Screening for lipoprotein[a] elevations in plasma and assessment of size heterogeneity using gradient gel electrophoresis


Lipid Metabolism Laboratory,* USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA; Framingham Heart Study, † National Heart, Lung, and Blood Institute Framingham, MA; Northwest Lipid Research Clinic and Department of Medicine,** University of Washington School of Medicine, Seattle, WA; and School of Life and Health Sciences, †† University of Delaware, Newark, DE

Abstract Plasma was screened for the presence of lipoprotein[a] using 2-16% nondenaturing, polyacrylamide gradient gel electrophoresis. Gels were scanned with a densitometer after staining with Sudan black B. Bands that migrated above low density lipoprotein bands were identified as lipoprotein[a] by immunoblotting with polyclonal and monoclonal antibodies to apolipoprotein[a]. Lipoprotein[a] was measured by gradient gel electrophoresis and by radioimmunoassay in 115 male patients with premature coronary artery disease and 132 control subjects. Lipoprotein[a] bands were detected in 96.7% of subjects with lipoprotein[a] values above 40 mg/dl; in 31.3% with values between 21 and 40 mg/dl, and in 6.5% with values below 20 mg/dl. This gel methodology is a simple and effective procedure for detecting elevated plasma lipoprotein[a] levels and for investigating size heterogeneity, but does not replace immunoassay for quantitation. — McNamara, J. R., H. Campos, J. L. Adolphson, J. M. Ordovas, P. W. F. Wilson, J. J. Albers, D. C. Usher, and E. J. Schaefer. Screening for lipoprotein[a] elevations in plasma and assessment of size heterogeneity using gradient gel electrophoresis. J. Lipid Res. 1989. 30: 747-755.

Supplementary key words coronary artery disease • apolipoprotein[a]

Lipoprotein[a] (Lp[a]) particles, first identified by Berg (1, 2), are similar to low density lipoproteins (LDL), containing apolipoprotein (apo) B and a similar lipid content. Additionally, however, these lipoproteins contain apo[a] and have a much higher content of sialic acid and carbohydrate than LDL (3–5). These particles are also known as sinking pre-beta lipoproteins due to their pre-beta mobility on agarose electrophoresis (3, 6, 7). Lp[a] presence has been identified as a genetically transmitted trait (8, 9), and elevated levels of Lp[a] have been associated with an increased risk of premature coronary artery disease (CAD) (6, 10–14). Some studies have indicated that Lp[a] may be catabolized through the LDL receptor pathway (15–17); others, however, have indicated an LDL receptor-independent mechanism (18, 19).

The presence of Lp[a] in several population studies has been assessed by both quantitative and qualitative methods, and the reported incidence has varied markedly in different studies (1, 9, 11, 20, 21). These variations have been due more to differences in the sensitivity of methods for Lp[a] determination, however, than to differences in population frequency. Due to their increased sensitivity, radial immunodiffusion (RID) (7, 9, 20) and radioimmunoassay (RIA) techniques (11) indicate the presence of Lp[a] in significantly higher percentages of the population (80–100%) (7, 9, 11, 20, 22), than do gel diffusion or agarose electrophoretic procedures (30–40%) (1, 20, 21). The immunological methods, however, have the potential disadvantage of being susceptible to antibody cross-reactivity with plasminogen (23, 24). None of the above methods have the ability to assess the physical heterogeneity of the particles.

In this study, we present methodology for the separation of Lp[a] particles from plasma using commercially available nondenaturing gradient gel. This gradient gel electrophoresis (GGE) procedure can be used to identify individuals with elevated Lp[a] levels, while simultaneously studying both Lp[a] and LDL size heterogeneity.

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; Lp[a], lipoprotein[a]; apo, apolipoprotein; CAD, coronary artery disease; RIA, radioimmunoassay; RID, radial immunodiffusion; GGE, gradient gel electrophoresis; FHS, Framingham Heart Study; C, cholesterol; TC, total cholesterol.

