Lp(a) Glycoprotein Phenotypes

Inheritance and Relation to Lp(a)-Lipoprotein Concentrations in Plasma

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Abstract

The Lp(a) lipoprotein represents a quantitative genetic trait. It contains two different polypeptide chains, the Lp(a) glycoprotein and apo B-100. We have demonstrated the Lp(a) glycoprotein directly in human sera by sodium dodecyl sulfate–gel electrophoresis under reducing conditions after immunoblotting using anti–Lp(a) serum and have observed inter- and intraindividual size heterogeneity of the glycoprotein with apparent molecular weights ranging from ~ 400,000–700,000 D. According to their relative mobilities compared with apo B-100 Lp(a) patterns were categorized into phenotypes F (faster than apo B-100), B (similar to apo B-100), S1, S2, S3, and S4 (all slower than apo B-100), and into the respective double-band phenotypes. Results from neuraminidase treatment of isolated Lp(a) glycoprotein indicate that the phenotypic differences do not reside in the sialic acid moiety of the glycoprotein. Family studies are compatible with the concept that Lp(a) glycoprotein phenotypes are controlled by a series of autosomal alleles ($Lp[a]^F$, $Lp[a]^B$, $Lp[a]^{S1}$, $Lp[a]^{S2}$, $Lp[a]^{S3}$, $Lp[a]^{S4}$, and $Lp[a]^0$) at a single locus. Comparison of Lp(a) plasma concentrations in different phenotypes revealed a highly significant association of phenotype with concentration. Phenotypes B, S1, and S2 are associated with high and phenotypes S3 and S4 with low Lp(a) concentrations. This suggests that the same gene locus is involved in determining Lp(a) glycoprotein phenotypes and Lp(a) lipoprotein concentrations in plasma and is the first indication for structural differences underlying the quantitative genetic Lp(a)-trait.

Introduction

The Lp(a) lipoprotein was first demonstrated in human plasma by Berg (1) as a genetic variant of low-density lipoprotein (LDL)[1] and believed to be transmitted as an autosomal dominant trait (1). Later investigators using quantitative immunochemical methods have provided evidence that the Lp(a) lipoprotein represents a quantitative rather than a qualitative genetic marker and is under polygenic control probably with a major gene effect for high Lp(a) concentrations (2–4). As early as 1967 Renninger et al. (5) recognized a positive association of Lp(a) lipoprotein with myocardial infarction. A series of subsequent investigations have confirmed and extended this observation (6–8). Therefore it has been postulated that high concentrations of the Lp(a) lipoprotein in plasma represent an independent risk factor for the development of coronary heart disease (8).

The Lp(a) lipoprotein is a spherical particle of 250 Å diameter that floats in a density range of ~ 1.05 to 1.1 g/ml. The lipid composition of Lp(a) lipoprotein closely resembles that of LDL. The protein moiety consists primarily of apo B-100 and the Lp(a) protein (9–13). The Lp(a) protein is a glycoprotein rich in neuraminic acid that stains strongly with periodic acid–Schiff and exhibits a high apparent molecular weight upon sodium dodecyl sulfate–gel electrophoresis (SDS-PAGE) (10, 12, 13). It is linked to apo B-100 by one or more disulfide bridges in the intact particle (12, 13). It is not known whether the Lp(a) glycoprotein interacts with lipids and is a true apolipoprotein. The presence of apo B-100 in the Lp(a) lipoprotein explains its immunochemical cross-reactivity with LDL whereas the Lp(a) protein is responsible for the specific immunochemical properties and the higher density of the Lp(a) lipoprotein. There are conflicting results on the binding of Lp(a) lipoprotein to the LDL (B/E) receptor (14–17).

Recent studies on isolated Lp(a) lipoprotein have demonstrated density heterogeneity of the lipoprotein (18, 19) and size heterogeneity of the Lp(a) glycoprotein (13, 19). In the present study we have investigated the heterogeneity of the Lp(a) protein in whole plasma and in fractions prepared by density gradient ultracentrifugation, using the Western blot method. This confirmed the remarkable size heterogeneity of the Lp(a) glycoprotein and moreover demonstrated that electrophoretic Lp(a) glycoprotein phenotypes are inherited and are associated with Lp(a) lipoprotein concentrations in plasma.

Methods

Plasma samples were collected in citrate from all unrelated volunteers (n = 247) attending the Plasmadienst Tirol GmbH in Innsbruck for plasmapheresis for a period of 2 wk during fall 1985 who fulfilled the medical requirements to donate plasma. Health examination of the plasma donors included a thorough physical examination, determination of blood pressure, and the following laboratory tests: total serum protein, hemoglobin, hematocrit, HBS Ag, serum glutamic pyruvic transaminase (SGPT), cardiolipin, HTLV III, serum electrophoresis, differential blood count, blood sedimentation rate, leukocyte count, urinary protein, glucose, and pH. Determination of Lp(a) concentrations and Lp(a) phenotyping was performed on all volunteers. A second blood sample was obtained from 18 of the above subjects between 1 mo and 1 yr later, and plasma was obtained under conditions minimizing proteolysis (19). From two subjects with double-band phenotypes and two with single-band phenotypes (see Results) blood was drawn repeatedly over a period of 1 yr and either plasma was obtained as previously described or serum was obtained by low-speed centrifugation after allowing the blood to clot

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1. *Abbreviations used in this paper:* DTE, dithioerythritol; LDL, low-density lipoprotein.

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for 1 h at room temperature. Lp(a) proteins of these subjects served as internal markers in the electrophoretic typing of Lp(a) protein patterns. Lp(a) glycoprotein phenotyping was performed in 22 members of three families. The probands for this study were selectively chosen according to various criteria either individually or in combination to obtain informative matings (e.g., high Lp(a) levels, a rare phenotype, presence of a double-band phenotype, etc.). Plasma or sera were used for Lp(a) analysis either within the first 2 d or were frozen immediately at −20°C until analyzed.

