Functional and metabolic differences between elastase-generated fragments of human lipoprotein[a] and apolipoprotein[a]

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Abstract We have previously shown that a functional free apolipoprotein[a] (apo[a]) can be isolated from its parent lipoprotein[a] (Lp[a]) by a mild reductive procedure. To shed further light on the properties of Lp[a] and apo[a] we subjected them to a limited proteolysis by porcine pancreatic elastase. This enzyme cleaved both at the Ile3520-Leu3521 bond in the linker between kringles IV-4 and IV-5 of apo[a] generating two fragments F1 and F2. In contrast to F1, which represented the N-terminal portion of apo[a] and was functionally inert, F2, representing the C-terminal domain, bound to lysine-Sepharose, fibrinogen, and fibronectin and formed a miniLp[a] particle when incubated with LDL. The proteolytic pattern by pancreatic elastase was also exhibited by human leukocyte elastase. F1, injected intravenously into normal mice, was rapidly cleared (T½, 2.9 h) and after 1 h fragments in the size range of 100–33 kDa were observed in the urine. In turn, F2 had a longer residence time (T½, 5 h) and was excreted in the urine only after 5 h as fragments of 70–45 kDa. Fragments in the same size range as found after F1 injection were also present in the urine after injection of apo[a] or Lp[a]. Moreover, apo[a] fragments of the size seen in mouse urine were spontaneously present in normal human urine and appeared derived from larger apo[a] fragments in the plasma. Our results indicate that enzymes of the elastase family cleave human apo[a] in vitro into two main fragments that differ in structural and functional properties and metabolic behavior. The comparable size of apo[a] fragments observed in the urine of rabbits and mice indicates the speculation that enzymes of the elastase family may play a role in the biology of Lp[a] in vivo. —Edelstein, C., J. A. Italia, O. Klezovitch, and A. M. Scanu. Functional and metabolic differences between elastase-generated fragments of human lipoprotein[a] and apolipoprotein[a]. J. Lipid Res. 1996. 37: 1786–1801.

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Lipoprotein [a] (Lp[a]) represents a class of lipoprotein particles containing apoB-100 linked by a single disulfide bridge to apolipoprotein[a] (apo[a]), a multi-kringle structure with a high degree of homology to plasminogen (for reviews, 1, 2). In the past, this chemical linkage prevented an investigation of the properties of free apo[a], which usually represents less than 0.5% of the total circulating Lp[a] (3). However, recently we have been able to isolate a water-soluble functional free apo[a] by subjecting Lp[a] to a mild reductive procedure in the presence of the lysine analogue ε-aminocaproic acid (EACA) (4). Moreover, we observed important differences in lysine and fibrinogen binding between free and Lp[a]-associated apo[a] and identified a domain which, in apo[a], is involved in fibrinogen and apoB-100 binding (5). In an attempt to better define the properties of this domain and in order to shed further light on the structural/functional relationships of the kringle domains in apo[a], we subjected apo[a] to limited proteolysis by using enzymes of the elastase family, shown by others to cleave in the interkringle regions of the five kringle-containing plasminogen (6). We initially focused on pancreatic elastase but later extended our investigation to leukocyte elastase, an ubiquitous enzyme of biological relevance (7–9). In our in vitro studies we compared the elastase digestion of free apo[a] and parent Lp[a] in order to assess whether the apo[a]–apoB-100 linkage might have an influence on the enzymatic reaction. We also examined the elastase-generated fragments for their capacity to bind to lysine, fibrinogen, fibronectin, and apoB-100 and studied their metabolic fate after injection into the mouse. The results

Abbreviations: Lp[a], lipoprotein[a]; apo[a], apolipoprotein[a]; LDL, low density lipoproteins; apoB-100, apolipoprotein B-100; F1, the N-terminal fragment of apo[a]; F2, the C-terminal fragment of apo[a]; R-miniLp[a], LDL reassembled with fragment F2; PAGE, polyacrylamide gel electrophoresis; EACA, ε-aminocaproic acid; DTE, dithioerythritol; PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropylfluorophosphate; KI, kallikrein inactivator; β-ME, β-mercaptoethanol; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SDS-PAGE, PAGE carried out in the presence of SDS; PBS, phosphate-buffered saline; K, kringle.

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of these in vitro and in vivo studies are the subject of this report.

MATERIALS AND METHODS

Chemicals and reagents

Materials were purchased from the following sources: cyanogen bromide (CNBr)-Sepharose 4B, EACA, Tween 20, phenylmethylsulfonyl fluoride (PMSF), diisopropylfluorophosphate (DFP), ethylenediaminetetraacetic acid (EDTA), L-lysine, L-proline, dithioerythritol (DTE), β-mercaptoethanol (β-ME), phosphate-buffered saline packets (PBS), porcine pancreatic elastase (EC 1.4.21.36) Type V, human leukocyte elastase (EC 3.4.21.37), human fibrinogen, and human plasma fibronectin from Sigma Chemical Co. (St. Louis, MO); kallikrein inactivator (KI) from Calbiochem (La Jolla, CA) and an enhanced chemiluminescent kit (ECL Western Blotting Detection kit) from Amersham (Arlington Heights, IL). All other chemicals were reagent grade.