**To whom correspondence should be addressed at: Lipid Metabolism Laboratory, USDA Human Nutrition Research Center on Aging at Tufts University, 711 Washington Street, Boston, MA 02111.
MATERIALS AND METHODS

Study population

A group of 518 CAD patients admitted to the New England Medical Center, Boston, MA, for elective coronary artery angiography had blood drawn in the fasting state for lipid and lipoprotein analysis. Plasma samples from a subset with severe disease were used in the present study. This cohort contained 115 males who were less than 60 yr of age (mean age, 50.9 ± 6.7 yr), and who had greater than 50% occlusion in at least one major coronary artery, no history of valvular heart disease, no myocardial infarction within 6 weeks prior to angiography, and with plasma triglyceride levels <400 mg/dl. A control group of 132 males from a random subset of offspring participants from the Framingham Heart Study (FHS) was used as previously described (25). These individuals (<75 yr of age) had a mean age of 50.9 ± 9.9 yr, no history of coronary disease, no history of valvular heart disease, and plasma triglyceride levels <400 mg/dl.

Lipoprotein analysis

Blood was drawn from subjects after a 12- to 14-h overnight fast in tubes containing EDTA (final concentration, 0.1%). Plasma was separated by centrifugation at 2500 rpm (1300 g) for 20 min at 4°C. Plasma cholesterol (TC), triglyceride, and HDL cholesterol (HDL-C) levels were determined on an Abbott Diagnostics ABA-200 bichromatic analyzer using enzymatic reagents (Abbott A-AGENT) (26) and dextran-Mg2⁺ precipitation (27). VLDL triglyceride, and HDL cholesterol levels were estimated for all subjects by use of the method of Friedewald, Levy, and Fredrickson (28). All lipid analyses were standardized through the Centers for Disease Control’s Lipid Standardization Program. Intermediate density lipoproteins (IDL) for one experiment were isolated in the density region of 1.006-1.019 g/ml by sequential ultracentrifugation at 39,000 rpm for 18 h in a Beckman L8-80 ultracentrifuge using a Beckman 50Ti rotor (Beckman Instruments, Inc., Pa10 Alto, CA).

Apolipoprotein analysis

Plasma Lp[a] levels were assayed using the RIA procedure and the polyclonal antibody of Albers, Adolphson, and Hazzard (11). This RIA procedure has been shown not to cross-react with plasminogen. The plasma apoA-I and apoB assays were performed with the use of a non-competitive, enzyme-linked immunosorbent assay as previously described (25, 29).

Gradient gel methodology

Lp[a] and LDL subfractions were separated by GGE with 2-16% non-denaturing polyacrylamide gels (Pharmacia Inc., Piscataway, NJ). The gels were loaded with fresh plasma, or plasma that had been stored at −70°C, which was combined 3:1 (vol/vol), with sucrose and bromphenol blue (25, 30). Particles were subjected to electrophoresis on a Pharmacia GE 2/4 apparatus for 20 min at 70 volts, followed by approximately 6 h at 450 volts for a total of 2700 volt-hours, as previously described (25, 30). The gels were stained for lipid with Sudan black B, dissolved with zinc acetate in ethylene glycol monoethyl ether (Cellosolve), and destained in a 50% solution of Cellosolve as previously described (25, 30). After restoration to original size in 25% Cellosolve, gels were scanned on an LKB Ultrascan XL laser densitometer (LKB Instruments Inc., Paramus, NJ). Integration of LDL bands (25, 30), and Lp[a] bands was performed with the GSXL software program (LKB) with manual identification of peaks and baselines. For most comparisons Lp[a] band area was corrected for gel-to-gel staining variability by comparing the area under the peak of Lp[a] bands with the area of the major LDL control band. Additionally, relative Lp[a] size was assessed by determining the distance above the major LDL control band. This LDL control pool was originally formulated to contain multiple LDL bands and was maintained in small, single-use aliquots at −70°C. It was routinely run in the two outside lanes of each gel.

Bands were transferred to nitrocellulose and immuno-blotted using the same polyclonal apo[a] antibody used in the RIA assay. Additionally, a monoclonal antibody to apo[a], that has also been shown to have no crossreactivity with plasminogen, was also used. This antibody, #1D1, developed by Dr. Usher, was produced by standard hybridoma technology, utilizing Lp[a] as isolated by the method of Fless, Rolih, and Scanu (31).