Acrylamide and (*N,N'*-methylene) bisacrylamide were from LKB Instruments Inc. (Bromma, Sweden). SDS was purchased from Bio-Rad Laboratories (Richmond, CA) and recrystallized from absolute ethanol. Goat anti-rabbit IgG were from Behring Werke (Marburg, FRG) or Bio Yeda (Rehovot, Israel). Bovine serum albumin was purchased from Boehringer Mannheim GmbH (Mannheim, FRG). Clostridium perfringens neuraminidase was from Sigma Chemical Co. (St. Louis, MO). High molecular weight protein standards were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden).

Lp(a)-lipoprotein and Lp(a)-protein isolation. The purification of Lp(a) was performed using density gradient ultracentrifugation of serum or plasma according to Redgrave et al. (20) with minor modifications (21). The distribution of Lp(a) lipoprotein in the gradient fractions was determined by electroimmunodiffusion. The Lp(a)-positive fractions from the density gradient were combined and 3 ml of the solution of $d \sim 1.06$ g/ml as determined by refractometry in a parallel gradient run without sample, were layered below 2 ml density solution of d 1.019 g/ml and 3 ml density solution of 1.035 g/ml. Below the Lp(a) fraction 4 ml of density solution of d 1.112 g/ml were layered. Centrifugation was performed in a SW 41-Rotor (Beckman) for 24 h at 4°C and 40,000 rpm. Fractions of 0.5 ml were collected from below from the continuous gradient that had formed during centrifugation. The Lp(a) lipoprotein was localized in the gradient fractions by electroimmunodiffusion using anti-Lp(a) serum. Purity of the Lp(a) lipoprotein was checked by PAGE (12) and by Ouchterlony double diffusion using anti-apo A-I and anti-Apo E antibodies.

In experiments designed to dissociate the Lp(a) lipoprotein, the isolated lipoprotein was incubated in the presence of 0.5 mM dithioerythritol (DTE) for 2 h at 37°C and centrifuged under the conditions described by Redgrave et al. (20) but using 3 ml of Lp(a) fraction instead of density solution 1.063 g/ml and 4 ml of density solution 1.21 g/ml instead of serum adjusted to 1.21 g/ml.

Cholesterol, apolipoprotein B, and the Lp(a) antigen were measured in the fractions. Purity of the Lp(a) protein was checked by SDS-PAGE (see Results).

Analytical and immunochemical methods. Cholesterol was measured with a commercial test kit (Boehringer Mannheim GmbH) and apolipoprotein B was determined by rocket immunoelectrophoresis. Lp(a) lipoprotein concentrations were determined by electroimmunodiffusion essentially as described by Krempler et al. (22) using polyclonal rabbit anti-Lp(a) and a purified human Lp(a) lipoprotein reference standard (Immuno AG, Vienna, Austria) prepared and calibrated essentially as described by Krempler et al. (22). Purified Lp(a) preparations from different phenotypes (F, S1, S3) with identical protein concentrations (Lowry determination) and apo B concentration (Laurell electrophoresis) yielded identical Lp(a) lipoprotein concentrations in this assay. Antisera were produced in rabbits using LDL and Lp(a) lipoprotein as antigens following the immunization procedure described by Berg (1). Antibodies were tested for specificity by reaction with human serum, isolated LDL, and Lp(a) lipoprotein in double diffusion experiments and by the Clark-Freeman technique as described (23). Antibodies produced against LDL exhibited only one precipitation line and reacted with apo B-100 in immunoblot experiments (anti-apo B). The antiserum against Lp(a) lipoprotein exhibited two precipitation lines, one of which showed complete identity with LDL and the other with Lp(a) but not LDL. The anti-Lp(a) serum was made specific by absorption with purified LDL and the γ-Globuline fraction was prepared by DEAE chromatography. Antibodies against rabbit IgG from goat were purchased from Behring Werke and labeled with ^{125}J (Amersham Buchler GmbH, Braunschweig, FRG sp act 13.6 mCi ^{125}J/μg) by the chloramine T method (24). Chloroauric acid was purchased from Sigma Chemical Co. The preparation of gold sol and the gold-IgG complexes was done according to Lin and Langenberg (25).

Electrophoretic procedures. 10 μl serum or plasma or equivalent fractions from density gradient ultracentrifugation that had been extensively dialyzed against 0.15 M NaCl; 0.05% EDTA, pH 7.4, was pipetted into 250 μl 5% SDS, 5 μl β-mercaptoethanol, and 10 μl 1.5% bromophenol blue in glycerol, and the solution was heated for 10 min in a boiling water bath. 50-μl aliquots of the mixture, corresponding to ~ 1.8 μl serum, were used as sample for electrophoresis. Higher sample loads resulted in overloading of gels and distortion of Lp(a) bands, thus making reliable phenotyping impossible. A reference plasma with a known double-band Lp(a) phenotype was included in each run. SDS-PAGE electrophoresis was routinely performed in 6.6% slab gels using the discontinuous buffer and gel system of Neville (26) and the electrophoresis equipment type SE 600 (Hoefer Scientific Instruments, San Francisco, CA). Gels were either fixed and stained with Coomassie Brilliant Blue R-250 or used for immunoblotting.

