Pankhurst et al., 2003).

Our studies of the HDL proteome of great apes have demonstrated that the similarities and differences in the apolipoprotein sequences determined from genomic data are consistent with our molecular mass measurements. *A priori*, one might assume that apolipoprotein molecular masses of the bonobo and the chimpanzee would be almost identical to their human counterparts, but only the chimpanzee apoA-I value is the same as humans and our molecular mass data indicate that both apoA-I and apoA-II of the bonobo are larger than the human apolipoproteins. On the other hand, the gorilla that diverged from the human-chimpanzee lineage between 7.7 and 9.4 million years ago has the same sequence for both apolipoproteins (Steiper et al., 2006). Interestingly, the orangutan, having the earlier time of divergence between 16.1 and 20.8 million years ago (Steiper et al., 2006), does not have the canonical 77 amino acid apoA-II.

As far as the involvement of the two apolipoproteins is concern, there is no reason to assume that their role will be different in great apes. However, the interaction of diet and metabolism represents potential sources for influencing evolutionary changes could give rise to different effects not seen in humans. The HDL are integrally involved in the metabolic process known as reverse cholesterol transport (Scanu and Edelstein, 2008). In this process the apoA-I containing HDL accept from extrahepatic tissue cholesterol which in turn is transesterified to cholesteryl ester through the action of lecithin cholesterol acyl transferase (LCAT) (Glomset, 1968; Scanu and Edelstein, 2008). This enzyme is activated by apoA-I. The eventual delivery of cholesteryl esters to the liver involves the interaction of apoA-I with the scavenger receptor SR-B1, located in the plasma membrane of hepatocytes where the hydrolyzed cholesterol is converted to bile acids. The bulk of the released fatty acids in this reaction will be polyunsaturated, having been involved in a previous LCAT reaction (Glomset, 1968). These, in turn, would be potential ligands for the peroxisome proliferator-activated receptors present in liver cells (Lee et al., 2003). Although phylogenetically related, the diets of the other great apes nevertheless are different from humans. Being primarily herbivorous and frugivorous, one would expect that the livers of the other great apes would be metabolizing a higher ratio of omega-3 vs omega-6 fatty acids from the uptake of both HDL cholesteryl esters and TG-rich lipoproteins. As more information is obtained on the respective genomes of the great apes, future studies might explore how these and other metabolic processes are influenced by diet.

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Puppione Fig. 1A

Puppione Fig. 1B

Figure 1.

Representative electrospray-ionization mass spectral profiles of chimpanzee and gorilla apoA-I (1A) and of bonobo and orangutan apoA-I (1B) separated by size-exclusion chromatography. Ordinate units of intensity are linear and arbitrary; the abscissa units of average molecular mass are in Daltons.

Orangutan apoA-II

Figure 2.

Representative electrospray-ionization mass spectral profile of orangutan apoA-II, showing masses for both the homodimer and the oxidized homodimer. Ordinate units of intensity are linear and arbitrary; the abscissa units of average molecular mass are in Daltons.

Table 1

Variations in great ape apoA-I

<u>Signal sequence</u>	MKAAVLT LAVLFLTGSQA
<u>Propeptide</u>	RHFWQQ
<u>The 243 amino acid mature Apolipoprotein A-I</u>	
DEPPQSPWDRVKDLATVYVDVLKDSGRDYVSQFEGSALGKQLNLKLLDNWDSV	53
T TSTFSKLREQLG PVTQEFWDNLEKETEGRLRQEMSKDLEEVKAKVQPYLDDFQK	106
KWQEEMELYRQKVEPLRAELQEGARQKLHEL	159
H QEKLSP LGEEMRDRARAHVDAL	
RTHLAPYSDEL RQRLAARLEALKENG GARLA EYHAKATEHLSTLSEKAKPALE	212
T DLRQGLLPVLESFKVSFLSALEEYTKKLNTQ	243
S	

Human apoA-I sequence is shown with five variations in the orangutan sequence indicated in red letters below. ApoA-I sequences of humans, chimpanzee and gorillas are identical.

Table 2

Variations in great ape apoA-II

<u>Signal sequence</u>	MKLLAATVLLLTICSLEG	
<u>Propeptide</u>	ALVRR	
<u>The 77 amino acid mature apolipoprotein</u>		
	<i>DN</i>	
QAKEPCVESLVSQYFQTVTDYGKDLMEKVKSP	ELQAEAKS	40
<i>P</i>	<i>Q</i>	
	<i>M</i>	
YFEKSKEQLTPLIKKAGTELVNFL SYFVELGTQPATQ		77
<i>N L</i>	-----	

Human apoA-II sequence is shown with three variations in the chimpanzee sequence indicated in green italicized letters above and with four in the orangutan sequence indicated in red letters below. The apoA-II sequences of humans and gorillas are identical.