Statistical analysis

The scientific package RS/1 (BBN Research Systems) allowed the various parameters measured in this study to be entered and stored in a VAX-780 computer (Digital Equipment Company, Maynard, MA). Pearson product moment correlation coefficients were computed to identify parameters having a significant correlation with the presence of a Lp[a] band. Paired t-test analysis compared band area as measured by the densitometer with Lp[a] concentration as measured by RIA. Unpaired t-test analyses compared the significance of the differences in mean values between CAD males and FHS males for: age, TC, LDL-C, HDL-C, triglycerides, apoA-I, apoB, Lp[a] concentration, LDL type, and Lp[a] presence by GGE. Stepwise multiple linear regression analyses, including forward selection, backward elimination, and maximum R-square improvement procedures, were used to examine significant contributions of these same parameters to the prediction of elevated levels of Lp[a]. Chi square analysis was used to compare the presence or absence of Lp[a] by GGE with
RESULTS AND DISCUSSION

When plasma is subjected to GGE under nondenaturing conditions, particles separate into bands according to their particle size, with the largest particles remaining near the origin, and smaller particles migrating farther down into the gel. We have previously studied LDL migration with this methodology (25, 30). Following GGE, distinct bands were observed above the LDL bands in some samples, as shown in lanes 1–3 of Fig. 1. These bands were stained readily with Sudan black B. We initially determined that these bands were not IDL by GGE of lipoproteins in the density range of 1.006–1.019 g/ml. Confirmation that the bands represented Lp[a] particles was obtained by immunoblotting with polyclonal (not shown) and monoclonal apo[a] antibodies (Fig. 2b). Gels loaded with identical plasma samples were subjected to GGE simultaneously. One gel was stained with Sudan black B (Fig. 2a); the other gel was immunoblotted to specifically identify the apo[a] protein (Fig. 2b). On the immunoblots, only bands containing apo[a] were stained.

Fig. 1. Six lanes of a 2–16% gradient gel that have been stained with Sudan black B. Lanes 1, 2, and 3 contain plasma from individuals with elevated plasma concentrations of Lp[a]; the Lp[a] bands are indicated. The bands below the Lp[a] are LDL bands of varying sizes. Lanes 4 and 5 contain plasma samples that have low levels of Lp[a] and show no Lp[a] bands. Lane 6 contains the plasma LDL control.

Fig. 2. Comparison of Lp[a] and LDL from several individuals, a: stained with Sudan black B; b: immunoblotted with monoclonal apo[a] #1D1; and c: rebotted with a polyclonal apoB. None of the LDL bands are stained by the apo[a] antibody (panel b), whereas both LDL and Lp[a] bands are stained by the apoB antibody (panel c). The apo[a] antibody has much greater sensitivity than the Sudan black B, as seen by comparing the Lp[a] bands in panel a with those in panel b.
These were primarily localized to positions directly above the LDL bands, with LDL bands not being stained. (Fless et al. (31) have also identified as Lp[a] a band migrating above LDL on 2.5–16% polyacrylamide gels.) However, not only was apo[a] staining observed in the bands migrating immediately above the LDL bands, but also in bands migrating well above LDL, near the origin (Fig. 2b), consistent with VLDL migration. This observation may support data by Bersot et al. (32) that apo[a] can be found in the triglyceride-rich lipoprotein density region. Caution must be taken, however, since staining in this area of the gels could simply represent aggregates of Lp[a] particles. Evidence of apo[a] presence in the HDL range was noted in three of the samples (Fig. 2b, lanes 2, 3, and 6), but whether the apo[a] is actually associated with these other lipoproteins will require further study. Fig. 2c shows the same immunoblot seen in Fig. 2b, but reincubated with a polyclonal antibody for apoB, showing the Lp[a] and the LDL bands. As we have previously observed with LDL bands (25), no differences in Lp[a] migration or staining intensity were noted when plasma samples were run fresh or after storage at −70°C.