The second dimension transfer of protein to nitrocellulose filter (BA 85, 0.45 μm, Schleicher & Schuell, Keene, NH) was performed according to Towbin et al. (27) using the Transblot cell (Bio-Rad Laboratories). Antigens were localized on the nitrocellulose using a double-antibody procedure involving rabbit anti-Lp(a) γ-globulins or anti-apo B antiserum as a first antibody and ^{125}J-labeled anti-rabbit IgG (100,000 cpm/ml) or gold-labeled IgG (E_{520} = 0.4) as the second antibody. Radioactive labeled protein bands were visualized by exposure to an X-Omat XAR5 X-ray film (Eastman Kodak Co., Rochester, NY) using a reflector Cronex Quanta IIF (DuPont Co., Wilmington, DE). The gold-labeled bands could be judged immediately or if further enhancement was needed a physical developer consisting of silver lactate and hydroquinone was employed (28). Molecular weight determination was done in the SDS-PAGE Systeme of Neville (26). The high molecular weight standard from Pharmacia Fine Chemicals and apo B-100 (mol wt 500,000) were used as calibration standards. Enzymatic digestion of Lp(a) glycoprotein with neuraminidase was done according to instruction of the purchasing company.

Statistical methods. Overall comparison of Lp(a) lipoprotein concentrations between phenotypes was done by the nonparametric Kruskal-Wallis test. Pairwise comparison of Lp(a) concentrations between phenotypes was performed by two-tailed Mann-Whitney test.

Results

Demonstration of Lp(a) glycoprotein from human plasma by immunoblotting. Lp(a) lipoprotein concentrations were determined by electroimmunodiffusion in the plasma of 247 healthy individuals. In agreement with previous studies (29) the distribution of Lp(a) lipoprotein concentrations in the group of plasma donors was of higher order (Fig. 1). Plasma samples from all blood donors were subjected to SDS-PAGE under reducing conditions, and the Lp(a) protein was demonstrated by immunoblotting using polyclonal rabbit anti-Lp(a) antibodies and either ^{125}J-labeled anti-rabbit IgG or gold-labeled anti-rabbit IgG as second antibody. For visualization of weak Lp(a) bands the gold-labeled blots were further developed by silver staining. Both methods had a similar sensitivity and detected amounts of Lp(a) protein corresponding to ~ 90 ng of Lp(a) lipoprotein. The concentration of Lp(a) lipoprotein in plasma clearly correlated with the intensity of major high molecular Lp(a)-positive bands detected by Western blotting. Subjects with high intermediate or unmeasurable Lp(a) lipoprotein concentrations in plasma exhibited strong, intermediate, or faint/absent high molecular weight Lp(a)-reactive bands, respectively, upon electrophoresis (Fig. 2). All plasma samples with Lp(a) lipoprotein concentrations > 7 mg/dl exhibited a Lp(a)-reactive band (or bands) upon electropho-

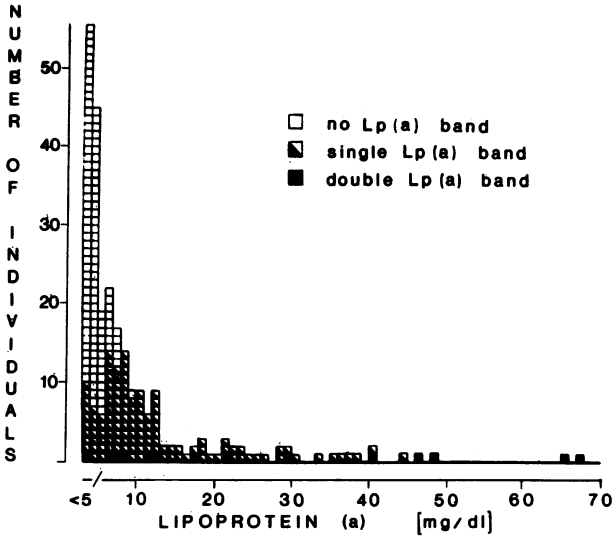

Figure 1. Distribution of Lp(a) concentrations in 247 plasma donors. Symbols denote presence or absence of Lp(a) protein upon Western blotting.

resis that occurred in a high molecular weight range (Figs. 2 and 3).

Of the 247 tested subjects, 77% had Lp(a) concentrations above the detection limit of electroimmunodiffusion and 51% exhibited an Lp(a) band(s) upon immunoblotting. 48% were positive with both techniques, whereas 31% were positive by one but negative by the other technique.

Some plasma specimen with Lp(a) concentrations under the detection limit of our electroimmunodiffusion assay still did exhibit a weak Lp(a) band(s) upon Western blotting (3%) whereas in other sera no Lp(a) band(s) were visible despite of the presence of low concentrations of immunochemically detectable Lp(a) antigen in plasma (29%). No low-molecular weight Lp(a)-specific bands were detected in such sera upon gradient gel electrophoresis (3–30%). Despite the discrepancies in the low concentration range that by all probability have technical reasons there was a good agreement between the results from both methods.

Size heterogeneity of Lp(a) glycoprotein. The mobility of the Lp(a) glycoprotein in SDS-PAGE differed considerably between individuals indicating size heterogeneity of the protein (Figs. 2 and 3). Some individuals exhibited two Lp(a) glycoprotein bands, but these bands did not always have the same intensities. Within one individual however the pattern remained constant with time, e.g., the relative positions and the number of Lp(a) bands were identical when blood samples were obtained repeatedly over a period of 1 yr (Fig. 4). Plasma or serum samples taken at different occasions from the same individuals always yielded identical patterns in a given subject. Hence proteases liberated during blood clotting do not affect the patterns. Moreover when plasma samples were recollected from 18 subjects under conditions minimizing proteolysis (19), each individual exhibited essentially the same Lp(a) protein pattern as determined in the original sample. This individual constancy and high reproducability of Lp(a) glycoprotein patterns make it unlikely that the major Lp(a) protein species are proteolytic fragments. We conclude that different individuals may have Lp(a) glycoprotein species of different primary structure or different degrees of posttranslational modification in plasma. According to their relative mobilities compared with apo B-100, Lp(a) glycoprotein patterns were categorized into phenotypes F (faster than apo B-100), B (similar to B-100), S1, S2, S3, and S4 (slower to different degrees than apo B-100), and into the respective double-band phenotypes (Fig. 3). The frequencies of these phenotypes in the plasma donors with a detectable Lp(a) band upon immunoblotting are given in Table I. Most subjects exhibited Lp(a) glycoprotein species with an apparent molecular weight higher than apo B-100, e.g., phenotypes S1 (14%), S2 (26%), S3 (26%) or S4 (20%), but occasionally bands with an apparent molecular weight similar to apo B-100 (phenotype B) were observed. None of the blood donors exhibited the F phenotype. This type was seen only twice in a large group of patients with hyperlipidemia and/or coronary heart disease and seems to be rare among Caucasians (H. G. Kraft, H. J. Menzel, T. Hopferwieser, G. Utermann, unpublished results).

