Apolipoprotein(a) Alleles Determine Lipoprotein(a) Particle Density and Concentration in Plasma

H.G. Kraft, C. Sandholzer, H.J. Menzel, and G. Utermann

The distribution of Lp(a) lipoprotein (Lp[a]) and genetic apolipoprotein(a) (apo[a]) isoforms in plasma samples from 29 healthy normolipidemic subjects of known apo(a) phenotype was evaluated by density gradient ultracentrifugation. The density of Lp(a) was directly related to the size of the apo(a) isoform, ranging from 1.043 g/ml for the LpF phenotype to 1.114 g/ml for the LpS4 phenotype. Heterozygotes had two distinct Lp(a) particles, each containing one of the respective isoforms in plasma. In each heterozygote, the concentration of the lighter Lp(a) species was higher than that of the denser Lp(a) population. These data suggest that apo(a) alleles determine the density and the metabolism and thereby also the concentrations of Lp(a) particles in plasma. (Arteriosclerosis and Thrombosis 1992;12:302–306)

KEY WORDS • lipoprotein(a) • apolipoprotein(a) isoforms • density gradient ultracentrifugation

Lipoprotein(a) (Lp[a]) is a quantitative genetic trait in human plasma that is associated with premature myocardial infarction and stroke.1 The lipoprotein floats in a density range from about 1.050 to 1.110 g/ml.2 The protein moiety of Lp(a) is composed of apolipoprotein B-100 (apo B-100) and the Lp(a)-specific apolipoprotein(a) (apo[a]). On treatment with thiol-reducing agents, Lp(a) dissociates into a particle very similar to low density lipoprotein (LDL), which contains apo B-100 and all lipids, and apo(a).3-5

Sequencing at the protein and cDNA levels as well as immunological studies has demonstrated a high degree of homology of apo(a) with plasminogen.6-9 Apo(a) exhibits a genetic size polymorphism, with individual isoforms ranging in apparent molecular weight from about 400 kd to >800 kd.10,11 The size differences are thought to result from a variable number of tandem repeats in the apo(a) gene that are homologous to kringle 4 from plasminogen.12,13 At least six different common isoforms of apo(a), which have been designated LpF, LpB, LpS1, LpS2, LpS3, and LpS4, are determined by codominant alleles at the apo(a) structural gene locus.10 The existence of 11 different isoforms has recently been reported,14 but their relation to the originally described genetic isoforms is presently unclear.

Systematic population and family studies have established a unique relation between the apo(a) size polymorphism and the quantitative Lp(a) trait10,11,15 and have demonstrated that the apo(a) structural gene is the major locus for Lp(a) concentration in plasma.16 In the population, an inverse relation exists between apo(a) isoform size and Lp(a) plasma concentration, i.e., smaller apo(a) isoforms are associated with higher Lp(a) levels in plasma. The mechanism by which apo(a) alleles affect Lp(a) plasma concentration is unknown. It has been suggested that a methylated DNA-binding protein that recognizes sequence motifs in the multiple repeats of the kringle 4 module in the apo(a) gene may be involved in the differential transcriptional control of apo(a) alleles.17

In the present study, we have investigated the density and concentration distribution of Lp(a) particles from subjects with known apo(a) phenotype. The following questions were addressed: 1) Do the different genetic isoforms of apo(a) reside on Lp(a) particles of different densities, and if so, 2) are the two apo(a) isoforms of a heterozygous subject present in similar quantities? and 3) Does the inverse relation between apo(a) size and Lp(a) concentration that has been found in the population also exist in a single subject? The results show that apo(a) alleles determine differences in the density and metabolism of Lp(a) particles.

Methods

For this investigation, plasma samples from 29 healthy normolipidemic Caucasian individuals with known apo(a) phenotypes were chosen from ongoing population and family studies. The criteria for their selection were 1) representation of all six common apo(a) isoforms in the sample, 2) a clear and unequivocal typing result on immunoblotting, and 3) availability of the proband for re-collection of a fresh plasma sample. Seventeen subjects showed only one apo(a) band on the immunoblot and are, therefore, either homozygotes for this isoform or are heterozygotes with one null allele.10 The remaining 12 subjects exhibited
two apo(a) bands on the immunoblot and thus were true heterozygotes.

Density gradient ultracentrifugation of human plasma was done according to Redgrave et al.\(^1\) with slight modifications. Sequentially, three density solutions (2 ml of \(d=1.006\) g/ml, 3 ml of \(d=1.019\) g/ml, and 3 ml of \(d=1.063\) g/ml), which were made up with solid potassium bromide, were layered into the tubes of an SW 41 rotor (Beckman Instruments, Palo Alto, Calif.). Finally, a solution of 4 ml plasma adjusted to \(d=1.21\) g/ml with solid potassium bromide was applied at the bottom of the centrifuge tube by using a long thin needle. Centrifugation was carried out in an SW 41 Ti rotor (Beckman) at 10°C at 40,000 rpm for 24 hours. Under the standard conditions of ultracentrifugation according to Redgrave et al.,\(^1\) lipoproteins do not reach equilibrium densities. To allow comparison of results with those obtained under isopycnic conditions, five samples (three single-band phenotypes and two heterozygotes) representing all isoforms were run in parallel for 24 and 48 hours. This allowed us to correct all densities from the 24-hour runs to isopycnic densities. These corrected densities are given throughout. After centrifugation, the tubes were punctured at the bottom and 0.5-ml fractions were collected by using the Beckman fraction recovery system and a fraction collector (LKB, Bromma, Sweden).

