Heterogeneity of human lipoprotein Lp[a]: cytochemical and biochemical studies on the interaction of two Lp[a] species with the LDL receptor

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Abstract Human Lp[a] can be fractionated into two species with different affinities for lysine-Sepharose. Forty to 81% of the total Lp[a] in the density fraction 1.055-1.15 g/ml from five individuals was retained by this affinity column. The remaining unretained Lp[a] species with no apparently functional lysine binding site was similar to the retained species in its electrophoretic mobility, lipid, protein, and apolipoprotein composition, and the heterogeneity was not related to apo[a] size polymorphism. Interaction of the two species with the low density lipoprotein (LDL) receptor was studied in human fibroblasts. Using gold-labeled lipoproteins and an immunochromatography procedure, both Lp[a] species could be located in clusters on the cell surface, but the extent of labeling was far lower than that seen with LDL. Both Lp[a] variants were less effective than LDL in 1) down-regulation of LDL-receptor activity; 2) suppression of cellular sterol synthesis; and 3) stimulation of cholesteryl ester formation in human fibroblasts. Although degradation of both species of Lp[a] by the perfused rat liver was stimulated after estrogen induction of hepatic LDL-receptor activity, the stimulation was to only a quarter of that seen with LDL. The heterogeneity of Lp[a] with respect to the ability to bind e-aminocarboxylic acid will need to be considered in studying the physiological role of this lipoprotein. Both Lp[a] species exhibited a similar interaction with the LDL-receptor in vitro, and confirmed previous investigations that Lp[a] is only a poor ligand for the LDL-receptor. - Armstrong, V. W., Harrach, H. Robenek, M. Helmhold, A. K. Walli, and D. Seidel. Heterogeneity of human lipoprotein Lp[a]: cytochemical and biochemical studies on the interaction of two Lp[a] species with the LDL receptor. J. Lipid Res. 1990. 31: 429-441.

Supplementary key words apo[a] • apoB-100 • lysine binding site • gold-labeled lipoproteins • immunocytochemistry

The lipoprotein Lp[a] is composed of an LDL-like lipoprotein to which the glycoprotein apo[a] is attached through disulfide bridging to apoB-100 (1-4). Apo[a] is structurally related to human plasminogen (5-7) and has presumably been derived from this plasma protease zymogen through gene duplication of the kringle 4 sequence of the latter. The complete primary sequence of an apo[a] isoform has been shown by cDNA analysis (6) to consist of a large number of krings (37 ± 2) all highly homologous to the kringle 4 sequence of plasminogen followed by a kringle 5 and a protease domain.

Several studies have addressed the question as to whether Lp[a] can be catabolized through the LDL-receptor pathway. While three groups of investigators (8-10) reported that Lp[a] can specifically bind to the LDL receptor, another group (11), after comparing the uptake of LDL and Lp[a] in fibroblasts from normal subjects and subjects with homozygous FH, concluded that Lp[a] is not a ligand for the LDL-receptor. We were able to shed some light on this controversy when we demonstrated that the ability of Lp[a] to bind to the receptor is strongly modulated by the presence of the apo[a] glycoprotein (3). After reduction of Lp[a] to remove apo[a] from the lipoprotein particle, the latter was bound, internalized, and degraded through the LDL-receptor pathway with the same affinity and efficiency as normal LDL, whereas native, unreduced Lp[a] was a much poorer ligand with a weaker affinity for the receptor. These results were confirmed by Zawadzki et al. (12), who also demonstrated that apo[a] modulates the immunochromatography properties of apoB-100 in the Lp[a] particle. Two in vivo studies of the plasma clearance of 125I-labeled human Lp[a] (13) and [3H]cholesteryl linoleyl ether-labeled human Lp[a] by the rat (14) also indicated a less efficient catabolism of Lp[a].

Abbreviations: LDL, low density lipoproteins; FCS, fetal calf serum; LDS, lipoprotein-deficient serum; PBS, phosphate-buffered saline; FCR, fractional catabolic rate.
by the LDL-receptor pathway in these animals.

When investigating the interaction of Lp[a] with the LDL-receptor, it is obviously of paramount importance that Lp[a] preparations are completely free of LDL. Furthermore, the potential in situ cleavage of apo[a] thereby generating an LpB particle must also be excluded. We therefore decided to investigate the binding of Lp[a] to the LDL-receptor by an immunocytochemical technique using highly purified Lp[a] prepared by affinity chromatography over lysine-Sepharose (5). In the course of this work we discovered that the lipoprotein Lp[a] was heterogeneous with regard to its ability to bind to lysine-Sepharose and that two lipoprotein species could be isolated. Both species have been partially characterized and their interaction with the LDL-receptor has been investigated.

MATERIALS AND METHODS

Purification of Lp[a]

Plasma (ca. 400 ml) freshly obtained from donors with Lp[a] concentrations >300 mg/l stabilized with 0.02% sodium azide, 1 mM EDTA, 10 U/ml aprotinin, and 0.2 mM PMSF. A lipoprotein density fraction of d 1.055-1.15 g/ml was then prepared by sequential ultracentrifugation.