Lp(a) phenotyping could also be performed on plasma or sera frozen at −20°C for several months. With some of our anti-Lp(a) antibodies all samples exhibited a minor band in the position of apo B-100 (compare Figs. 2 and 4 with Figs. 3 and 9). In plasma with low Lp(a) concentration, presence of this band might suggest a B phenotype or a double-band phenotype. However this band was even present in plasma without im-

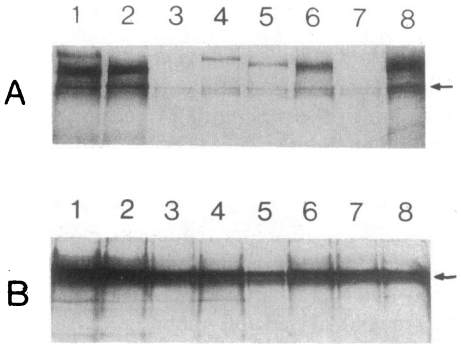

Figure 2. Demonstration of Lp(a) protein and apo B in individual sera of subjects with varying Lp(a) lipoprotein concentrations by immunoblot analysis using anti-Lp(a) (*A*) and anti-apo B (*B*). Sera (1.8 μl) were subjected to SDS-PAGE under reducing conditions in 7.5% polyacrylamide gels, and proteins were transferred to nitrocellulose by electroblotting. Lp(a) protein and apo B were demonstrated by incubation of the nitrocellulose sheets with the respective rabbit antisera followed by ^{125}J-labeled goat anti-rabbit IgG. Sera had the following Lp(a) lipoprotein concentrations: lane *1*, 78 mg/dl; lane *2*, 58 mg/dl; lane *3*, 3 mg/dl; lane *4*, 3.5 mg/dl; lane *5*, 5 mg/dl; lane *6*, 9.5 mg/dl; lane *7*, 3 mg/dl; lane *8*, 29 mg/dl. Arrows denote position of apo B-100. Note also the double Lp(a) band in lane *1*.

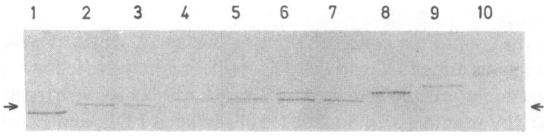

Figure 3. Demonstration of different Lp(a) glycoprotein phenotypes in individual plasma samples by immunoblot analysis. Plasma (1.8 μl) was subjected to SDS-PAGE under reducing conditions in 6.6% polyacrylamide gels. Proteins were transferred to nitrocellulose by electroblotting Lp(a) glycoprotein was demonstrated by incubating the nitrocellulose sheets with rabbit anti-Lp(a) γ-globulins followed by gold-labeled anti-rabbit IgG. Lane *1*, F-type; lanes *2* and *3*, B-type; lanes *4*, *5*, and *7*, S1-type; lane *6*, S1/S2-type; lane *8*, S2-type; lane *9*, S3-type; lane *10*, S4-type. Arrows indicate relative position of B-100.

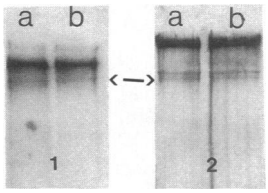

Figure 4. Demonstration of Lp(a) protein (7.5% acrylamide gels) by immunoblotting in sera from two individual subjects (*1* and *2*). Samples were obtained from each subject at a 1-yr interval (*a* and *b*). Note differences in mobility of Lp(a) protein between the individuals and the constancy of the pattern within the same subject. Arrow indicates position of apo B-100.

munochemically detectable Lp(a) antigen and was clearly distinct in position from the B phenotype (Fig. 2). When present, this band occurred in the same position in all samples regardless of the mobility of the major Lp(a) species and regardless of whether there was a single- or double-band phenotype. Upon density gradient ultracentrifugation this material was found preferentially unassociated with lipoproteins in the bottom fractions of the gradient (data not shown). This indicates that this material either represents an unspecificity or a constant degradation product of Lp(a) not related to the size polymorphism. This band if present was therefore ignored in the phenotyping of Lp(a) glycoprotein patterns. However it proved helpful as an internal marker for alignment and comparison of Lp(a) patterns.

Dissociation of Lp(a) glycoprotein from apo B-100 lipoprotein. Lp(a) lipoprotein was isolated by density gradient ultracentrifugation. The isolated lipoprotein (2 mg protein/12 ml) was incubated with 0.5 mM DTE at 25°C for 2 h and subjected to density gradient ultracentrifugation (Fig. 5). A second aliquot of Lp(a) lipoprotein was incubated and centrifuged in the absence of DTE and served as control. Analysis of density gradient fractions revealed that the DTE-treated lipoprotein had dissociated into two components, one with the density and electrophoretic mobility of LDL that contained exclusively apo B-100 and all cholesterol, and another that occurred in the bottom fractions of the gradient and contained Lp(a) glycoprotein but neither apo B-100 nor cholesterol (Figs. 5 and 6). The isolated Lp(a) glycoprotein had the same electrophoretic pattern as the authentic material in total plasma (Fig. 6).

