Factors influencing Lp[a]- particle size as determined by gradient gel electrophoresis

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Abstract This study examined factors influencing the particle diameter of Lp[a], the low density lipoprotein (LDL)-like moiety of Lp[a], in 26 subjects chosen to provide a range of Lp[a] and triglyceride levels. Lp[a] and LDL fractions were isolated by vertical density ultracentrifugation. Lp[a] was further purified using a lysine-Sepharose affinity column and Lp[a]- obtained by incubating Lp[a] with dithiothreitol. Lp[a], LDL, and Lp[a]- fractions were run on 3–13% gradient gels to determine particle diameter. Lp[a] size correlated positively with LDL size (r = 0.62; P < 0.0001), but the association between Lp[a]- size and LDL size was stronger (r = 0.82; P < 0.0001). Log triglyceride level correlated inversely with Lp[a]- size (r = -0.72; P < 0.0001) and LDL size (r = -0.69; P < 0.0001). HDL cholesterol level correlated positively with Lp[a]- size (r = 0.67; P < 0.0005) and LDL size (r = 0.64; P < 0.0005). The strong correlation between LDL size and Lp[a]- size may be due to extracellular utilization of circulating LDL in the production of Lp[a] or may reflect the same metabolic processes influencing both these particles once Lp[a] has been formed. O'Neal, D., G. Grieve, D. Rae, G. Dragicevic, and J. D. Best. Factors influencing Lp[a]: particle size as determined by gradient gel electrophoresis. J. Lipid Res. 1996. 37: 1655–1663.

Supplementary key words lipoprotein[a] • lipoprotein[a]- • low density lipoproteins

First described by Berg in 1963 (1), lipoprotein[a] or Lp[a] consists of a glycoprotein apo[a] linked to apolipoprotein B-100 (apoB-100) on an LDL-like particle Lp[a]- (2). Both apo[a] and apoB-100 moieties are synthesized primarily within hepatocytes (3, 4) though it remains uncertain as to where they are assembled to form Lp[a]. Lp[a] has been reported to be a risk factor for ischemic heart disease (5–8), cerebrovascular disease (9, 10), and peripheral vascular disease (11). Based on the number of repeat sequences of cysteine-rich, internally looped structures known as kringles, apo[a] can be separated into different isoforms (12). Concentration of Lp[a] has been shown to vary inversely with apo[a] isoform size (13–16). Consequently, epidemiological studies of the contribution of Lp[a] to cardiovascular risk have generally focused their attention upon the apo[a] moiety, with Lp[a]- being relatively ignored (5–11).

Our aim was to determine factors influencing the particle diameter of Lp[a] and, in particular, of Lp[a]-, the LDL-like moiety of Lp[a] that is depleted of apo[a]. The relationship of the size of Lp[a]- to LDL particle size was of major interest. The structural similarity between Lp[a]- and LDL raises the possibility that there may be inter-individual variation in the particle size of Lp[a]-, a heterogeneity that has been extensively documented for LDL particles (17–19). We also wanted to investigate the relative effects of metabolic influences on each of these lipoprotein classes. Previous studies have shown that there is a strong inverse correlation between the diameter of the LDL particle and the degree of hypertriglyceridemia within an individual (19–21). Size heterogeneity of Lp[a]- particles is of potential clinical relevance as Austin et al. (21, 22) have shown that LDL particle size, determined by nondenaturing gradient gel electrophoresis, is related to athereogenic risk. Smaller, more dense particles are thought to confer an increased risk. By convention, Phenotype A refers to those individuals with an LDL particle size distribution containing predominantly larger particles with a peak size ≥25.5 nm, while Phenotype B refers to those individuals with an LDL particle size distribution containing predominantly smaller particles (<25.5 nm). Recent electron cryomicroscopy studies have indicated that Lp[a] particles are roughly spherical in shape (23), which would make nondenaturing gradient gel electrophoresis a suitable method by which to determine their size. If Lp[a]- particle size displayed an inter-individual variation simi-

Abbreviations: LDL, low density lipoprotein; Lp[a], lipoprotein[a]; Lp[a]-, LDL-like moiety of Lp[a]; apo, apolipoprotein; CV, coefficient of variation; HDL, high density lipoprotein; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; DTT, dithiothreitol.