Lysine-Sepharose affinity chromatography

Lysine-Sepharose 4B (Pharmacia, Sweden) in which lysine had been coupled to the matrix via its α-amino group was used in these experiments. The lipoprotein density fraction 1.055-1.15 g/ml was extensively dialyzed against 0.1 M phosphate, pH 7.4, 1 mM EDTA and then applied to a column (7 × 2 cm) of lysine-Sepharose 4B equilibrated with the same buffer. The column was washed with this buffer until the A280 eluate had returned to baseline. The Lp[a] bound to the lysine-Sepharose was released by introducing 50 mM 6-aminohexanoic acid into the phosphate buffer. This material will be referred to as Lp[a]lys+. Since it contains a functional lysine binding site, it is referred to as Lp[a]lys+. When investigating the interaction of Lp[a] with the LDL-receptor, it is obviously of paramount importance that Lp[a] preparations are completely free of LDL. Furthermore, the potential in situ cleavage of apo[a] thereby generating an LpB particle must also be excluded. We therefore decided to investigate the binding of Lp[a] to the LDL-receptor by an immunocytochemical technique using highly purified Lp[a] prepared by affinity chromatography over lysine-Sepharose (5). In the course of this work we discovered that the lipoprotein Lp[a] was heterogeneous with regard to its ability to bind to lysine-Sepharose and that two lipoprotein species could be isolated. Both species have been partially characterized and their interaction with the LDL-receptor has been investigated.

Immunosorbent chromatography

An immunosorbent column was prepared by coupling 86.5 mg of goat anti-apo[a] immunoglobulins to 15 g of CNBr-Sepharose 4B (Pharmacia, Sweden) according to Gaubatz, Cushing, and Morrisett (15). This immunosorbent was then packed onto a 25 × 2 cm column above a layer of Sephadex G-50 (10 × 2 cm) and the column was equilibrated with 0.5 M Tris, pH 7.4, 0.15 M NaCl, 1 mM EDTA. The unretained fraction from the lysine-Sepharose chromatography step was applied to the immunosorbent column and the latter was washed with column buffer until the A280 eluate had returned to baseline. Bound material was released from the column by washing with 15 ml 3 M KSCN followed by the column buffer. The released lipoprotein was immediately desalted in the Sephadex G-50 layer and eluted from the column in front of the thiocyanate peak. It was dialyzed against column buffer to ensure complete removal of all traces of KSCN. This material, which does not apparently possess a functional lysine binding site, is referred to as Lp[a]lys+. The released lipoprotein was immediately desalted in the Sephadex G-50 layer and eluted from the column in front of the thiocyanate peak. It was dialyzed against column buffer to ensure complete removal of all traces of KSCN.

Analytical techniques

Apo[a] isoforms of the different Lp[a] species were characterized by immunoblot analysis after SDS-PAGE in 5.1% polyacrylamide gels as described previously (7). Quantification of Lp[a] was carried out with a polyclonal ELISA that showed less than 0.02% cross-reactivity to plasminogen. Agarose gel lipoprotein electrophoresis was performed with a commercially available test kit (Lipidophor, Immuno, Austria). The purity of each Lp[a] preparation was checked by fast protein liquid chromatography using the Pharmacia system and Mono-Q HR 5/5 strong ion exchanger column as previously described (3). Briefly, samples of Lp[a] (ca. 100 µg protein/ml) were applied to the Mono Q column which was eluted with a linear gradient of 0-0.4 M NaCl in 0.02 M Tris, pH 8.2, at a flow rate of 0.2 ml/min. Protein/lipoprotein eluting from the column was monitored by its absorbance at 280 nm and peak area was automatically integrated by the LCC-500 liquid chromatography controller system. Both Lp[a] variants eluted at 0.34 M NaCl as opposed to 0.24 M NaCl for LDL. The Lp[a] preparations used for these studies were over 99% pure in terms of total peak area of the chromatogram.

Total cholesterol, free cholesterol, phospholipids, and triglycerides were determined by standard enzymatic test kits (3). Protein was measured by the method of Lowry et al. (16). The sialic acid content of Lp[a] was determined with a commercially available test kit based on enzymatic analysis (Boehringer Mannheim).

Antisera

Antisera for the immunocytochemical studies were raised in rabbits using purified Lp[a]. The anti-B-100 immunoglobulins were separated by affinity chromatography over an LDL-Sepharose immunosorbent column and the anti-Lp[a] immunoglobulins were separated over an Lp[a]-Sepharose immunosorbent. To check the specificities of the antibodies, purified apo[a] was prepared as described (3) as well as a narrow LDL density cut of 1.03-1.05 g/ml from a donor with low serum Lp[a] levels (12 g/l). In double gel diffusion experiments, immunoprecipitation bands were only observed between anti-Lp[a] and
apo[a] and between anti-B-100 and LDL. For the immunocytochemical binding studies, anti-B-100 and anti-Lp[a] were used at dilutions of 1:100.

Cultured human fibroblasts

Fibroblasts were cultured from skin biopsies of healthy, normolipidemic individuals and were maintained in Dulbecco's minimal essential medium containing 25 mM NaHCO₃, 20 mM HEPES, pH 7.4, and 10% fetal calf serum (FCS). The culture medium was supplemented with 100 U penicillin/ml and 100 μg streptomycin/ml. Cells were seeded after eight through ten passages into 20-mm plastic dishes containing 2–3 ml of culture medium. The medium was changed after 3 days and then on the fifth day 1 ml of medium containing 10% lipoprotein-deficient serum (LDS) in place of the FCS was added to the cells. After a further 48 h experiments were started.