Table I. Frequencies of Lp(a) Phenotypes in Plasma Donors

Phenotype	No.	Frequency
		% positives
Single-band		
F	0	0
B	4	3
S1	18	14
S2	33	26
S3	33	26
S4	25	20
Double-band		
BS_1	1	0.8
BS_2	2	1.5
BS_3	1	0.8
S_1S_2	1	0.8
S_2S_3	4	3.1
S_2S_4	1	0.8
S_3S_4	3	2.2
No band detectable	121	49
Total	247	

To determine whether the differences in electrophoretic mobility of Lp(a) bands are due to differences in the sialic acid content of the sialic acid–rich protein (10), we incubated the isolated glycoproteins from subjects with phenotypes F, B, S1/S2, and S3/S4 with neuraminidase. Lp(a) glycoprotein treated with neuraminidase had a lower apparent molecular weight by ~ 50,000 D than the untreated protein (shown for phenotypes F and S1/S2 in Fig. 7). In Lp(a) glycoprotein preparations with a double-band phenotype both bands were shifted to a lower molecular weight range. But most importantly, the differences between the phenotypes were not abolished by treatment with neuraminidase (Fig. 7). Hence it seems unlikely that the differences between Lp(a) phenotypes reside in the sialic acid moiety of the glycoprotein.

Association between Lp(a) phenotypes and Lp(a) lipoprotein concentration. Analysis of Lp(a) lipoprotein concentrations in healthy individuals with different single-band phenotypes suggested an association of Lp(a) glycoprotein phenotype with Lp(a) lipoprotein concentrations in plasma (Table II). However for each phenotype the Lp(a) values measured covered a wide range. Lp(a) concentrations at the lower end of the total range are common whereas those at the upper end are rare, giving rise to an asymmetric, nonnormal distribution (Fig. 1). Hence nonparametric tests have been applied to compare Lp(a) concentrations of different phenotypes. Overall comparison demonstrated highly significant differences between Lp(a) concentrations of the different phenotypes (Kruskal-Wallis test, $P < 0.005$). Pairwise comparison by two-tailed Mann-Whitney test showed that the four frequent phenotypes may be split up into two pairs (S1/S2 and S3/S4) with nonsignificant differences between the Lp(a) concentrations within a pair, e.g., S1 vs. S2 and S3 vs. S4, but significant differences between Lp(a) values of phenotypes in different pairs, e.g., S1 vs. S3 ($P \sim 0.05$), S1 vs. S4 ($P \sim 0.05$), S2 vs. S3 ($P \sim 0.001$), and S2 vs. S4 ($P \sim 0.002$). Hence phenotypes S1 and S2 and possibly also B are associated with high concentrations and phenotypes S3 and S4 with low Lp(a) concentrations in plasma. Phenotype B was not included in the pairwise comparison due to the small number of individuals in this group, but it seems to be associated with high Lp(a) concentrations.

Together these data indicate that Lp(a) lipoprotein concentrations in plasma are inversely related to apparent molecular weight of the Lp(a) glycoprotein. There was however considerable overlap between Lp(a) concentrations of the different phenotypes, and it is not possible to predict Lp(a) concentrations from Lp(a) phenotype or vice versa (see Table II).

Family studies: results and interpretation. Pedigrees of the three families studied are shown in Fig. 8 and a respective immunoblot in Fig. 9. The family material is still limited and contains only a few of the many possible mating types. Nevertheless the data obtained are highly indicative of a genetic transmission of Lp(a) glycoprotein phenotypes. The following relevant observations were made: (*a*) No Lp(a) glycoprotein species occurred in offspring that were not present in one of the parents. (*b*) Lp(a) species that are rare in the population (e.g., B-phenotypes; see Table I) were seen regularly among offspring of individuals with such phenotypes. (*c*) Among the offspring of a parent with a double-band phenotype and a spouse without detectable Lp(a), only the respective single-band phenotypes were present.