We compared plasma Lp[a] levels determined by RIA with the Lp[a] band area measured on the gradient gels (Table 1). The correlation was significantly positive for both CAD patients \((r = 0.7123, P < 0.0001)\) and FHS controls \((r = 0.7779, P < 0.0001)\) (Fig. 3). Chi square analysis comparing only presence or absence of a band with the presence or absence of an elevated Lp[a] level \((>40 \text{ mg/dl})\) also showed a significant positive association in both groups \((P < 0.0001)\). In some samples, however, the area under the peak following band integration and the concentration by RIA, did not correspond well. Most of the discrepancies have been found to be technical in nature. Integration of lanes containing two Lp[a] bands appear to have overestimated band area, probably due to a doubling of background color. Over-staining of a few gels caused an increase in the background noise of the scans, also causing overestimation of peak area. There were a few discrepancies, however, that could not be explained through technical means. Two possible explanations for these other cases might be: 1) that these particles have a higher lipid:apo[a] ratio, so that there is an underestimation of the mass concentration (and increased staining, perhaps), resulting in higher band area compared to RIA concentration and, conversely, 2) that apo[a] associated with lipoproteins other than LDL is present in some plasma samples at levels that cause overestimation of Lp[a] concentration compared to band area.

Our GGE data showed a sensitivity threshold of approximately 40 mg/dl. Below 20 mg/dl, a band was observed in only 6–8% of individuals as shown in Table 1. In the combined populations \((n = 247)\), GGE produced bands in 96.7% of individuals where Lp[a] values exceeded 40 mg/dl and in 31.3% of those with Lp[a] values between 21 and 40 mg/dl. There were nine (5.8%) cases where a band was observed in the 155 individuals with Lp[a] levels below 20 mg/dl. Conversely, there were only two (3.3%) cases where the Lp[a] levels were greater than 40 mg/dl with no band seen. Although the mean ages of the CAD patients and FHS controls were identical, the age ranges were somewhat different between groups (28 to 60 yr for CAD patients and 28 to 75 yr of FHS controls). We therefore compared Lp[a] levels in CAD patients and FHS controls of matched ages. Our data indicate that age does not affect Lp[a] levels.

We observed considerable variation in Lp[a] band location (particle size) among individuals. Lp[a] bands of different individuals did not migrate into specific, defined regions, however, and therefore could not be classified in the same manner as LDL (25). Of the 77 individuals with observable plasma Lp[a] bands, 81% had one band, and 19% had two bands, with no difference in frequency observed between CAD patients and FHS controls. Several investigators have now documented that there is heteroge-
Fig. 3. Relative Lp[a] band area (Lp[a] band area/LDL control area) is plotted against Lp[a] concentrations (mg/dl) for: A) the CAD patient population ($y = 0.00272 - 0.01080, P < 0.0001$), and B) the FHS control population ($y = 0.00411 - 0.00826, P < 0.0001$). In most cases no band was seen on the gradient gels when apo[a] concentrations were below 40 mg/dl.

Heterogeneity in the size of the apo[a] protein as well as in the lipid composition of these particles (31, 33-35); Utermann et al. (33) have indicated that the size may be determined by a series of autosomal alleles at a single locus; and McLean et al. (24) have suggested that the apo[a] heterogeneity may be due to varying numbers of the amino acid repeats that contain homology with kringle 4 of plasminogen. These findings may explain the variability in particle size that we have observed. We found no significant association between Lp[a] particle size and other parameters measured in this study, but that may be due to the relatively small number of individuals with elevated Lp[a]. In the 42 CAD patients and 35 FHS controls where Lp[a] bands were visualized, particle size was not related to Lp[a] levels and was not significantly associated with LDL size (CAD: $r = 0.0904$, FHS: $r = 0.2979$). In the CAD group, however, there was a trend toward smaller particles (Fig. 4). Whether these smaller sized Lp[a] particles are related to the smaller LDL previously observed in patients with CAD (36, 37) will require further study.