Ultrastructural detection of lipoprotein receptors

Preparation of protein-gold complexes. Monodisperse gold sols of 12 nm diameter were prepared by reduction of chloroauric acid with sodium citrate as reducing agent (17). Protein A was purchased from Sigma and protein A-gold complexes were prepared according to the method of Roth, Bendayan, and Orci (18). The gold sols could be stored at 4°C for up to 4 weeks in PBS-1% BSA-0.02% azide with no loss of immunoreactivity. LDL and Lp[a] were conjugated to the colloidal gold particles as follows (19, 20). The pH of the gold sols prepared with sodium citrate as reducing agent was 5.3. Since this is close to the pIs of Lp[a] (pI 5.0) and LDL (pI 5.6), further adjustment of the pH was not necessary. For the coupling procedure, lipoprotein solutions containing 1.5–2.0 mg protein/ml in 0.1 M phosphate, pH 7.4, 0.001 M EDTA were diluted with distilled water to reduce the salt concentration to 0.005 M or below (20-30-fold dilution). The amount of lipoprotein needed to stabilize 1 ml of gold sol was then determined in a linear series by addition of increasing amounts of the diluted lipoprotein solution to the gold sol. The optimum amount of protein was that at which the colloid no longer flocculated after addition of 10% NaCl solution, flocculation being judged visually by a color change from red to blue. This was found to be approximately 15 μg protein/ml gold sol for both LDL and the two Lp[a] variants. A 10% excess of lipoprotein was then used to prepare the gold-labeled conjugates for the binding experiments. Unconjugated lipoproteins were removed by centrifugation (2 × 30 min at 12,000 g). The quality of the resulting pellets of conjugated lipoproteins was checked routinely by negative staining; the gold-Lp[a] conjugates and the gold-LDL conjugates were monodisperse and the gold particles were covered by five to eight lipoprotein particles. Less than 1% of the lipoprotein particles was unconjugated. Lipoprotein-gold conjugates were used on the day of preparation and there was no sign of destabilization during the incubation period as would be indicated by a change of color from red to blue.

Visualization of lipoprotein receptors. We employed two principally different approaches to visualize lipoprotein receptors. The first method was based on the selective binding of gold-labeled lipoproteins LDL and Lp[a] to directly localize the corresponding receptors at the cell membrane surface. Human fibroblasts plated on plastic dishes were washed with ice-cold medium and incubated with the respective gold-labeled lipoprotein for 1 h at 4°C. The lipoprotein concentrations used in these binding studies were 30 μg LDL protein/ml and 40 μg Lp[a] protein/ml. There appears to be general agreement that in Lp[a] apoB-100 comprises about 65–70% of total protein (3, 4, 21, 22); so by using 33% more total protein in the case of Lp[a] we will have ensured approximately similar concentrations of apoB-100 in these binding studies, i.e., 30 μg LDL apoB-100/ml and 26–28 μg Lp[a]-apoB-100/ml. The experiments were terminated by thorough washing with ice-cold buffer. The cells were then fixed with Karnovsky's fixative (23) and processed for electron microscopy. In control experiments the specificity of the method was confirmed by exposing the cells to gold-labeled lipoproteins in the presence of a 25-fold excess of the same unlabeled ligand. In these experiments the cell surface was completely devoid of gold label.

The second method for visualizing lipoprotein receptors involved indirect immunocytochemical labeling of receptor-bound lipoproteins. Precoupled fibroblasts were exposed to unlabeled lipoproteins (30 μg LDL protein/ml, 40 μg Lp[a] protein/ml) for 1 h and then, after thorough washing, to antibodies directed against either apoB-100 or apo[a] for an additional 1 h. Finally, lipoprotein-bound antibodies were detected by incubation with gold-labeled Protein A. All incubation and washing steps were carried out at 4°C. Specificity of the immunostaining was controlled by omitting either the lipoprotein or the primary antibody. After the last wash in ice-cold buffer, the cells were fixed and processed for electron microscopy.

Processing for electron microscopy

For surface replication, fixed cells were dehydrated and critical-point dried. Small pieces (1 × 1 cm) from the bottom of the petri dish covered with fibroblasts were cut out using a soldering iron. Platinum-carbon replicas of the cell culture surfaces were made in a Balzers BA 300 freeze-etching apparatus (Balzers AG, Liechtenstein), equipped with electron gun evaporators and a quartz crystal thickness monitor. Replicas were obtained by shadowing the cell surface with platinum-carbon at an angle of 38°, followed by carbon at 90°. The replicas were cleaned overnight in household bleach and washed in distilled water. They were then picked up on 200-mesh copper grids and examined in a Phillips EM 410 60 kV.
Down-regulation of LDL-receptor activity

Monolayer cultures of skin fibroblasts were incubated for 48 h in medium containing LDS to up-regulate LDL-receptor activity. Fresh medium (1 ml) containing LDL (20 μg protein/ml) or Lp[a] (30 μg total protein/ml or 19.5-21 μg apoB-100/ml) was then added to the cultures. In a control experiment, medium without lipoprotein was added to the cells. After a further 24-h incubation, LDL-receptor activity was estimated by adding 125I-labeled LDL (20 μg protein) for 5 h. The amount of LDL protein that had been bound, internalized, and degraded was determined as described previously (3). All incubations were carried out in triplicate at 37°C.