Antisera to purified preparations of Lp[a] and low density lipoprotein (LDL) were raised in the rabbit. Antibodies to apo[a], Lp[a], and LDL were affinity-purified as previously described (10). Anti-Lp[a] was shown to be devoid of immunoreactivity to LDL and plasminogen; anti-LDL was unreactive to apo[a]. Monoclonal antibodies to apo[a] KV were prepared in our laboratory.

Buffers

Buffer A was 10 mM phosphate containing 1 mM EDTA, 0.02% NaN3, pH 7.5. Buffer B was 10 mM phosphate containing 1 mM EDTA, 0.02% NaN3, and 100 mM EACA, pH 7.5. All other buffers were prepared as described in the text.

Human subjects

The subjects used for the preparation of Lp[a] and LDL were healthy donors with plasma Lp[a] protein levels in the range of 15–45 mg/dl with a known apo[a] phenotype and genotype. Their plasma was obtained by plasmapheresis performed in the Blood Bank of the University of Chicago. The steps for Lp[a] and LDL isolation were carried out immediately after blood drawing using the procedure outlined below. We used an additional 10 subjects for studying the apo[a] fragments in their plasma and urine. These were also healthy subjects with a known phenotype and genotype. Their plasma Lp[a] protein levels varied between 0.1 and 10 mg/dl and their size isoforms varied between 300 and 600 kDa. Aliquots of 3 ml of plasma were floated at d 1.21 g/ml in a TLA-100.3 rotor in a tabletop TL-100 ultracentrifuge (Beckman Instruments, Palo Alto, CA) for 18 h at 100,000 rpm. The top fraction containing Lp[a] and the bottom fraction containing apo[a] were quantitated by ELISA using monospecific apo[a] antibodies and also analyzed by Western blots of SDS-PAGE. In each subject, 3–4 h urine samples were collected, centrifuged for 15 min at 3000 rpm and either used fresh or frozen immediately at -80°C. Before use, they were thawed, concentrated 200-fold in microconcentrators (Amicon Corp. Beverly, MA) and then separated by SDS-PAGE. All of the subjects used in the study gave a written informed consent.

Phenotyping and genotyping of apo[a]

Apo[a] phenotyping was performed on isolated apo[a] samples by SDS-PAGE followed by immunoblotting using anti-Lp[a] (4). The mobility of the individual apo[a] bands was compared with isolated apo[a] isoforms of known molecular weights (11). For apo[a] genotyping, DNA plugs were prepared from blood mononuclear cells, subsequently fractionated by pulsed field gel electrophoresis and the blots were developed with an apo[a] specific probe essentially as described earlier (12).

Preparation of human Lp[a] and LDL

To prevent lipoprotein degradation, the plasma obtained by plasmapheresis was adjusted with 0.15% EDTA, 0.01% NaN3, 10,000 U/L KI and 1 mM PMSF. Lp[a] were isolated by sequential ultracentrifugation and lysine-Sepharose chromatography as previously described (11). The purity of the product was assessed by mobility on precast 1% agarose gels (Ciba-Corning, Palo Alto, CA) and Western blots of SDS-PAGE, utilizing anti-Lp[a] and anti-apoB-100. The LDL preparations used in this study were isolated at d 1.030–1.050 g/mL by sequential flotation as previously described (13).

Isolation of apo[a] from Lp[a]

Apo[a] was isolated from Lp[a] essentially as described previously (4). Briefly, Lp[a], 1 mg/mL protein, was incubated with DTE at a final concentration of 1.5–2 mM. EACA to a final concentration of 100 mM was then added and the mixture incubated at room temperature for 1 h under nitrogen gas. After dialysis against buffer B, an equal volume of 60% sucrose in the same buffer was added and the resulting mixture was placed into a TLA 100.3 titanium rotor and spun in a table-top TL100 ultracentrifuge at 10°C, 412,160 g for 18 h. After cen-
trifugation, the top 0.5-mL fraction contained LDL free of apo[a], [Lp[a-]] and unreacted Lp[a]. The bottom 1.0 mL fraction contained free apo[a] in pure form. The latter was stored in the sucrose solution at -80°C.