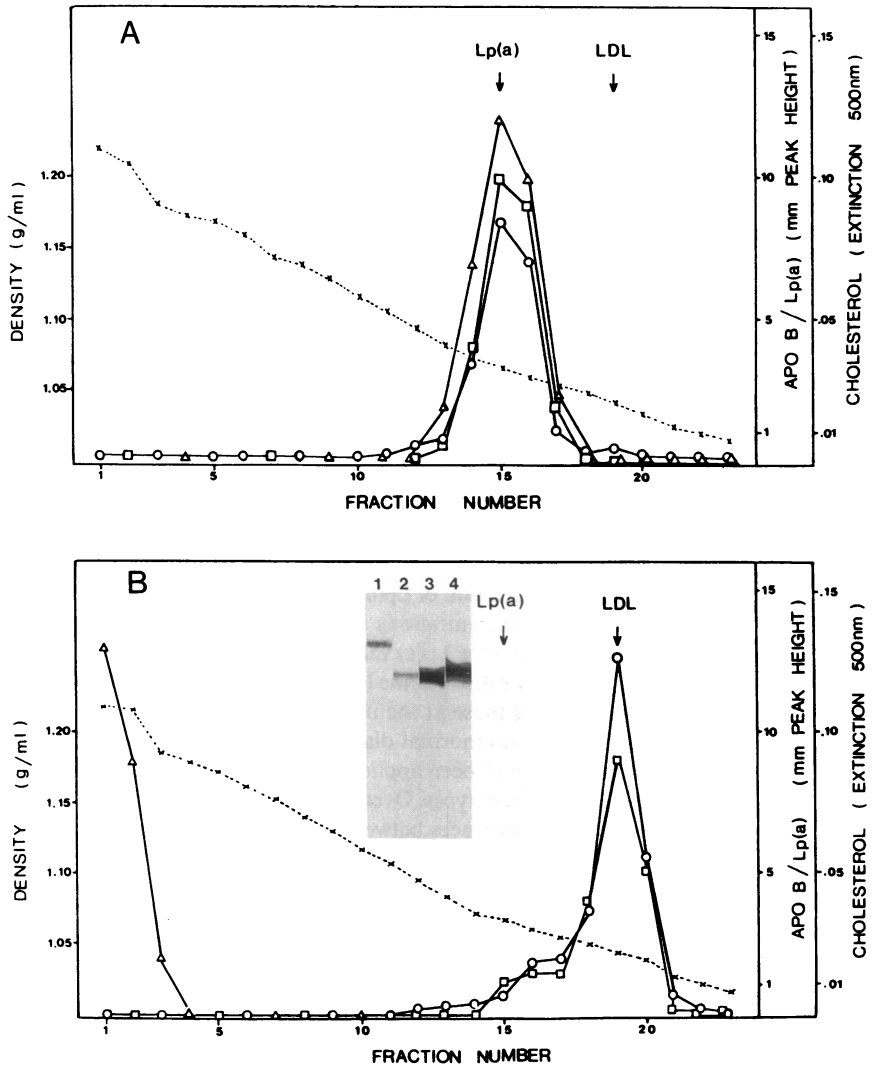

Figure 5. Density gradient centrifugation profiles of native (*A*) and DTE-treated Lp(a) lipoprotein (*B*). Density, (×); Lp(a), (△); apo B, (□); and cholesterol, (○). Insert shows PAGE of prestained lipoprotein fractions in 3.75% gels: lane *1*, Native Lp(a) fraction 15, diagram A; lane *2*, DTE-treated Lp(a) fraction 19, diagram B; lanes *3* and *4*, LDL before and after treatment with DTE, respectively.

Whereas each of these findings may be explained by chance, all observations together are highly indicative of a genetic transmission of Lp(a) protein species and may be explained by a model where a series of autosomal codominant alleles at a single locus designated $Lp(a)^F$, $Lp(a)^B$, $Lp(a)^{S1}$, $Lp(a)^{S2}$, $Lp(a)^{S3}$, $Lp(a)^{S4}$ and an allele operationally defined as a null allele ($Lp[a]^0$) control Lp(a) glycoprotein polymorphism. According to this concept individuals with a single-band phenotype may be either homozygotes or heterozygotes carrying the respective expressed allele plus a "null" allele. This explains the occurrence of different single-band phenotypes in a parent and offspring, e.g., B and S2 in the Du family and also the occurrence of "null" phenotypes (genotype $Lp[a]^0/Lp[a]^0$) in offspring of this family. According to our interpretation both parents in the Du family are heterozygotes, one being $Lp(a)^B/Lp(a)^0$ and the other $Lp(a)^{S2}/Lp(a)^0$, thus allowing for the occurrence of B, S2, B/S2, and O-phenotypes among their offspring. The postulate of a $Lp(a)^0$ allele is in keeping with the population data where a high frequency of individuals present with a "null phenotype." Hence $Lp(a)^0$ has to be considered the most frequent allele in our population. Because the definition of a "null phenotype" depends at least in part on the sensitivity of our assay we use the term "operational null allele" to account for this fact. Therefore individuals with a "null phenotype" may well express Lp(a) glycoprotein species even though under the detection limit of our immunoblot procedure.

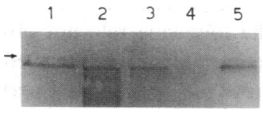

Figure 6. Immunoblot of the Lp(a) protein in serum and density gradient fractions (see Fig. 5) from the same individual (6.6% gels). Lane *1*, serum of subject with an F phenotype; lane *2*, Lp(a) lipoprotein isolated by density gradient ultracentrifugation; lane *3*, Lp(a) lipoprotein recentrifugated in absence of DTE (control); lane *4*, LDL fraction after DTE treatment, and lane *5*, bottom fraction of Lp(a) after DTE-treatment. Arrow indicates position of B-100.

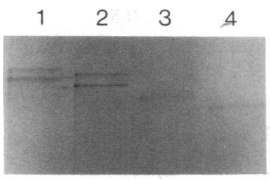

Figure 7. SDS-PAGE (6.6% gels) of isolated native and neuraminidase-treated Lp(a) protein. Coomassie staining. Lane *1*, S1/S2-type Lp(a) protein; lane *2*, same S1/S2 Lp(a) protein treated with neuraminidase; lane *3*, F-type Lp(a) protein; and lane *4*, same Lp(a) protein treated with neuraminidase.

Table II. Lp(a) Lipoprotein Concentrations in Plasma Donors with Different Single-band Lp(a) Phenotypes

Lp(a) phenotype	No.	Mean	SD	Median	Range
B	4	28.0	13.4	31.0	10–40
S1	18	16.3	14.1	13.5	0–46
S2	33	15.5	9.8	12.0	0–44
S3	33	8.2	4.2	8.0	0–22
S4	25	8.0	5.4	8.0	0–28

Concentrations measured in milligrams per deciliter. Significance of overall differences between phenotypes, $P < 0.005$ (Kruskal-Wallis test).

Figure 9. Immunoblots of Lp(a) glycoprotein from the plasma of individual members of the Du family (see Fig. 8). After SDS-PAGE (6.6% gel) and electroblotting to nitrocellulose Lp(a) glycoprotein was demonstrated by incubation with rabbit anti–Lp(a) antibodies followed by gold-labeled anti–rabbit IgG. *C* denotes a reference plasma, and *F* and *M* the father and mother, respectively. Immunoblots from children 1–9 (same order as in pedigree) are shown. Phenotypes are indicated. Note different band intensities of B and S2 protein within the same individual.