Cellular sterol synthesis

Monolayer cultures of fibroblasts were incubated in LDS for 48 h and then either LDL or Lp[a] in 1 ml of DME containing 10% LDS was added. After a further 6 h, medium was decanted and the cells were thoroughly washed with PBS containing 0.2% BSA. Fresh LDS medium (1 ml) containing 0.8 μCi of [14C]acetate and unlabeled carrier acetate (0.2 μmol/ml, sp act 4 μCi/μmol) was added to each dish and incubation was continued for 2 h at 37°C (24). Cells were then washed twice with PBS/BSA medium and three times with PBS alone before being lysed with 2 ml 0.1 mM NaOH and ultrasonified. A portion of the homogenate was removed for measurement of radioactivity, while a further 1 ml of the homogenate was added to glass, screw-capped vials containing 1 ml of 100% ethanol and 0.2 ml of 90% KOH. The mixture was saponified at 80°C for 3 h, diluted with 1.5 ml water, and nonsaponifiable lipids were extracted into 2.5 ml hexane. After washing with 2.5 ml of 0.1 mM sodium acetate, the hexane phase was collected and evaporated and radioactivity was determined. Incorporation of [14C]acetate into sterols is expressed as pmol acetate incorporated into nonsaponifiable lipids. To confirm that the major sterol synthesized was cholesterol, the hexane phase was fractionated by thin-layer chromatography on silica gel G plates with petroleum ether-diethyl ether-acetic acid 80:20:1 (v/v/v). A band that comigrated with an unlabeled cholesterol standard was scraped from the plate and radioactivity was measured. Cellular protein was measured in the aqueous homogenate of the fibroblast cultures by the method of Lowry et al. (16). Cholesteryl oleate synthesis is expressed as pmol cholesteryl oleate/mg cell protein.

Liver perfusion

Livers were obtained from male Wistar SPF rats (Mus rattus, Bruultal, FRG) weighing 200–300 g. The animals were kept in a 12-h dark–light schedule and were fed ad libitum with standard rat chow (diet 1324, Altromin International, Lage, FRG). Hepatic LDL-receptor activity was stimulated by subcutaneously injecting rats with 17α-ethinyl estradiol at a daily dose of 5 mg/kg body weight. On the sixth day, livers were excised for perfusion experiments. Livers were perfused using a previously described technique (25) using Krebs-Ringer bicarbonate medium containing 2.5% albumin, and 10% hemoglobin in the form of human erythrocytes. Perfusion was begun with 120 ml of medium and the first 20 ml was allowed to pass through the liver and was then discarded. The remaining 100 ml was then continuously recirculated through the liver. Bile was collected throughout the perfusion. After a 30-min equilibration, 125I-labeled LDL or 125I-labeled Lp[a] (1 mg protein, ca. 1 × 10^7 cpm) was added to the perfusion medium and TCA-soluble radioactivity (corrected for free iodide) was monitored in the plasma over a period of 3 hr.

RESULTS

Heterogeneity of the lipoprotein Lp[a]

We had originally envisaged purifying Lp[a] through its reported affinity to lysine-Sepharose (5). A lipoprotein density fraction of 1.055–1.15 g/ml was therefore applied to a column of lysine-Sepharose in 0.1 M phosphate buffer, pH 7.4, and the column was washed with the same buffer until no further A280-absorbing material was recorded in the eluate. The Lp[a] bound to the column was then released by including 50 mM 6-amino-hexanoic acid in the
The amounts of Lp[a] lipoprotein in the density fraction applied to the affinity column and in both Lys+ (the fraction retained on lysine-Sepharose) and Lys− (the unretained fraction) from the column were quantified by an ELISA procedure using a commercial Lp[a] standard (Immuno AG, Austria).

### TABLE 1. Proportion of Lp[a] bound to lysine-Sepharose after elution of the d 1.055-1.15 g/ml fraction over the affinity column

<table>
<thead>
<tr>
<th>Proband</th>
<th>1.055-1.15 g/ml</th>
<th>Lys+</th>
<th>Lys−</th>
<th>Total Recovery</th>
<th>Percentage Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Density Fraction</td>
<td>mg</td>
<td>mg</td>
<td>mg</td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>30.78</td>
<td>20.51</td>
<td>4.77</td>
<td>25.28</td>
<td>82.1</td>
</tr>
<tr>
<td>2</td>
<td>49.11</td>
<td>14.36</td>
<td>21.34</td>
<td>35.70</td>
<td>72.7</td>
</tr>
<tr>
<td>3</td>
<td>21.09</td>
<td>8.23</td>
<td>8.91</td>
<td>17.14</td>
<td>81.3</td>
</tr>
<tr>
<td>4</td>
<td>67.59</td>
<td>36.14</td>
<td>27.76</td>
<td>63.90</td>
<td>94.5</td>
</tr>
<tr>
<td>5</td>
<td>67.91</td>
<td>40.54</td>
<td>19.20</td>
<td>59.74</td>
<td>88.0</td>
</tr>
</tbody>
</table>