**Limited proteolysis of Lp[a] and apo[a]**

To optimize the conditions for limited proteolysis, we conducted experiments in which Lp[a] and apo[a], containing KI (200 U/ml), were incubated at 22°C with porcine pancreatic elastase at various molar ratios of Lp[a] or apo[a] protein to enzyme (5, 10, 25, 50, 100 to 1) for time periods of 1 to 24 h. The digestion mixtures were then examined on Western blots of SDS-PAGE probed with anti-apo[a]. Our end point for limited proteolysis was the production of two major bands of 220 kDa and 170 kDa. Minor amounts (<10%) of smaller molecular weight bands were also observed. The final conditions used in the subsequent studies were as follows: Lp[a] or apo[a] in 50 mM Tris-HCl, 100 mM NaC1, pH 8.0, KI (200 U/ml) were digested with pancreatic elastase at a molar ratio of 25:1 (protein:enzyme) at 22°C for 2 h and the reaction was terminated by the addition of 5 mM DFP with further incubation for 20 min. Limited proteolysis with human leukocyte elastase (1 U = 1 nm p-nitrophenol/sec from N-1BOC-L-Ala p-nitrophenyl ester) was conducted after 1500-fold dilution of the diluted enzyme was incubated per µg of apo[a] protein at 37°C for 1 h. The reaction was terminated with 5 mM DFP for 20 min at 22°C.

**Reassembly studies**

To quantitate the amount of Lp[a] reassembled, apo[a] or elastase-generated fragments (1 µg) were incubated with LDL in buffer A at an apoB-100:apo[a] molar ratio of 50:1 in a total volume of 175 µL using a shaking water bath at 37°C for 5 h in the presence of 50 μM β-hydroxytoluene, KI (10,000 KI units/L) and 1 mM PMSF under nitrogen gas. In some experiments, the apo[a] was incubated with 100 mM EACA for 1 h at 37°C before the addition of LDL and the mixture was then incubated for 5 h. An aliquot of the reaction mixture was diluted with an equal volume of 60% sucrose containing 200 mM EACA and centrifuged as previously described (4). Subsequently, the top fraction was removed and quantitated by ELISA as described (4). In the studies directed at defining the properties of reassembled Lp[a], apo[a] fragments were incubated with LDL and the reassembled products were isolated by a combination of centrifugation and lysine-Sepharose chromatography according to the previously described procedure (4).

**Lysine-Sepharose chromatography**

CNBr-activated Sepharose 4B was coupled to the α-amino group of L-lysine essentially according to the instructions supplied by Pharmacia-LKB. The amount of L-lysine crosslinked to the beads was assessed according to Wilkie and Landry (14) and ranged between 16 and 21 μmol of L-lysine per mL bead suspension. Chromatography was performed at 22°C on a Bio-Rad Econo Chromatography system (Bio-Rad, Hercules, CA). Columns were packed with lysine-Sepharose at a ratio of 5 mL of packing material to 1 mg of Lp[a] protein and equilibrated with PBS containing 1 mM EDTA and 0.02% NaN3. After loading, the column was washed with at least 3 column volumes of equilibrating buffer followed by 3 column volumes of 500 mM NaCl in order to elute non-specifically absorbed material and with 200 mM EACA for elution of specifically bound components.

**Molecular sieving chromatography**

To separate undigested apo[a] from the elastase digest, 0.2-ml aliquots were applied onto a Superose 6 prepacked HR 10/30 column (Pharmacia Biotech Inc., Piscataway, NJ) previously equilibrated with 10 mM Tris-HCl, pH 8.0, containing 150 mM NaCl. Chromatography was conducted with an FPLC system (Pharmacia Biotech) at a flow rate of 0.3 ml/min.

**Fibrinogen and fibronectin binding assay**

The wells of microtiter plates (Beckman, Fullerton, CA) were coated with 10 µg/ml (100 µl/well) of fibrinogen or fibronectin in TBS buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) and incubated overnight at 22°C. Nonspecific binding sites were blocked with 2% BSA and 0.02% Tween-20, various concentrations of Lp[a] and the derived fragments were added to the wells in TBS buffer with or without 200 mM EACA and incubated for 2 h at 22°C. After washing with TBST buffer (TBS supplemented with 0.1% BSA and 0.02% Tween-20), various concentrations of Lp[a] and the derived fragments were added to the wells in TBS buffer with or without 200 mM EACA and incubated for 2 h at 22°C. After washing with TBST buffer, rabbit anti-apo[a] antibody was added and incubated for 1 h at 22°C. At this time, the wells were washed with TBST buffer and the goat anti-rabbit IgG conjugated to alkaline phosphatase was added for 1 h at 22°C. After washing with TBST buffer, p-nitrophenyl phosphate (1 mg/ml in diethanolamine buffer, pH 9.8) was added and the color development followed at 405 nm on a microplate reader, Biomek 100 (Beckman, Fullerton, CA). The results obtained, as the change of absorbance per minute, were then transformed into moles by using standard curves established for each component. The maximum
number of moles bound at saturation was defined as \( B_{\text{max}} \). Analysis of the binding data was performed on the assumption of single-site binding. Dissociation constants \((K_d)\) were derived from the slope of the linearized expression of the Langmuir equation (15).

\[
[F_n - X] = \frac{[F_n_0] K[X]}{[X][1 + K[X]]}
\]

where \([F_n_0]\) represents the total number of fibrinogen or fibronectin binding sites, \([F_n]\) the number of moles adsorbed on fibrinogen or fibronectin, \([X]\) refers to Lp[a], apo[a], mini-Lp[a] or apo[a] fragments, and \(K\) the association constant.