Notably Lp(a) phenotypes seem to be associated with Lp(a) concentrations even within the large Du family. Mean Lp(a) concentrations in the different phenotypes (genotypes) were B/S2 = 60 mg/dl, B(B/O) = 47 mg/dl, S2(S2/O) = 30 mg/dl, and O(O/O) = 6 mg/dl. This observation may indicate that Lp(a) alleles affect Lp(a) concentrations in an additive manner.

Discussion

Previous studies on the protein moiety of the Lp(a) lipoprotein have shown that it contains two major protein components, apo B-100 and the Lp(a) glycoprotein linked by disulfide bridge formation in the intact particle (12, 13). Here we show that reductive cleavage of disulfide bonds in isolated Lp(a) lipoprotein by DTE or 2-mercaptoethanol followed by electrophoresis or ultracentrifugation allows for the separation of the Lp(a) protein from a LDL-like particle (see Figs. 5 and 6). While this work was in progress Armstrong et al. (17) and Fless et al. (30) reported similar findings. The Lp(a) glycoprotein isolated by ultracentrifugation is water soluble (Kraft, H. G., H. J. Menzel, and G. Utermann, unpublished observation) and identical in apparent molecular weight with the authentic glycoprotein in plasma. In the present study we used SDS-PAGE under reducing conditions followed by immunoblotting to demonstrate Lp(a) glycoprotein directly from the plasma or sera of unrelated individuals and in family studies. This revealed a striking inter- and intraindividual size heterogeneity of the Lp(a) glycoprotein. The patterns obtained from plasma blots were identical with those obtained from iso-

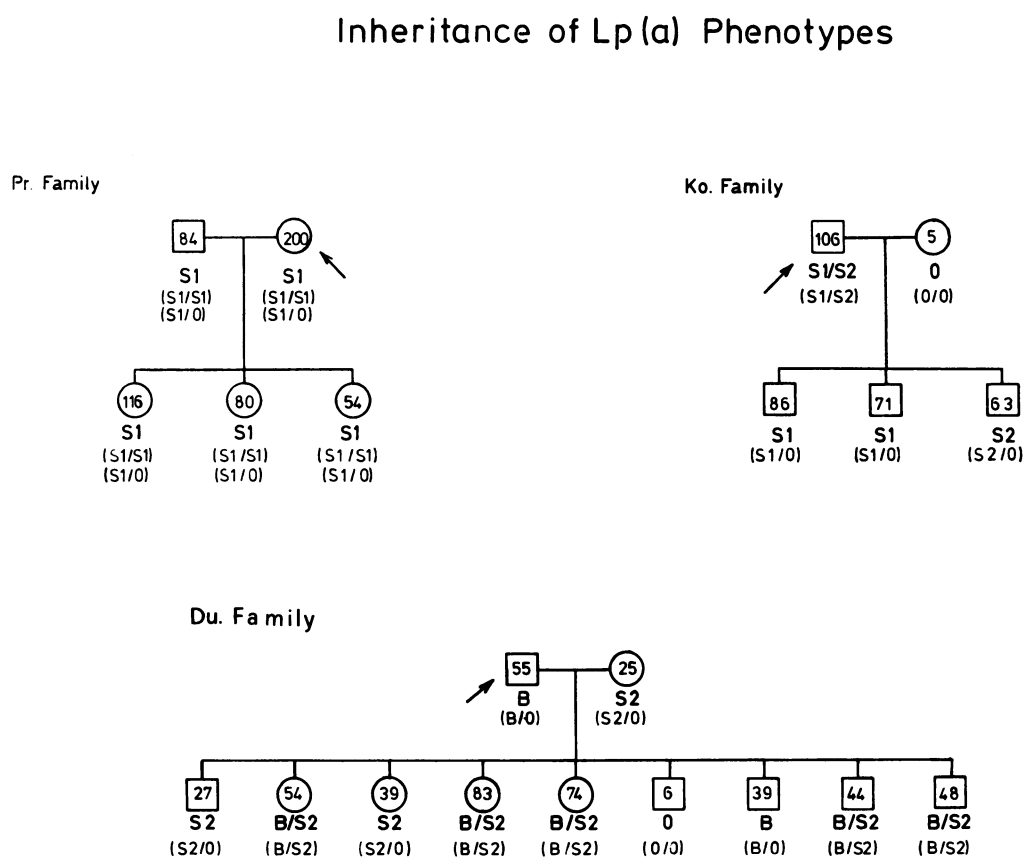

Figure 8. Pedigrees of three families. Lp(a) phenotypes and Lp(a) genotypes (in brackets) are indicated under the symbols. Lp(a) concentrations in plasma (mg/dl) are indicated within the symbols. Arrows denote propositi.

lated Lp(a) lipoprotein or Lp(a) glycoprotein and the quality of the serum blots was identical or even superior to those obtained from ultracentrifugally isolated Lp(a) lipoprotein (Fig. 6). Serum, citrate plasma, and EDTA plasma containing proteinase inhibitors all yielded the same Lp(a) pattern in a given individual, demonstrating that size heterogeneity also is not likely to be caused by proteolysis. Our observations confirm and extend those of Fless et al. (19) who reported on size heterogeneity of Lp(a) protein in isolated Lp(a) lipoprotein preparations. Here we show that the same Lp(a) glycoprotein band(s) seen in the isolated lipoprotein is present in plasma. Hence the observed size heterogeneity is not due to modifications that might have occurred during isolation of the lipoprotein.