*The amounts of Lp[a] lipoprotein in the density fraction applied to the affinity column and in both Lys+ (the fraction retained on lysine-Sepharose) and Lys− (the unretained fraction) from the column were quantified by an ELISA procedure using a commercial Lp[a] standard (Immuno AG, Austria).

phosphate buffer and the amounts of Lp[a] in both the retained and unretained fractions were determined with an ELISA. Although total recovery from the column was good, a significant proportion of the Lp[a] was found to be in the unretained fraction (Table 1) amounting to 19-60% of the total Lp[a] recovered. When the unretained fraction was rechromatographed, less than 5% of the Lp[a] still in this fraction was retained, demonstrating that this phenomenon was not due simply to an inadequate capacity of the column. The unretained fractions from probands 4 and 5 were further purified over an anti- [a]-Sepharose column. Analysis of this material showed it to be a lipoprotein with all of the characteristics of Lp[a]. It had pre-β mobility on agarose gel electrophoresis, as did its lysine binding counterpart (Fig. 1A) and immunoelectrophoresis showed that both apoB-100 and apo[a] were associated with this lipoprotein (Fig. 1B). Both Lp[a] species eluted at the same salt concentration of 0.34 M NaCl in 0.02 M Tris, pH 8.2, from a Mono-Q strong ion exchange column (3). The lipid–protein compositions (Table 2) of the two species of Lp[a] were similar and comparable to those already published (3). The two Lp[a] species from proband 4 contained similar amounts of sialic acid while in the case of proband 5 Lp[a]-lys− had a higher sialic acid content than Lp[a]-lys+. However, the sialic acid content of the two Lp[a] species from proband 5 were 2- and 3-fold higher than their respective counterparts in proband 4. Finally, there were no differ-

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**Fig. 1.** A) Agarose gel electrophoresis of the two Lp[a] species; lane 1, Lp[a]-lys+; lane 3, Lp[a]-lys−; lanes 2 and 4, normolipidemic human serum. After electrophoresis the lipoproteins were visualized by polyanion precipitation. B) Immunoelectrophoresis of the two Lp[a] species; lane 1 Lp[a]-lys+; lane 2 Lp[a]-lys−.
TABLE 2. Comparison of the lipid-protein compositions and the sialic acid content of Lp[a] fractions

<table>
<thead>
<tr>
<th>Proband</th>
<th>Lp[a]</th>
<th>Free Cholesterol</th>
<th>Cholesterol Ester</th>
<th>Triacylglycerol</th>
<th>Phospholipid</th>
<th>Protein</th>
<th>Sialic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Lys +</td>
<td>5.5</td>
<td>37.0</td>
<td>10.2</td>
<td>17.6</td>
<td>28.7</td>
<td>31.5</td>
</tr>
<tr>
<td>4</td>
<td>Lys -</td>
<td>6.6</td>
<td>36.8</td>
<td>7.8</td>
<td>19.7</td>
<td>29.1</td>
<td>28.8</td>
</tr>
<tr>
<td>5</td>
<td>Lys +</td>
<td>7.0</td>
<td>40.0</td>
<td>5.0</td>
<td>21.2</td>
<td>26.8</td>
<td>64.7</td>
</tr>
<tr>
<td>5</td>
<td>Lys -</td>
<td>6.7</td>
<td>40.3</td>
<td>4.9</td>
<td>20.2</td>
<td>27.5</td>
<td>107.3</td>
</tr>
</tbody>
</table>

ences in the apo[a] isoforms of each Lp[a]; for example, although proband 5 possessed two different molecular weight apo[a] isoforms, both isoforms were found to be present in each of the two Lp[a] species (Fig. 2).

Cytochemical and immunocytochemical labeling

To study the binding of Lp[a] to fibroblast cell surfaces at the ultrastructural level, two approaches were used: 1) direct labeling with gold-labeled LDL or Lp[a]; 2) indirect immunocytochemical labeling. The results presented are derived from three independent sets of experiments for each approach in which a suitable large number of cells (ca. 200) were examined. Figs. 3 and 4 are representative for average cells in the respective populations, although there was great variability in labeling intensity. In the first approach, colloidal gold-labeled Lp[a] were allowed to bind to fibroblasts at 4°C and the cell surfaces were then visualized by electron microscopy. For comparison, gold-labeled LDL were also used in a set of separate experiments. After incubation of the gold-labeled ligands with fibroblasts, gold particles were identified on the cell surfaces through their high intrinsic electron density. The gold-labeled LDL particles were not uniformly distributed over the cell surface but were found in clusters (Fig. 3A) as has been reported (19). In agreement with a previous study (26), gold-labeled Lp[a]lys + particles were located in clusters on the cell surface (Fig. 3B); a similar labeling pattern was observed with Lp[a]lys − (data not shown). However, the extent of labeling with LDL was far in excess of that observed with either species of Lp[a], even though similar amounts of lipoprotein apoB-100 had been used in these experiments (compare Fig. 3A with Fig. 3B).