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lar to that described for LDL, then small dense Lp[a]- could have particular importance in the genesis of atherosclerosis.

MATERIALS AND METHODS

Subjects

Twenty-six subjects (17 male/9 female, aged 57 ± 3 years) were selected from the staff and outpatient population of St. Vincent's Hospital. As there is a structural and biochemical similarity between Lp[a]- and LDL, and in the light of the reported relationship between LDL size and triglyceride level, the subjects were chosen to display the effect of a broad range of triglyceride levels on Lp[a] and Lp[a]- size (Table 1). A wide range of apo[a] levels was also desirable in order to demonstrate the effect of apo[a] on Lp[a] particle size. Sixteen subjects were normal volunteers and 11 subjects had Type II diabetes mellitus. All subjects were clinically stable without any intercurrent illness. None of the subjects was receiving β-blockers, lipid modifying medication, estrogen, or corticosteroid therapy. Informed consent was obtained and the study was approved by the St. Vincent's Hospital Human Research Ethics Committee.

Lipoprotein analysis

Ten ml of blood was collected in an EDTA-containing tube from all subjects, after a 12-h overnight fast. Plasma was separated by centrifugation at 1300 g for 15 min at 4°C. Total plasma cholesterol (cholesterol oxidase, Trace Scientific, Melbourne, Australia) and total plasma triglyceride (glycerol-3-phosphate oxidase, Trace Scientific, Melbourne, Australia) determinations were performed as part of a routine automated laboratory enzymatic assay (24) on an Olympus AU5031. The interassay coefficient of variation (CV) for cholesterol was 1.6% and 0.9% at 3.2 mmol/l and 7.8 mmol/l, respectively.

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<th>Lp[a] Size</th>
<th>Lp[a]- (Lp[a]- Size</th>
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For triglycerides, the CV was 1.9% at 1.0 mmol/l and 1.8% at 2.2 mmol/l. HDL cholesterol was assayed after polyethylene glycol 6000 precipitation of non-HDL lipoproteins (25). CV was 5.5% at 2.03 mmol/l and 4% at 0.97 mmol/l. LDL cholesterol was calculated using the equation of Friedewald, Levy, and Fredrickson (26) when the total triglyceride level was ≤4.5 mmol/l. For higher levels, VLDL was isolated by ultracentrifugation and cholesterol was measured separately. In these cases LDL cholesterol was calculated by subtraction of HDL and VLDL cholesterol from total cholesterol levels. Assay of apo[a] levels was performed by IRMA (27) using a commercial kit (Pharmacia, Uppsala, Sweden). Interassay CV was 14% at 112 U/I and 9% at 380 U/I.

Identification of Lp[a] bands

Validation studies were performed using plasma from four of the subjects. Preparation of Lp[a] was based on an adaptation of the method of Chung et al. (28). After a 12-h fast, 10 ml of whole blood was collected from subjects in an EDTA-containing tube and was centrifuged at 1500 g for 10–15 min to separate the plasma. The density of 4 ml of the plasma was adjusted to 1.21 g/ml by the addition of 1.31 g of KBr. This plasma was then pipetted into an ultracentrifuge tube and overlayed with 1.006 g/ml solution. The tubes were sealed and placed in a Beckman VTi65.1 rotor and spun for 90 min at 65000 rpm at 7°C with acceleration and deceleration parameters set at 6. After ultracentrifugation, LDL formed a visible band in the middle of the centrifuge tube with HDL remaining at the bottom of the tube. The tubes were then fractionated using a fractionator and fraction collector (ISCO Lincoln, NB). The ultracentrifuge tubes were punctured at their base without disturbing the LDL layer and a dense immiscible liquid (Maxidens, Nycomed, Oslo, Norway) was pumped by a syringe pump (Razel, Stanford, CT) into the base of the tube displacing its contents upwards. The fraction collector was programmed to collect 13 drops per fraction (400 μl).