On the basis of their respective mobilities in SDS-PAGE, we have distinguished six Lp(a) glycoprotein species with apparent molecular weight ranging from ~ 400,000 to 700,000, designated F, B, S1, S2, S3, and S4. Individual sera contained either one or two of these Lp(a) glycoprotein bands, resulting in several different Lp(a) phenotypes occurring with different frequencies in the population (Table I). The most common Lp(a) species observed in the plasma donors were the S2 and S3 forms. However it should be noted that these frequencies were determined in a truncated distribution. 49% of sera did not exhibit visible Lp(a) bands upon Western blotting. Harvie and Schultz (2) have claimed that there are no true Lp(a)-negative sera. We do not know which Lp(a) species are present in those samples where Lp(a) concentrations are under the detection limit of our immunoblot method but where Lp(a) antigen was detected by electroimmunodiffusion. Our observation that Lp(a) glycoprotein forms are associated with Lp(a) concentrations indicates that the frequencies of the different Lp(a) species may be different among the negative samples in our assay. Attempts to concentrate Lp(a) from "negative" plasma samples by ultracentrifugation have failed so far. Hence, the frequencies that we have determined may not be identical to those in the total population. With our present methods, however, we can not test this hypothesis.

Another possible source of error is that a minor Lp(a) component in plasma with a major Lp(a) species might be present that is not detected due to its low concentration. This may result in an overestimation of single-band phenotypes and those Lp(a) species associated with high Lp(a) concentrations. We therefore envisage our frequency determinations as first approximations.

The fact that no individual had more than two major Lp(a) species in plasma suggested to us that these phenotypes may be genetically controlled. Our family studies do indeed strongly support this concept and suggest that Lp(a) glycoprotein phenotypes are controlled by a series of autosomal alleles at a single locus. Inherited Lp(a) glycoprotein phenotypes are associated with Lp(a) lipoprotein concentrations in plasma. This can be clearly demonstrated by a comparison of Lp(a) lipoprotein levels in voluntary healthy plasma donors of different single-band Lp(a) glycoprotein phenotypes. Further support for the association of Lp(a) concentration with Lp(a) phenotypes has been obtained in a study of patients with hyperlipidemia and/or coronary heart disease (Kraft, H. G., H. J. Menzel, T. Hopferwieser, and G. Utermann, unpublished data).

Since the original discovery of the Lp(a) system by Berg (1), it has been known that Lp(a) concentrations in plasma are genetically controlled, and it is generally accepted that Lp(a) lipoprotein represents a quantitative genetic trait (2–4). Although the genetics of the quantitative Lp(a) trait remained unclear it has been postulated that one major gene is responsible for high Lp(a) concentrations. We show here that Lp(a) phenotypes B, S1, S2 are associated with high and phenotypes S3 and S4 with low Lp(a) concentrations. The simplest explanation for this is that the same genes are involved in determining both electrophoretic Lp(a) phenotypes and Lp(a) lipoprotein concentrations in plasma. Differences in allele frequencies then could be responsible for the skewed distribution of Lp(a) concentrations. Obviously other interpretations are possible but seem much more unlikely to us.

Krempler et al. (31) have demonstrated by in vivo turnover studies that Lp(a) lipoprotein concentrations in plasma are mainly determined by the rate of synthesis rather than by differences in catabolism of the Lp(a) lipoprotein. Hence our findings indicate that Lp(a) glycoprotein structure relates to the synthesis, assembly or secretion of Lp(a) lipoprotein. Bersot et al. (32) very recently reported that chylomicrons induced by fat feeding in humans contain an Lp(a) protein species with a higher apparent molecular weight than the Lp(a) protein in Lp(a) lipoprotein. This finding challenges our conclusion that Lp(a) protein heterogeneity may be genetically determined. However, in similar experiments we could not find differences in size between Lp(a) proteins from chylomicrons and Lp(a) lipoprotein of the same individuals (Seitz, C., and G. Utermann, unpublished observations). The reason for this discrepancy is not yet clear, but it should be pointed out that Bersot et al. (32) compared Lp(a) proteins from different individuals.

The structural differences between Lp(a) glycoprotein species are presently unknown. The differences in apparent molecular weight between Lp(a) species are enormous, ~ 100,000 D. It seems unlikely that these differences truly reflect differences in molecular mass of Lp(a) species. It is known that glycoproteins may exhibit anomalous mobility upon SDS-PAGE (33) and that differences in carbohydrate moiety, especially in sialic acid content, may result in differences in apparent molecular weight of glycoproteins far exceeding those expected from actual differences in molecular mass between differently glycosilated proteins.

The Lp(a) lipoprotein is rich in carbohydrate, containing about six times more sialic acid than LDL (10). This is mainly or exclusively due to the presence of the Lp(a) glycoprotein in the lipoprotein complex. Treatment of Lp(a) glycoprotein with neuraminidase expectedly reduced the apparent molecular weight of the protein by ~ 50,000 D. However it did not abolish differences between the phenotypes. Therefore it seems unlikely that the different Lp(a) species result from differences in sialylation. Also the results from our family study are difficult to interpret assuming that differences between Lp(a) species are primarily due to differences in posttranslational modifications, e.g., glycosilation. However differences in the primary structure of the Lp(a) protein might secondarily effect glycosilation, thus explaining the unusual large apparent differences between Lp(a) species. A detailed analysis of the carbohydrate structures of Lp(a) species is necessary to clarify this point. The Lp(a) system is unique in that the concentration of a complex composed of a lipoprotein resembling normal low-density lipoproteins and of a high–molecular weight glycoprotein is under strong genetic control, probably from a major gene locus. The elucidation of the structural differences between Lp(a) species and studies on their synthesis and secretion in cell culture may help us understand the qualitative basis underlying the quantitative Lp(a) lipoprotein trait. Such studies are presently underway in our laboratory.

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