The second approach was designed to ascertain whether or not intact Lp[a] could bind specifically to the cell surface. In these experiments unlabeled LDL or Lp[a] were initially incubated with fibroblasts at 4°C. The respective antigens apo[a] and apoB-100 were then located on the cell surface by incubation with anti-Lp[a] or anti-B-100 followed by gold-labeled Protein A, and subsequent electron microscopy. Typical results obtained with LDL and Lp[a]lys + are illustrated in Fig. 4. Using anti-B-100, LDL were again found to be located in clusters on the cell surface (Fig. 4A), although the degree of binding with this immunocytochemical technique was lower than that seen with gold-labeled LDL. In the control experiment in which anti-Lp[a] was used in the second incubation step, only minor, unspecific background

Fig. 2. Immunoblotting of the apo[a] isoforms from the two Lp[a] species obtained from proband 5; lane 1, apo[a] isoforms from Lp[a]lys +; lane 2, apo[a] isoforms from Lp[a]lys −.
labeling was observed, the gold particles being randomly distributed over the cell surface (Fig. 4B). In the case of Lp[a], small clusters of gold particles could be seen irrespective of whether anti-B-100 (Fig. 4C) or anti-Lp[a] (Fig. 4D) was used as the antibody in the second incubation step. It would therefore appear that intact Lp[a] can bind to specific sites on the cell surface and that cleavage of the apo[a] from the lipoprotein particle is not a prerequisite for binding.

Down-regulation of LDL-receptor activity

The ability of the two Lp[a] species to down-regulate LDL-receptor activity were compared to that of LDL. After 48 h in LDS to up-regulate LDL-receptor activity, the cells were exposed to the respective lipoprotein for 24 h. Finally, labeled LDL were added to the cells and the amounts of LDL specifically bound, internalized, and degraded after a 5-h incubation were determined. The results are presented in Table 3. Preincubation of the fibroblasts with LDL led to a 56–68% suppression in LDL-receptor activity as determined in the individual steps of the LDL receptor pathway, whereas the two Lp[a] species were less effective at equimolar concentrations in suppressing LDL-receptor activity; the ability to suppress receptor activity was as follows: LDL > Lp[a]lys+ > Lp[a]lys-.

Suppression of cellular sterol synthesis

The conversion of [14C]acetate into cellular sterols (primarily cholesterol) was compared after incubation of fibroblasts with different concentrations of either LDL or Lp[a]. A strong concentration-dependent suppression of cellular sterol synthesis was found (Fig. 5) on incubation of the cells with LDL, a maximum suppression of ca. 87% being obtained at 20 μg LDL cholesterol/ml. Both species of Lp[a] were less efficient in their ability to suppress cellular sterol synthesis, and even at an Lp[a] cholesterol concentration of 50 μg/ml, maximum suppression of sterol synthesis had not been reached.
Stimulation of cellular cholesteryl ester formation

The two species of Lp[a] were compared to LDL in their ability to stimulate the esterification of cholesterol in cultured human fibroblasts. Both Lp[a]lys+ and lys− showed a similar concentration-dependent stimulation of cholesteryl ester formation (Fig. 6), but even at Lp[a] cholesterol concentrations of 100 μg/ml a plateau had not been reached, and the stimulation of cholesteryl ester formation was far below that achieved with similar concentrations of LDL-cholesterol.

Liver perfusion studies

The LDL-receptor activity of the normal rat liver is low but it can be stimulated by treatment of the animals with
TABLE 3. Down-regulation of LDL-receptor activity in human fibroblasts on incubation with LDL or either of the two Lp[a] species

<table>
<thead>
<tr>
<th>Incubation Conditions</th>
<th>Heparin-Releaseable Surface Binding</th>
<th>Internalization</th>
<th>Degradation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDS</td>
<td>192</td>
<td>1142</td>
<td>5764</td>
</tr>
<tr>
<td>30 µg/ml Lp[a]lys +</td>
<td>196</td>
<td>885</td>
<td>3278</td>
</tr>
<tr>
<td>30 µg/ml Lp[a]lys -</td>
<td>143</td>
<td>739</td>
<td>4479</td>
</tr>
<tr>
<td>20 µg/ml LDL</td>
<td>97</td>
<td>403</td>
<td>2463</td>
</tr>
</tbody>
</table>

After a 24-h incubation with LDL or Lp[a], 125I-labeled LDL was added and the amount of label specifically bound, internalized, and degraded through the LDL-receptor pathway was determined after a further 5 h incubation (3).

17α-ethinyl estradiol. The degradation of Lp[a] and LDL was therefore compared in the isolated, perfused rat liver from normal and estrogen-treated animals. The time courses for the degradation of these lipoproteins by the rat liver are summarized in Fig. 7. The amount of LDL protein degraded in 3 h increased from 3.5 µg in the normal liver to 17.6 µg in the estrogen-stimulated liver. The corresponding amounts of Lp[a]lys+ protein degraded in a 3-h perfusion were 1.8 µg in the normal liver and 5.4 µg in the estrogen-stimulated liver. Thus Lp[a] was only degraded with 50% of the efficiency of LDL in the normal, perfused rat liver. Stimulation of hepatic LDL-receptor activity with 17α-ethinyl estradiol increased the amount of LDL protein degraded by a value of 14.1 µg, whereas the increase in the degradation of Lp[a]lys+ protein amounted to only 3.6 µg. Similar results were obtained when Lp[a]lys− was substituted for Lp[a]lys+ in the liver perfusion experiments.