Lp[a] band presence was positively associated with LDL-C ($P < 0.004$), TC ($P < 0.003$), and apoB ($P < 0.05$), and inversely associated with the use of beta blockers (BB) ($P < 0.05$) in the CAD patient group. It was also associated with LDL-C ($P < 0.02$) and TC ($P < 0.04$) in the FHS controls (Table 2). Similar associations were observed for Lp[a] concentrations measured by RIA.
Lp[a] levels (mean ± SD) for CAD patients were significantly higher overall (34.0 ± 33.6 mg/dl) than those of the FHS controls (19.0 ± 27.3 mg/dl), (P < 0.001). These data show a consistent trend toward higher Lp[a] levels in the CAD population and support previous studies in which investigators have assessed Lp[a] presence in individuals relative to coronary disease (6, 10-14, 21). We did, however, observe an inverse association in CAD patients for both Lp[a] band presence and Lp[a] levels with the use of beta blockers. With the number of relationships being assessed, the level of association, (P < 0.05), should probably not be considered significant, but it does show a trend. The mean Lp[a] level in 122 normal controls was 19 ± 27 mg/dl; that of 32 CAD patients not using beta blockers was 45 ± 35 mg/dl (P < 0.001). The mean value of 83 patients on beta blocker therapy, however, was only 30 ± 33 mg/dl (P < 0.05). At the time of sampling, 72% of CAD patients in the study were using beta blockers. These data suggest that beta blocker therapy may reduce Lp[a] con-
centrations, resulting in lower Lp[a] band areas and lower Lp[a] levels in these individuals. We have previously shown a beta blocker effect on LDL particle size (38). Other evidence also suggests that Lp[a] levels can be modulated through pharmacological means. Gurarak et al. (39) noted Lp[a] reductions of 24% with neomycin and 45% with neomycin plus niacin, with similar decreases in LDL-C; Albers et al. (40) reported a 65% reduction in Lp[a] with the use of the anabolic steroid, stanozolol, in women with osteoporosis, without any concomitant reduction of apoB levels. Vessby et al. (41), however, saw no change in Lp[a] concentration following administration of cholestyramine even though there was a 4–17% reduction in apoB levels and an 11–26% reduction in LDL-C concentration. Changes in Lp[a] size, concentration, and particle heterogeneity could potentially be followed over the course of treatment using GGE, although subtle concentration changes could be difficult to document with this procedure.

Since the presence of elevated concentrations of Lp[a] in plasma indicates an increased risk of CAD, identification of individuals with elevated levels is important, and quantitative assays for measuring Lp[a] and/or apo[a] concentration will eventually be available on a routine basis. GGE methodology should not be viewed as a replacement for immunoassays, but rather as additional methodology for the study of Lp[a]. Analysis of Lp[a] using GGE has the qualitative advantage of allowing assessment of particle size and particle heterogeneity in individuals and family groups with elevated levels. At the same time, assessment of LDL sizes can be made. With the use of various apo[a] antibodies and immunoblotting techniques, this methodology can also be expanded to compare heterogeneity within the protein moiety with that of the intact particle. Currently, Lp[a] presence and Lp[a] and LDL size heterogeneity are being assessed in our laboratory for the entire Framingham Heart Study Offspring population, using small aliquots of plasma and this GGE methodology.  

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REFERENCES


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**TABLE 2. Relationship of Lp[a] band presence with other lipoprotein parameters**

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<tr>
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<th>CAD Patients</th>
<th>FHS Controls</th>
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<tr>
<td></td>
<td>With Lp[a]</td>
<td>Without Lp[a]</td>
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<tr>
<td></td>
<td>Band (n = 33)</td>
<td>Band (n = 72)</td>
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<tr>
<td></td>
<td>With Lp[a]</td>
<td>Without Lp[a]</td>
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<td></td>
<td>Band (n = 63)</td>
<td>Band (n = 97)</td>
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<tr>
<td>TC</td>
<td>mg/dl</td>
<td>mg/dl</td>
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<td>227.0 ± 49.3</td>
<td>205.0 ± 35.5**</td>
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<td>TG</td>
<td>mg/dl</td>
<td>mg/dl</td>
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<td>195.0 ± 53.6</td>
<td>177.6 ± 69.5</td>
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<td>LDL-C</td>
<td>mg/dl</td>
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<td>156.6 ± 43.4</td>
<td>137.9 ± 34.0**</td>
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<td>HDL-C</td>
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<td>31.3 ± 8.2</td>
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<tr>
<td>Lp[a]</td>
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<td>67.0 ± 31.7</td>
<td>14.4 ± 13.2**</td>
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<tr>
<td>ApoA-I</td>
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<td>98.3 ± 21.6</td>
<td>96.1 ± 22.9</td>
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<tr>
<td>ApoB</td>
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<td>108.5 ± 31.8</td>
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<td>BB%*</td>
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<td>79.2*</td>
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*Percent of individuals using beta blockers.

**Significant at P < 0.05 by t-test analysis; **, significant at P < 0.001 by t-test analysis; ****, significant at P < 0.0001 by t-test analysis.
pre-B lipoprotein, hyperlipoproteinemia, and apolipoprotein B. *Metabolism.* 24: 1047–1054.