**Electrophoretic methods**

SDS-PAGE, (4% polyacrylamide or gradients of 4–12%), was performed on a Novex system (Novex, San Diego, CA) for 1.5 h at constant voltage (120 V) at 22°C. The samples were prepared by heating at 95°C for 5 min in sample buffer that consisted of 94 mM phosphate buffer, pH 7.0, 1% SDS, and 2 M urea with or without 3% β-ME. Immediately after electrophoresis, the gels were placed onto Immobilon-P sheets (Millipore Corp., Bedford, MA) that were previously wetted with a buffer containing 48 mM Tris, 99 mM glycine, pH 8.9. Blotting was performed on a horizontal semi-dry electroblot apparatus (Pharmacia-LKB, Piscataway, NJ) at 0.8–1 mA/cm² for 45 min at room temperature.

**Immunoblotting**

After electroblotting, the Immobilon-P sheets were blocked in PBS containing 5% non-fat dry powdered milk and 0.3% Tween 20 followed by incubation with anti-apo[a] or anti-apoB antibody. In specified cases, monoclonal antibodies directed against kringle V of apo[a] were used. The blots were washed and incubated with anti-rabbit or anti-mouse horseradish peroxidase-labeled IgG. Subsequently, the blots were developed with the ECL Western Detection Reagent according to the manufacturer's instructions.

**Amino-terminal sequence analyses**

Apo[a] fragments (10–30 µg) were electrophoresed under reducing conditions as outlined above. After electrophoresis, the gels were electroblotted onto Immobilon PSQ sequence grade membranes (Millipore Corp., Bedford, MA) as described above in the immunoblotting section. The gels were rinsed in distilled water, stained with Coomassie Blue R250 (0.025% in 40% methanol) and destained with 50% methanol. The stained bands were cut from the membrane, further washed with 40% methanol, and allowed to air dry. Reduction with DTT and alkylation with iodoacetamide was performed directly on the PSQ membrane which was then subjected to automated Edman degradation on an Applied Biosystems 477A unit using procedures recommended by the manufacturer.

**Electron microscopy**

Solutions containing lipoproteins at 0.05 mg/mL protein in 10 mM NH₄HCO₃ were transferred to Formvar carbon-coated copper grids. Lipoproteins were allowed to adhere, and the excess was removed by touching the edge of the grids with filter paper. After washing the grids twice with deionized water, each grid was coated with one drop of 1% phosphotungstic acid. The excess phosphotungstic acid was removed and the grids were air dried and examined in a Philips CM 10 electron microscope at an accelerating voltage of 100 kV.

**Lipoprotein and apolipoprotein analyses**

Lp[a] and LDL protein were quantitated by a sandwich ELISA essentially as previously described (10) except that anti-Lp[a] and IgG was used as the capture antibody and anti-apoB-100 IgG conjugated to alkaline phosphatase as the detection antibody. For the ELISA quantitation of apo[a], anti-apo[a] IgG conjugated to alkaline phosphatase was used as the detection antibody. Subsequently, an extinction coefficient \( (ε_{278} = 1.31 \text{ mL mg}^{-1} \text{ cm}^{-1}) \) was established for apo[a] in the 30% sucrose solution. Quantification of kringle V in apo[a] and the fragments was carried out with a sandwich ELISA in which anti-kringle V was the capture antibody and anti-apo[a] IgG conjugated to alkaline phosphatase was the detection antibody. Protein determinations were performed by the Bio-Rad DC Protein Assay.