The density of the fractions was estimated by measuring the refractive index in a parallel gradient that contained a potassium bromide density solution \((d=1.21\) g/ml) instead of the plasma sample. Cholesterol content of the fractions was determined by employing a commercial test kit (Boehringer-Mannheim, Mannheim, FRG). Before sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the density gradient fractions were extensively dialyzed against phosphate-buffered saline.

Apo(a) phenotyping of the plasma samples as well as of the gradient fractions was performed by immunoblotting exactly as described,\(^10\) employing the monoclonal antibody 1A\(^2\).\(^19\) Quantification of Lp(a) was done by electroimmunodiffusion (intra-assay coefficient of variation [CV], 4.6%; interassay CV, 7.6%) as described by Kremlper et al.,\(^20\) using a commercially available standard serum from Immuno (Vienna, Austria). In several experiments, Lp(a) was additionally quantified by a sandwich-type enzyme-linked immunosorbent assay (ELISA) (intra-assay CV, 2.5%; interassay CV, 4.4%) based on a specific monoclonal antibody.\(^21\)

### Results and Discussion

Plasma samples from 29 individuals of known apo(a) phenotype (including all common isoform types) were subjected to density gradient ultracentrifugation. The resulting fractions were analyzed for cholesterol concentration, Lp(a) concentration, and apo(a) isoform by immunoblotting. Apo(a) phenotype and Lp(a) lipoprotein concentration of the 29 investigated individuals are summarized in Table 1.

Evaluation of gradients from all 29 individuals demonstrated that in each subject, >80% of apo(a) was present in the density interval 1.04–1.13 g/ml, representing Lp(a) lipoprotein. A clear correlation of apo(a) isoform with the density of the respective Lp(a) species emerged (Table 2). The densities of the Lp(a) particles increased with the size of the isoform. Lp(a) particles containing the F isoform had, on average, the lowest density (mean, 1.043 g/ml), being closest to the density of LDL (mean, 1.036 g/ml), whereas Lp(a) of the S4 type had the highest density (mean, 1.114 g/ml).

There was, however, some overlap between the isoform groups. The overlap between Lp(a) particle densities of the different phenotypes may be explained by different lipid contents of the Lp(a) particles and may also be related to the genetically determined density and size heterogeneity of LDL.\(^2\)\(^\text{22}\)

The prototypical immunoblot of density gradient fractions shown in Figure 1 illustrates the occurrence of two Lp(a) particle populations in heterozygotes. This result is not unexpected if the molar ratio of apo B-100 to apo(a) in Lp(a) is 1:1, with only one apo(a) isoform...
TABLE 2. Density of Lipoprotein(a) With Different Apolipoprotein(a) Isoforms

<table>
<thead>
<tr>
<th>Apo(a) isofom</th>
<th>No.*</th>
<th>Lp(a) density (g/ml)</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>4</td>
<td>1.043</td>
<td>1.040-1.049</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>8</td>
<td>1.068</td>
<td>1.058-1.079</td>
<td></td>
</tr>
<tr>
<td>S1</td>
<td>4</td>
<td>1.079</td>
<td>1.066-1.086</td>
<td></td>
</tr>
<tr>
<td>S2</td>
<td>11</td>
<td>1.091</td>
<td>1.083-1.102</td>
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<tr>
<td>S3</td>
<td>5</td>
<td>1.100</td>
<td>1.093-1.108</td>
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<tr>
<td>S4</td>
<td>9</td>
<td>1.114</td>
<td>1.096-1.131</td>
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Apo(a), apolipoprotein(a); Lp(a), lipoprotein(a).

Number of Lp(a) isotypes from different individuals for which the density was determined. The total number of 41 apo(a) isofoms results from 17 homozygous subjects plus 12 heterozygous subjects, with each contributing two isofoms.

in the complex. Such a stoichiometry for Lp(a) particles is in good agreement with both observations: the increase in density of the Lp(a) particle with the increasing size of the apo(a) isofon present in the complex and the presence of two and not three Lp(a) density peaks in heterozygotes that contain two apo(a) isofoms (Figure 1).

Fless et al2 have calculated that the molar ratio of apo B to apo(a) in Lp(a) is 1:2. However, the molecular weight of apo(a), as determined in their study by sedimentation equilibrium in 6 M guanidine hydrochloride, probably was too low (280 kd). The absence of a third “intermediate” Lp(a) particle population in heterozygotes strongly argues against an apo B to apo(a) stoichiometry of 1:2. With such a stoichiometry, occurrence of particles heterodimeric for apo(a) can be expected.