DISCUSSION

While purifying Lp[a] from the density fraction 1.055-1.15 g/ml by chromatography over lysine-Sepharose, we observed that a significant proportion of the Lp[a] did not bind to this column and could be detected immunochemically in the unretained eluate. The relative proportion of Lp[a] in this fraction showed interindividual variation ranging from 19% to 60% of the total Lp[a] in the five subjects so far investigated. After further purification by immunosorbent chromatography over anti-apo[a] Sepharose, this material was identified as a lipoprotein with the characteristic properties of Lp[a]. We have termed this species Lp[a]lys− since it does not possess a functional lysine binding site in contrast to Lp[a]lys+, the species that binds to lysine-Sepharose. Both Lp[a] species were similar with regard to their electrophoretic mobility (pre-B) in agarose, their retention by a Mono-Q strong ion exchange resin, and their lipid/protein composition. The heterogeneity was not related to the known size polymorphism of apo[a] (27). Even individuals with a single apo[a] isoform displayed both species of Lp[a], and in an individual possessing two different molecular weight isoforms of apo[a], both isoforms were present in each of the two Lp[a] species.

Fless, Rolih, and Scanu (28) were able to demonstrate heterogeneity of Lp[a] by rate zonal ultracentrifugation. However, this heterogeneity was related to the apo[a] isoform, the more dense Lp[a] species possessing the larger apo[a] isoforms. A recent publication (29) reported that two clearly defined variants of Lp[a] could be separated by chromatofocusing, both variants containing similar sized apo[a] isoforms. Whether these two variants correspond to the two Lp[a] species that we have observed remains to be clarified.

The chemical basis for the observed heterogeneity with regard to the lysine binding site is not apparent from this present study. Chemical modification of the kringle 4 fragment from plasminogen has shown (30) that the residues Arg-71 and Asp-57 (using the kringle numbering scheme from ref. 31) are essential for the binding of 6-aminocarboxylic acids. While both amino acids have been retained in kringle 1 from plasminogen, Asp-57 is not present in kringles 2 and 3 and Arg-71 has been lost in kringle 5, although it has been replaced by another...
basic amino acid, Lys, in the latter. More recent crystallographic analysis and energy minimization studies (31) have suggested that, in addition to the above two residues, Arg-34 and Asp-55 are also required for ligand binding; the former is only present in kringle 1 and may account for stronger binding of lysine to this domain, whereas the latter is present in all five kringles from plasminogen.

Inspection of the complete structure published for an apo[a] isoform (6) reveals that all kringles contain the residues Asp-55 and Arg-71. However, only kringle 37 of apo[a] has also retained Asp-57 and thus bears all of the known lysine binding residues present in kringle 4 of plasminogen. Nevertheless, four other kringles, 32 to 35, have another acidic amino acid, Glu, in place of Asp at position 57 and kringle 35 additionally contains Arg-34. Whether all or only some of the above mentioned kringles from apo[a] possess lysine binding properties remains to be clarified as well as the possibility that apo[a] variants of similar apparent molecular weight may exist in which these essential lysine binding site amino acids have been lost in all kringles. Another perhaps more likely explanation for the observed heterogeneity might lie in differences in the glycosylation of apo[a]. The affinity of plasminogen to lysine-Sepharose is modulated by glycosylation and two plasminogen species can be separated by affinity chromatography over lysine-Sepharose (32, 33). Since apo[a] is highly glycosylated (7, 21, 34, 35) it is feasible that differences in the degree of glycosylation, particularly in the region of kringles 32–37, might alter the binding properties of apo[a] to 6-aminocarboxylic acids. In our present study we were able to determine the sialic acid content of the Lp[a] variants from two individuals. There was a greater interindividual variation in the sialic acid content between similar Lp[a] species from the two probands than there was between the two Lp[a] species from the same individual. Wide variation in the amount of glycosylation of Lp[a]/apo[a] has been reported (7, 21, 34–36) and the range of sialic acid values that we found were similar to those in the literature. This rather limited evidence does not suggest that differences in the degree of glycosylation are responsible for the lysine binding heterogeneity of Lp[a]. However, in view of the limited number of kringles with potential lysine binding sites, the site of glycosylation rather than the extent of glycosylation may be paramount in determining the lysine binding ability of Lp[a].

The reason for the heterogeneity of Lp[a] notwithstanding, the existence of Lp[a] variants with differing affinities for 6-aminocarboxylic acids may be of physiological relevance since the lysine binding sites on plasminogen are necessary for the binding of this protein to fibrin. Investigations into the potential interactions between Lp[a] and the fibrinolytic system will have to take the observed heterogeneity into account.