Apo[a] and cholesterol levels were assayed on all fractions to identify the density of Lp[a] particles in the ultracentrifuge tube relative to LDL and HDL particles. In addition, all fractions were subjected to non-denaturing gradient gel electrophoresis. The method used was a modified form of that described by Krauss and Burke (18). Approximately 80 μl was added to 20 μl of tracking dye and 60 μl of Tris-borate buffer (pH 8.4) and run at 180 volts for 12 h on a 2–9% non-denaturing polyacrylamide gradient gel. All fractions were run in duplicate on identical gels, with one stained for protein and the other immunoblotted with antibody to apo[a]. This step was done to identify the Lp[a] bands and their relative positions on the gels. The gels stained for protein were first fixed with a 10% 5-sulfosalicylic acid solution for 1 h and then stained with Coomassie blue by immersion in stain for 3–4 h. Next, the gels were destained in a 5% acetic acid solution for 24–48 h. Western blotting (29) was performed on the corresponding gels by the transfer of proteins to a nitrocellulose membrane (0.45 nm pore size) using a Bio-Rad semi-dry electrophoretic for 50 min at 15 volts. The membrane was immersed in 5% skimmed milk powder solution for 1 h at room temperature to block unbound sites. The membrane was then incubated overnight in a 5% skimmed milk solution in TBS (Tris-buffered saline: 20 mM Tris, 50 mM NaCl, pH 7.5) containing 1:500 dilution of mouse monoclonal antibody against apo[a]. The next morning, after extensive washes with TBS and Tween TBS (0.05% Tween 20 detergent in TBS), the membrane was incubated for at least 2 h with the secondary antibody. After further washes with Tween TBS and TBS, the membrane was placed into a color development solution containing the substrate 5-bromo-4-chloro-3-indoyl-phosphate and nitroblue tetrazolium until the bands developed.

Sizing of Lp[a], Lp[a]-, and LDL

Lp[a] was initially isolated as described above using single vertical spin density gradient ultracentrifugation with aspiration of the Lp[a] fraction. We had previously established that the Lp[a] peak was situated between LDL and HDL (see below), usually not visible to the naked eye. In some subjects where the apo[a] level was greater than 800–1000 U/I, the Lp[a] fraction became visible, first as a faint band which then became progressively stronger with increasing levels.

Because of significant contamination of the aspirated Lp[a] with LDL, a further purification step was necessary and was achieved using a lysine-Sepharose affinity column. The aspirated sample was injected onto a Pharmacia XK 500 column containing 15 g of lysine-Sepharose equilibrated in phosphate-buffered saline (PBS). Elution of any contaminating LDL and salts was achieved by washing the column out with PBS. After the contaminants were removed, the Lp[a] was then eluted with 5.5 g/l of ε-aminoacaproic acid in PBS. The eluent was monitored at both 234 nm, which detects salts, lipid and protein and gave greater sensitivity for detection of the Lp[a] peak, and 280 nm to detect predominantly protein (Fig. 1). The Lp[a] peak was collected and then concentrated by dialysis against polyethylene glycol 8000 in a 10,000 MW cut-off filter resulting in an approximately 20-fold concentration. In subjects with apo[a] levels greater than 1000 U/ml, 4 ml of plasma yielded a sufficient amount of Lp[a] to permit sizing, but in those subjects with apo[a] levels less than 100 U/ml, up to 44 ml of plasma was required.
1.2

1.1

.7

1

4

0

1000

2000

3000

4000

5000 6000

time
(Kcs.)