Harvie and Shultz23 were the first to note intraindividual heterogeneity of Lp(a) particle density by analytical ultracentrifugation. Later, Fless et al5 distinguished three different forms of apo(a) and found them to be associated with Lp(a) particles of different densities. These observations are consistent with our findings. However, these authors found only three isofoms in their population as well as more than two Lp(a) density peaks in the plasma from a single individual. It is, therefore, unclear how their isofoms relate to the genetic apo(a) types described by us. Fless et al5 compiled the density profiles of 90 individuals and revealed six major Lp(a) density peaks that might correspond to our six apo(a) isofoms.

Twelve of the subjects in our study were heterozygotes, and 17 had only one apo(a) species in plasma. All subjects with a single apo(a) isofom also had a single Lp(a) species in the density gradient. We have observed Lp(a) in multiple density peaks only in some dyslipidemic subjects (C. Sandholzer and G. Utermann, unpublished observations).

Notably, Lp(a)s of different isofom composition differ not only in density but also in concentration in the plasma of single heterozygous individuals. In the case of the F/S4 heterozygote shown in Figure 1, >77% of Lp(a) is in fractions of mean density 1.042 g/ml containing the F isofom and only <10% is in fractions of mean density 1.097 g/ml representing the S4-containing Lp(a) species. The relative concentrations of Lp(a) particles in different heterozygotes are given in Table 1.

On average, 8±3% (mean±SD) of apo(a) is detected in fractions at opposite ends of the density gradient: 5±4% of apo(a) is found in fractions of density <1.006 g/ml, and 3±2% is found in the lipid-free >1.21 g/ml density fraction. The identity of the immunoreactive material in fractions of density <1.006 g/ml and >1.21 g/ml with apo(a) was confirmed by immunoblotting. The intensities of the apo(a) bands on the immunoblots from the three apo(a)-containing fractions corresponded well with the concentrations determined by immunochemi-
The striking differences in Lp(a) concentration between heterozygotes on immunoblots suggested that genetic apo(a) isoforms occur in different concentrations also in the plasma of single donors. A similar finding was obtained very recently by Gaubatz et al., who also found that the lower M₄ band was more intense than the higher one in a majority of cases. The results presented here clearly show that apo(a) isoforms of different size occur in different particles and with different concentrations. Qualification of Lp(a) in the density gradient fractions from heterozygotes showed that more dense Lp(a) particles containing the larger apo(a) isoforms were consistently less abundant than their lighter counterparts with smaller isoforms. Hence, the general relation between apo(a) size and Lp(a) concentration that was established in population studies is reflected in single subjects, too.

It might be argued that the apparent differences in Lp(a) particle concentration reflect differences in immunoreactivity of the apo(a) species rather than true differences in particle mass. There may be more identical antigenic determinants recognized by the antibody in a large apo(a) species if this antibody is directed against the multiple kringle 4 repeats in apo(a). However, this would result in an overestimation rather than an underestimation of those particles containing large isoforms. On the other hand, differences in mass of the various apo(a) isoforms might result in an overestimation of Lp(a) concentration in subjects with smaller isoforms at the expense of those with larger isoforms. The striking differences in Lp(a) concentration between and within subjects with different apo(a) isoforms cannot, however, be explained by such relatively minor potential sources of error. Also, the same plasma concentrations and quantitative distributions in the density gradients were obtained by electroimmuno diffusion by using a polyclonal antibody and by a sandwich ELISA that uses a monoclonal antibody for detection (Table 1). Moreover, the ratio of the two Lp(a) species in heterozygotes, as determined by immunochromatographic quantification in density gradient fractions, agreed well with the ratio of the two apo(a) isoforms in total plasma as determined by densitometric scanning of the immunoblots (G. Utermann, unpublished data).

Together, our results demonstrate that genetically different Lp(a) particles behave metabolically differently even in the same subject. Either they differ in their rate of synthesis, assembly, or secretion, or they are catabolized at different rates. A first indication that this regulation takes place at the transcriptional level in humans was very recently given by Koschinsky et al., who found a higher abundance of the smaller apo(a) mRNA compared with the larger variety in three heterozygous individuals. The results of turnover studies by Krempler et al. also demonstrated that differences in synthesis rather than catabolism may explain the variations in Lp(a) plasma concentration among subjects.

On the gene level, the distinct apo(a) isoform sizes result from different numbers of tandemly repeated kringle 4 units in the apo(a) gene. It will be interesting to see how the apo(a) allele size differences determine differences in the concentrations of the respective Lp(a) particles in plasma. The effects might be a direct consequence of the variations in the size of the apo(a) DNA, mRNA, or protein. However, there could also be linkage disequilibrium between the structural kringle 4 domains and regulating elements in the apo(a) gene.

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