Two previous groups have studied the binding of Lp[a] to cell surface receptors by a morphological approach. Hesz et al. (26) compared the binding of Lp[a]-gold and LDL-gold particles to human skin fibroblasts at 37°C. They observed that the Lp[a]-gold and LDL-gold particles were concentrated in discrete clusters on the cell surface and, using a differential labeling technique, they could demonstrate the presence of both LDL and Lp[a] in coated pits. Although the authors suggested that the distribution pattern of LDL and Lp[a] binding sites may not be completely identical, they found no qualitative differences in the specificity nor in the site of binding of Lp[a] to the cell surface as compared with LDL. Our studies on the binding of Lp[a]-gold particles to human skin fibroblasts are in general agreement with those of Hesz et al. (26), both Lp[a] species binding in discrete clusters to the cell surface. However, as can clearly be seen in Fig. 3, when similar concentrations of LDL-apoB-100 and Lp[a]-apoB-100 were used in the assay, the extent of labeling with LDL-gold was far in excess of that seen with Lp[a]-gold.

No differences were observed between Lp[a]-lys+ and Lp[a]-lys – in the extent and site of labeling.
In another investigation, Havekes et al. (9) used immunofluorescence microscopy and immunoelectron microscopy to visualize the binding of Lp[a] in coated pits. However, the primary antiserum employed was anti-apoB-100 and not anti-Lp[a]. We therefore compared the binding of Lp[a] and LDL to human skin fibroblasts using either anti-apoB-100 or anti-Lp[a] as the primary antiserum. The surface-bound immunoglobulins were then localized with Protein A-gold. Irrespective of which antiserum was used, Lp[a] demonstrated a weak binding to the cell surface with evidence of clustering in specific regions on the cell surface. When the binding of LDL to the cell surface was studied with anti-Lp[a], only a low, unspecific background labeling could be seen with the few gold particles randomly scattered over the cell surface, whereas using anti-apoB-100 the discrete clustering of gold particles was once again evident.

While these experiments indicate that Lp[a] can bind as an intact particle to the LDL-receptor, they nevertheless highlight the fact that the affinity of this lipoprotein for the receptor is much weaker than that of LDL, confirming our previous studies with [3H]labeled Lp[a] (3). Further confirmation of these data was obtained from biochemical experiments into the effects of Lp[a] on cellular cholesterol metabolism. Floren, Albers, and Biermann (10) had found that Lp[a] at a cholesterol concentration equivalent to LDL was less effective in increasing cell cholesterol content and stimulated cholesterol esterification only half as much as did LDL. We measured an even lower stimulation of cellular sterol synthesis with our highly purified Lp[a] preparations. At 20 μg lipoprotein cholesterol/ml cell medium, the stimulation of cholesterol esterification with the two Lp[a] species was only 10-14% that of LDL. Both Lp[a] species were also less effective in suppressing cellular sterol synthesis, implying that they were not able to down-regulate HMG-CoA reductase activity with the same degree of efficiency as LDL.

Finally, the degradation of Lp[a] by perfused livers from 17α-ethyl estradiol-treated rats was fivefold lower than that of LDL and the stimulation of hepatic LDL-receptor activity by 17α-ethyl estradiol did not stimulate the degradation of Lp[a] to the same degree as LDL. Similar findings were reported by Harkes et al. (13) who observed that the serum decay of LDL in rats was greatly increased by induction of hepatic LDL-receptor activity with 17α-ethyl estradiol, whereas the serum decay of Lp[a] was only moderately increased. Using [1H]cholesterol ether-labeled human Lp[a], Ye et al. (14) found that the tissue sites of degradation of human Lp[a] in the rat were similar to those for human LDL, but the residence time of Lp[a] in the circulation was much longer than that of LDL, suggesting a less efficient catabolism of Lp[a] by the LDL-receptor pathway. Taken together, the present and previous findings demonstrate that Lp[a] is only a poor ligand for the LDL-receptor, apo[a] presumably modulating the receptor binding sites on apoB-100 either through steric hindrance or through allosteric mechanisms. The results are in accord with the fact that serum Lp[a] levels are not affected by drugs such as cholestyramine (37) or the HMG-CoA reductase inhibitor simvastatin (38) that are known to effectively reduce LDL concentrations by increasing LDL receptor activity. In the only in vivo turnover study so far, Krempler et al. (8) observed that the FCR for Lp[a] in normolipidemic individuals was highly correlated with the FCR for LDL. However, in one individual with familial hypercholesterolemia (FH), the FCR for Lp[a] was greater than that for LDL, in contrast to the normolipidemics in whom the FCR for Lp[a] was always less than that for LDL. In a study into the kinetics of the return of Lp[a] and LDL concentrations to baseline levels after an acute reduction in their plasma pools through HELP-LDL-apheresis (39), we calculated the first order disappearance constants for Lp[a] and LDL according to Apstein et al. (40). The first-order disappearance constants for LDL were 0.082, 0.231, and 0.43 in individuals with homozygous FH, heterozygous FH, and normocholesterolemia, respectively, values in keeping with their receptor activity. The corresponding values for Lp[a] were 0.158, 0.142, and 0.199, respectively, and, in contrast to LDL, did not therefore appear to be influenced by the LDL-receptor status of these individuals. From the in vitro and in vivo data at present available it would seem that the LDL-receptor pathway does not play a major role in the catabolism of Lp[a]. However, final clarification is needed from turnover studies on larger numbers of individuals with different LDL-receptor activities.

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