Fig. 1. Elution profile from the lysine-Sepharose affinity column, demonstrating separation of LDL from Lp[a] in subject 17, whose apo[a] level was 1030 U/l (see Table 1). Absorbances were set at (a) 234 nm, which detects salts, lipid and protein and gave greater sensitivity for detection of the Lp[a] peak, and (b) 280 nm, which detects predominantly protein. The initial large peak indicates the elution of salts and LDL. Subsequently Lp[a] was eluted with &-amine caproic acid. The arrow indicates the beginning of the Lp[a] peak.

Lp[a]- was generated by incubating the isolated Lp[a] with 0.01 M dithiothreitol (DTT) at room temperature for 1 h (30), thus cleaving the disulphide link between apoB-100 and apo[a]. LDL was isolated by the same ultracentrifugation technique (28) used for isolation of Lp[a]. After ultracentrifugation, the LDL was visible as a band in the middle of the ultracentrifuge tube, which was aspirated by direct puncture.

Lp[a], Lp[a]-, and LDL particle diameters were determined as previously described (18, 31) using commercially available 3–13% non-denaturing native gels (Gradipore, Sydney, Australia). All gels used in this study were from the same production run. Markers used were 29 nm latex beads (Duke, Palo Alto, CA) and Pharmacia high molecular weight standards (Pharmacia, Piscataway, NJ). The gels were scanned by a Tracktel Video Densitometer Scanner (Vision System Ltd., Adelaide, Australia) to provide a quantitative measurement of the size of the peak and its distance from the origin. Particle diameter was obtained by plotting the log of the known diameter of the standards (y axis) against their positions on the scanned gel (x axis). A statistical package was used to plot a regression and derive a formula allowing unknown samples to be sized. Coefficient of variation on a 26.1 nm quality control sample run on every gel was 0.8%.

To establish that the lysine-Sepharose column did not alter Lp[a] particle size, we selected two individuals with Lp[a] levels high enough for a band to be visible after ultracentrifugation. This band was aspirated and an aliquot was removed before further purification by the column. Both the direct aspirate and the sample run through the column for each subject were run in adjacent lanes on a 3–13% gradient gel and the size was calculated.

It was also important to establish that Lp[a]- size was not significantly altered by the incubation with DTT. Because of the similarity of LDL and Lp[a]-, this question was tested by running 21 samples of LDL with and without incubation with DTT in adjacent lanes on sizing gels.

Apo[a] phenotyping

Fifteen to thirty ml of plasma in EDTA was used to determine apo[a] phenotype by established methods (12, 15). Isoform standards used were designated B, S1, S3, S4, *S4 (Lp[a] phenotyping kit, Immuno-Ag, Vienna, Austria). Both samples and standards were mixed with reducing buffer, incubated at 100°C for 5 min, cooled,
particles in nonfasting subjects with hypertriglyceridemia. A dominant band is shown, but in addition a more faint band was noted on immunoblot (Fig. 3). Subject 25 (Table 1) was the one of the subjects. In subject 25 (Table 1) two co-dominant bands were seen (Fig. 5a) and the Lp[a] particle size used in this case represented the mean of the two peaks. The particle sizes of both Lp[a] and Lp[a]-displayed considerable heterogeneity among different individuals. There was no association of Lp[a] or Lp[a]-size with either the sex or the age of the subjects.

A significant correlation between LDL particle size and Lp[a] particle size (Fig. 6) was noted (r = 0.62; P < 0.001). With the removal of the apo[a] moiety and correlation of LDL particle size with Lp[a]-particle size, this relationship was strengthened considerably (r = 0.82; P < 0.0001). There was, however, a small but significant difference in the mean size of LDL and Lp[a]-with Lp[a]-being larger than LDL (26.1 ± 0.1 nm vs. 25.9 ± 0.1 nm; P < 0.05). There was no association between log apo[a] level and Lp[a]-size (P = 0.7).