**Metabolic studies in mice**

Balb/c female mice (10–12 weeks) from Jackson Laboratories (Bar Harbor, ME) were used. All mice were housed in individual cages under normal light. The evening before the experiment, the mice were given a 10% sucrose solution to drink ad libitum in place of water. The following morning the mice were anesthetized with Metafane and 25–250 µg of either Lp[a], apo[a], or the fragments obtained from the elastase digestion in a volume of 200 µL were injected into the tail vein. The mice were then placed in metabolic cages and given access to standard lab chow and the 10% sucrose solution. Blood samples were withdrawn from the orbital vein into heparinized hematocrit tubes at the specified time points and immediately iced. Urine was
collected at 0–3, 3–5, and 5–24 h. ELISA quantitation, sensitive to >0.0015 mg/dl of apo[a], was performed on the urine samples to determine the levels of apo[a] reacting material. These results indicated to what degree the sample was to be concentrated for electrophoretic detection which was estimated to be >0.03 mg/dl. The urine was concentrated in Amicon Centriprep filters and the extent of concentration for each representative sample is stated in the legends to the figures. Mouse plasma and urine were analyzed for the levels of apo[a], Lp[a], and kringle V by a sandwich ELISA using monospecific antibodies as described above. The decay of Lp[a], apo[a] or fragments in plasma was expressed as:

\[
\text{percent of injected dose} = \left( \frac{\text{mg/dl}_t}{\text{mg/dl}_i} \right) \times 100
\]

where \(t\) was the concentration at a given time and \(i\) was the concentration in plasma at 1 min after injection. The log of the percent of the injected dose was plotted against time and the half-time (T_{1/2}) of the injected sample in the intravascular compartment was determined from the slope of the linear portion of this curve. The plasma and urine specimens were also analyzed by SDS-PAGE.

Molecular modeling

Modeling was performed on a molecular graphics workstation from Silicon Graphics Inc. using the modeling system Insight II v.95.0 and the programs Builder, Biopolymer and Discover (Biosym/MSI, San Diego, CA). As crystallographic coordinates are not available for the linker regions we used the amino acid sequence deduced from the cDNA sequence (16) and built each amino acid sequentially in a linear fashion. The secondary structure was then constructed based on the algorithms of Chou and Fasman (17) and Garnier, Osguthorpe, and Robson (18) and the model was subjected to energy minimization.

RESULTS

Limited digestion of Lp[a] and apo[a] by pancreatic elastase

Lp[a] and apo[a] were digested with pancreatic elastase under conditions of limited proteolysis and the digests were subjected to 4% SDS-PAGE immunoblot analysis using a rabbit anti-apo[a] polyclonal antibody. Figure 1A shows immunostained gels run under unre-
Fig. 2. Western blot analyses of the products separated from Lp[a] digests by ultracentrifugation. Lp[a] was digested with porcine pancreatic elastase as described in the legend to Fig. 1A and the digests were separated by ultracentrifugation (see Results) and the top and bottom fractions were isolated and analyzed by 4% SDS-PAGE as described in Materials and Methods. Rabbit anti-apo[a] polyclonal antibody was used to probe the blot. On the left are the unreduced samples: lane Lp[a], control Lp[a] before digestion with pancreatic elastase; lane 1, unfractionated Lp[a] digest; lane 1, top d 1.127 g/ml fraction; lane 3, bottom d 1.127 g/ml fraction; lane 4, purified fragment F2 obtained from the mild reduction of the d 1.127 g/ml top fraction. The arrow marks the position of the band migrating faster than Lp[a]. Right, reduced gel, samples as defined in lanes 1-4 of the unreduced blot were reduced with 3% β-ME and analyzed.

duced (left) and reduced (right) conditions. Lanes 1 and 4 represent the control undigested Lp[a] and apo[a], respectively. In the unreduced gels, digested Lp[a] and apo[a] (lanes 2 and 3, respectively) exhibited a common migrating band, designated as F1. On reduced gels, the banding patterns of digested Lp[a] and apo[a] were identical (lanes 2 and 3). F1 had an apparent mass of 220 kDa and was associated with a second band with an apparent mass of 170 kDa, designated F2, and a set of faint bands differing in size by approximately 20 kDa, which is the apparent size of a single kringle. On reduced gels, apo[a], F1, F2 and the repeating bands migrated with slower mobilities than under nonreduced conditions, likely due to the effect of the reducing agent on the conformation of each product. The limited proteolysis of Lp[a] and apo[a] was also carried out by another enzyme of the elastase family, human leukocyte elastase. On anti-apo[a] immunostained reduced gels, the banding pattern of the leukocyte elastase digest was compared to that of the digest from pancreatic elastase (Fig. 1B, lanes 1 and 2, respectively). Leukocyte elastase digestion of Lp[a] and apo[a] and subsequent isolation of the proteolytic products gave results comparable to those obtained with pancreatic elastase (data not shown). Based on these results, in the subsequent studies we utilized pancreatic elastase as this enzyme was readily available to us in a highly purified form and in relatively large quantities.