Apo[a] also contributed to Lp[a] size, as shown by the inverse relationship (r = -0.44; P < 0.05) between Lp[a] size and log apo[a] level using a simple linear regression. A further measure of the contribution of apo[a] to Lp[a] particle size was derived by subtracting Lp[a]-size from Lp[a] size to give Lp[a] - {Lp[a]-} size. This analysis revealed a significant inverse relationship between the log of the apo[a] level and Lp[a]-{Lp[a]-} size (r = -0.55; P < 0.005). Table 1 shows apo[a] level, triglyceride level, Lp[a] diameter, Lp[a]-{Lp[a]-} diameter, and apo[a] phenotype in each of the 26 subjects studied.

A significant negative association of LDL particle size with log triglyceride level (r = -0.69; P < 0.0001) and positive association with HDL cholesterol level (r = 0.64; P < 0.0005) was seen. Similar associations were noted for Lp[a] and Lp[a]-. Figure 6 demonstrates the association of Lp[a]-size with total triglyceride and HDL cholesterol levels. There was a significant inverse association (r = -0.52; P < 0.01) between Lp[a] diameter and log triglyceride level which was strengthened after the cleavage of the apo[a] moiety by DTT and formation of Lp[a]- (r = -0.72; P < 0.0001). A positive correlation (r = 0.44; P < 0.05) was also noted between HDL cholesterol and

RESULTS

Identification of Lp[a] band

In all four subjects, peak apo[a] levels assayed in the ultracentrifuged fractions were between the LDL and HDL cholesterol peaks in the density range 1.050–1.090 g/ml (Fig. 2). On the gradient gels stained with Coomassie blue, fractions in which the apo[a] levels peaked produced a dominant well-defined band that did not travel as far into the gel and hence had a greater diameter than LDL. This band was confirmed as Lp[a] by immunoblot (Fig. 3). Subject 17, whose immunoblot is shown, was a heterozygote with the phenotype S1/S3 (Table 1). A dominant band is shown, but in addition a more faint band was noted on immunoblot (see fractions 16 and 17 in Fig. 3). Although it has been reported that apo[a] can also be associated with triglyceride-rich particles in nonfasting subjects with hypertriglyceridemia (32), our results are in keeping with previous findings that most of this apolipoprotein is associated with an LDL-like particle in the fasting state (2).

Sizing of Lp[a], Lp[a]-, and LDL

There was no significant effect of DTT on LDL size with a mean particle diameter in the DTT-treated LDL of 25.8 (±0.2) nm and for the native LDL of 25.8 (±0.2) nm (r = 0.99; P < 0.0001). In the two individuals where Lp[a] aspirated directly was run beside Lp[a] eluted from the lysine-Sepharose column on gradient gels, no differences were seen after protein staining and sizing (results not shown).

One dominant Lp[a] band and, after incubation with DTT, one dominant Lp[a]-band (Fig. 4a) were observed in all but one of the subjects. In subject 25 (Table 1) two co-dominant bands were seen (Fig. 5a) and the Lp[a] particle size used in this case represented the mean of the two peaks. The particle sizes of both Lp[a] and Lp[a]-displayed considerable heterogeneity among different individuals. There was no association of Lp[a] or Lp[a]-size with either the sex or the age of the subjects.

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**DISCUSSION**

In a group of subjects with a wide range of apo[a] levels, we have isolated and sized Lp[a], demonstrating a lipoprotein particle that has both greater density and size than LDL. The density range in which peak Lp[a] levels were detected in our study is consistent with the 1.050–1.100 g/ml reported by previous investigators (33, 34). There was considerable size heterogeneity of Lp[a] as reported previously by McNamara et al. (35), who first made use of nondenaturing polyacrylamide gradient gels to identify individuals with elevated Lp[a] levels. In this study we have extended the technique further by incorporating standards that have allowed us to assign a particle diameter to Lp[a] and we have used this technique to determine the size of the LDL-like moiety of Lp[a] known as Lp[a]-.