**Ultracentrifugal separation of the products obtained by digestion of Lp[a] with pancreatic elastase**

Digested Lp[a] (Fig. 2, lane 1) was diluted 1:1 (v/v) with 60% sucrose in buffer A containing 200 mM EACA to a final density of 1.127 g/ml and the mixture was centrifuged in a TL 100 tabletop ultracentrifuge at 15°C, overnight. Two fractions were collected; a top (0.5 ml), and a sedimenting one (1 ml). In the anti-apo[a] immunostained unreduced gels, the top fraction contained a band representing undigested Lp[a] and a second one that migrated faster than Lp[a], (see arrow Fig. 2, lane 2, unreduced gel). Both bands were also detected with an anti-apoB-100 antibody (data not shown). Upon reduction, these bands disappeared and were replaced by a faint apo[a] band and one migrating in the position of F2 (lane 2, reduced). The latter was unreactive to the anti-apoB-100 antibody (data not shown). The bottom fraction contained F1 and a few faint bands with a faster mobility.
Fig. 3. Fractionation by lysine-Sepharose of the products from the limited digestion of apo[a] with pancreatic elastase and electrophoretic analyses of the products. Panel A: lysine-Sepharose elution profile. The apo[a] digest was dialyzed against buffer A before loading onto a 7-ml column. The sample was applied at a flow rate of 7.8 ml/h. After washing with three column volumes each of PBS, the column was further washed with 500 mM NaCl to elute nonspecifically bound material; thereafter, the bound component was eluted with 200 mM EACA at a flow rate of 15 ml/h. Panel B: Western blot analyses of the fractions isolated by lysine-Sepharose chromatography. The fractions were reduced, separated on 4-12% gradient SDS-PAGE, and probed with anti-apo[a] antibody. Lane 1, apo[a] before digestion; lane 2, apo[a] digest applied to the column; lane 3, fraction F1, eluted in the PBS wash; lane 4, fraction F2, eluted with EACA.

Migration (lane 3 unreduced and reduced gels). In addition, the bottom fraction was unreactive to anti-apoB-100 antibody and, based on a specific ELISA, did not contain KV (data not shown).

The d 1.127 g/ml top was treated with 1.5 mM DTE in the presence of 100 mM EACA and centrifuged in 30% sucrose according to the method utilized by us for the isolation of free apo[a] (4). The sedimenting lipid-free fraction contained a small amount of undigested apo[a] and F2. The latter was purified by molecular sieving on Superose 6. By electrophoresis, the isolated F2 exhibited a band with an apparent mass of 170 kDa, as detected by an anti-apo[a] antibody (Fig. 2, lane 4, unreduced and reduced gels). Based on specific ELISAs, F2 contained KV but not apoB-100 (data not shown). Thus, the limited proteolysis of Lp[a] by elastase produced two fractions: one of them, referred to as F1, was lipid-free, sedimented at d 1.127 g/ml, and reacted against anti-apo[a] but not against anti-KV or anti-apoB-100; the second fraction was lipid-rich, floated at d 1.127 g/ml, and reacted against anti-apoB-100, anti-apo[a], and anti-KV. Mild reduction of this fraction generated a 170 kDa band, F2, reactive against anti-apo[a] and anti-KV and nonreactive against anti-apoB-100. We interpreted the above results to indicate that the d 1.127 g/ml floating fraction is a lipoprotein particle containing apoB-100 covalently linked to F2. We called this lipoprotein, miniLp[a]. This nomenclature was recently proposed by Huby et al. (19) to designate a lipoprotein particle that they obtained by digesting Lp[a] with thermolysin. In those studies thermolysin caused hydrolysis of apoB-100. In the current studies elastase also caused a partial cleavage of apoB-100 in Lp[a] and the resulting fragments remained associated with the lipoprotein particle (see section on Functional studies).

Thus, limited elastase digestion cleaves Lp[a] into a lipid-free fraction, F1, and a miniLp[a] particle in which F2 is linked covalently to LDL containing a partially hydrolyzed apoB-100.

Isolation by lysine-Sepharose chromatography of the products obtained by limited digestion of apo[a] with pancreatic elastase

Free apo[a] digested with pancreatic elastase (Fig. 3B, lane 2) was applied to a lysine-Sepharose affinity column which was then washed with three column volumes of PBS, 500 mM NaCl, and 200 mM EACA (Fig. 3A). Two major peaks were observed, one eluting with PBS and one with EACA. Electrophoretic analyses on reduced gels (4-12%) probed with anti-apo[a] showed that the unbound fraction eluting with PBS represented F1 migrating in the 220 kDa position (Fig. 3B, lane 3) and the fraction eluting with EACA represented F2 migrating in the 170 kDa position (lane 4). Of the two fractions, only F2 reacted against anti-KV (data not shown).

Amino terminal sequence analyses of the proteolytic fragments obtained from digested Lp[a] and apo[a]

Figure 4 shows the partial NH2-terminal sequences of F1 and F2 obtained from the elastase digestion of Lp[a] and apo[a]. By aligning these sequences with those of apo[a], using the program ClustalW, we located the
cleavage site at the Ile3520-Leu3521 bond in the linker region between kringles IV-4 and IV-5. From these data we concluded that the apo[a] fragment that eluted from the lysine-Sepharose column with PBS (Fig. 3A) was identical to the ultracentrifugal d 1.227 g/ml bottom fraction (Fig. 2, lane 3) and corresponded to the N-terminal portion of apo[a] comprising KIV-1 through KIV-4. In turn, the fraction that eluted with EACA (Fig. 3A) was identical to F2, the apo[a] fragment obtained by mild reduction of elastase digested Lp[a] (Fig. 2, lane 4). This fraction corresponds to the C-terminal fragment of apo[a] containing kringles IV-5 through IV-10, kringle V, and the protease region. The electrophoretic migration of F1 varied according to apo[a] size, an indication of its dependence on the number of KIV-2 repeats. In contrast, F2 exhibited a constant size which, based on amino acid composition, we calculated to be 113,030.

Thus, whether starting from Lp[a] or free apo[a], elastase cleavage produces two main apo[a] fragments, F1 and F2, representing the N- and C-terminal components of apo[a], respectively. A schematic diagram of the structure of Lp[a] and the derived fragments is shown in Fig. 5.

Functional studies

LDL binding. We have previously shown that the incubation of apo[a] with LDL results in an Lp[a] particle with properties indistinguishable from those of native Lp[a] (4). In the current studies, the incubation of F2 with LDL at a molar ratio of 50:1 (LDL:F2) for 5 h at room temperature, produced an F2-LDL covalent complex. This was not the case for F1. The yields of the reassembled complex with F2 were comparable to those obtained when apo[a] was incubated with LDL (Table 1). Similarly, the assembly was inhibited by either EACA or proline. In the unreduced form, the F2-reassembled particle migrated electrophoretically as a single band that was immunostainable by both anti-apo[a] and anti-apoB-100 (Fig. 6A and B, lane 3, unreduced gel). On reduced gels, anti-apo[a] staining revealed a band corresponding to fragment F2 (Fig. 6A, lane 3, reduced gel); in turn, immunostaining with anti-apoB-100 showed one band that migrated in the position of apoB-100 (Fig. 6B, lane 3, reduced gel). From these results we came to recognize that by combining F2 with LDL we had generated a particle differing from Lp[a] by the absence of the N-terminal fragment, F1. Thus, we called this particle, R-miniLp[a] to differentiate it from the miniLp[a] that we generated by the cleavage of Lp[a] with elastase. MiniLp[a], obtained from the elastase digestion of Lp[a] (Fig. 6A and B, lane 4, unreduced gels), exhibited fragments of apoB-100 (Fig. 6B, lane 4, reduced gel). Therefore, in our functional studies we utilized R-miniLp[a] particles where the fragmentation of apoB-100 was absent. By electron microscopy (Fig. 7), native Lp[a], miniLp[a], and R-miniLp[a] had comparable mean diameters of 24.2 ± 4.2 (n = 184), 24.8 ± 3.7 (n = 190), and 20.2 ± 3.7 (n = 190), respectively.

Fig. 4. Alignment of the partial amino acid sequences of F1 and F2. F1 and F2 were purified from the elastase digests of Lp[a] and apo[a] as outlined in Results and prepared for microsequencing as described in Materials and Methods. Apo[a] is composed of repeats of KIV numbered 1-10, one KV and a protease domain (P) according to the nomenclature of Scam and Edelstein (1). The first 20 amino acids obtained by sequencing F1 were aligned to the amino acid sequence of the mature apo[a] without the signal peptide sequence. The amino acid sequence of F2 began with leucine at position 3521. The elastase cleavage site was determined to be in the linker region between KIV-4 and 5. The dashed lines refer to undetermined amino acids and the dotted lines are continuation of sequences upstream and downstream of the protein sequences. Alignments to the deduced amino acid sequence of apo[a] (16) were performed with the ClustalW sequence alignment program.
25.1 ± 3.7 nm (n = 202), respectively. However, R-miniLp[a] appeared somewhat more homogeneous in size than native Lp[a] or miniLp[a].

Binding to lysine-Sepharose. Like native Lp[a] and apo[a], R-miniLp[a] and F2 bound specifically to lysine-Sepharose in that they were eluted from it with EACA. In contrast, F1 representing the N-terminal portion of apo[a], the unfractionated apo[a] digest, and F1 the fastest. By following the clearance of the unfractionated elastase digest of apo[a] on reduced electrophoretic gels probed with anti-apo[a] we observed that F1 and F2 had different removal rates (Fig. 9B). Three hours after injection, the bands corresponding to F1 were no longer visible, whereas, F2 was still present up to the 5 h time point.