Our data indicate that Lp[a]- is smaller in size than Lp[a] and that there is a very close correlation between LDL and Lp[a]- particle diameter, a finding that invites comparison of these two cholesterol- and apoB-100-rich particles. It has been known for some time that elevated triglyceride levels and decreased HDL cholesterol levels, or factors that determine these levels, correlate with smaller, more dense LDL particles and that smaller, more dense LDL particles are associated with an increased risk of atherosclerosis (20, 21). Our findings confirm this relationship between lipid parameters and LDL size. In addition, it is now apparent that triglyceride and HDL cholesterol levels, or factors that determine these levels, may also play an important role in determining Lp[a] size. As with triglyceride level, this relationship was strengthened ($r = 0.67; P < 0.0005$) with removal of the apo[a] moiety to produce Lp[a].
size through an influence on the size of the Lp[a]-moiety.

Rainwater et al. (36), using density gradient ultracentrifugation, have reported that apo[a] isoform accounted for approximately 80% of variation in Lp[a] density. Residual Lp[a] density (presumably related to the Lp[a]-moiety) correlated with LDL density, HDL size, and triglyceride level. The authors concluded that small, dense Lp[a] particles are found under conditions leading also to small, dense LDL particles and to small, dense HDL particles. Our findings of a strong correlation between Lp[a]-particle size and LDL particle size, as well as with HDL cholesterol level and the inverse of log triglycerides, are entirely consistent with this recently published study.

In an earlier study, Fless, ZumMallen, and Scanu (37) used analytical equilibrium ultracentrifugation to examine the relative properties of Lp[a], Lp[a]-, and LDL particles. They found that the molecular weight of Lp[a]- was approximately 10% greater than LDL in the two subjects studied. Using the molecular weight and density of LDL and Lp[a]- of the two subjects from that study and making the assumption that these particles are spherical, the calculated difference in diameter is of the order of 2% in both subjects. We also found that the mean diameter of Lp[a]- in our subjects was greater than the diameter of LDL, with a maximum difference of 2.3% and a mean difference of 0.8%.

Although the focus of this investigation was directed at the Lp[a]- portion of Lp[a], we have also addressed the contribution heterogeneity of apo[a] makes to variation in Lp[a] size. The study of Rainwater et al. (36) in a large number of patients has established the dominant contribution of apo[a] phenotype to Lp[a] density. Our data indicate the contribution of apo[a] to Lp[a] size by measuring Lp[a] and Lp[a]- particle sizes and relating the difference in size to apo[a] level and phenotype. The results indicate that both apo[a] and Lp[a]- moieties make independent contributions to Lp[a] diameter.

Thus it appears that the same metabolic factors that determine LDL size also influence Lp[a]-. This influence may occur prior to the association of apo[a] and Lp[a]-, with the latter being drawn from the pool of circulating LDL. Support for this mechanism comes from results of studies with a line of mice expressing the human apo[a] gene, which suggest that human apo[a] may be secreted into the plasma free of lipoprotein and subsequently associate with LDL in the circulation (38). In addition, data from White, Rainwater, and Lanford (39) indicate that baboon hepatocytes secrete apo[a] in the free form and that association between apo[a] and apoB-100 occurs after secretion. However, it also remains possible that the Lp[a]- moiety is not drawn from the circulating LDL pool but, once formed, circulating Lp[a] is subject to modification by the same metabolic influences that determine LDL particle size.

A hypothesis that arises from this study is that the atherogenic risk associated with Lp[a] may not be determined by the apo[a] level alone but also by the nature...
of the Lp[a]- moiety. We suggest that in hypertriglyceridemic individuals, small dense Lp[a] particles may increase cardiovascular risk in a manner analogous to that described for small dense LDL particles (22). Those at particular risk would include patients with Type II diabetes (40) and patients with renal failure treated by peritoneal dialysis (31, 41). Thus individuals may be at greater risk of vascular disease if their apo[a] glycoprotein is associated with small dense Lp[a]- particles compared with other individuals whose apo[a] is associated with larger, less dense Lp[a]- particles. With reference to this last point, it is of interest that several previous studies have failed to show an association between apo[a] levels and atherosclerotic vascular disease in diabetic patients (42, 43) where small dense LDL and presumably small dense Lp[a] particles predominate.

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