Metabolic studies in mice

The clearance rates of intravenously injected Lp[a] and the derived products were based on ELISA quantitation of mouse plasma collected up to 24 h after injection to insure that more than 95% of the injected material was removed from the circulation. Figure 9A shows a semi-log plot of the percent of injected dose as a function of time. The slopes of the curves between 3 and 7 h could be separated into three groups: R-miniLp[a] with the slowest clearance, Lp[a] and F2 intermediate, and apo[a] the fastest. By following the clearance of the unfractionated elastase digest of apo[a] on reduced electrophoretic gels probed with anti-apo[a] we observed that F1 and F2 had different removal rates (Fig. 9B). Three hours after injection, the bands corresponding to F1 were no longer visible, whereas, F2 was still present up to the 5 h time point. Table 3 lists the plasma half-times (T½), calculated from the slope of the linear

<table>
<thead>
<tr>
<th>Incubation Mixture</th>
<th>No Additive</th>
<th>EACA</th>
<th>L-Proline</th>
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<tbody>
<tr>
<td>Apo[a] + LDL</td>
<td>70</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>F2 + LDL</td>
<td>60</td>
<td>10</td>
<td>20</td>
</tr>
</tbody>
</table>

All incubations were performed using apo[a] or fragment F2 and human LDL at an apoB-100:apo[a] molar ratio of 50:1 for 5 h at 37°C. After centrifugation overnight in sucrose at d 1.127 g/ml, the floating fraction was analyzed for the presence of apoB-100:apo[a] complexes by ELISA (see Materials and Methods).

⁎EACA or proline (100 μmol) was incubated with apo[a] or fragment F2 for 1 h at 37°C before incubation with LDL.
portion of each decay curve between 4 and 24 h. The apparent $T_{1/2}$ of R-miniLp[a] is significantly longer than that of Lp[a] (8.3 and 5.1 h, respectively). In turn, the $T_{1/2}$ of F1 is shorter than that of F2 (2.9 and 5.0 h, respectively). Of note, the $T_{1/2}$ of apo[a] is identical to that of the unfractionated digested apo[a] (3.7 h).

We also examined, on anti-apo[a] immunostained blots of reduced 4% gels, the plasma samples taken 1 h after injection of Lp[a] and derivatives into the mouse. Compared to the electrophoretic patterns before injection of Lp[a] and apo[a] (Fig. 10A, lanes 1 and 3, respectively), those after injection contained new bands (lanes 2 and 4) in addition to the major apo[a] component, suggesting that a degradation process had occurred in vivo. It should be noted that the extent of Lp[a] and apo[a] degradation in plasma was less than 5%, and that the majority of the injected material remained intact. The banding pattern of F1 before and after injection (lanes 5 and 6, respectively) was comparable in banding pattern but increased in band intensity. On the other hand, after injection, F2 exhibited an additional faster migrating band designated by an arrow (compare lanes 7 and 8). We next examined the urine of mice 0–5 h after injection of Lp[a] and derivatives by anti-apo[a] immunostained blots of reduced 4–12% gradient gels. The urinary pattern of Lp[a], apo[a] and F1 (lanes 2, 3 and 4, respectively) showed a broad spectrum of bands, three of which were comparable, migrating at 85, 57, and 33 kDa, respectively. In contrast, the urinary pattern after injection of F2, exhibited two major bands migrating in a more narrow size range (70 to 62 kDa) (lane 5). Of interest, by a KV specific ELISA, none of the urine samples contained KV.

In order to rule out artifactual contributions to the formation of fragments, the collected urine samples from injected mice were incubated at 37°C overnight and compared to freshly collected urine kept on ice. Western blot analyses showed no formation of new fragments. Moreover, the mobility of the bands on the gels was unaffected by changing the pH of the urine (pH 3 to 8). No fragments were observed when the urine of a control uninjected mouse was incubated with intact Lp[a] or its derivatives. It is particularly interesting to note that when apo[a] was digested with pancreatic elastase for prolonged periods of time (12–24 h), the fragments observed, on analysis of the digest by SDS-PAGE were closely comparable to the patterns produced on gel analyses of urine samples obtained from mice injected with apo[a] (data not shown).

The above data indicate important differences between the urinary patterns of injected F1 and F2 both in qualitative and quantitative terms. The F1 pattern was characterized by several bands that were observed as early as 1 h after injection and by ELISA, represented less than 0.5% of that injected. In comparison, the F2 pattern was characterized by fewer bands that appeared later (5 h) and represented less than 0.05% of the injected material.

![Fig. 6](image-url) Western blot analysis of R-miniLp[a] and miniLp[a]. Panel A: anti-apo[a] immunoblot of unreduced and reduced samples in 4% SDS-PAGE. Lane 1, control Lp[a]; lane 2, purified F2 used for the incubation with LDL; lane 3, R-miniLp[a] purified by lysine-Sepharose affinity chromatography; lane 4, miniLp[a] purified from Lp[a] previously digested by pancreatic elastase. Panel B: anti-apoB-100 immunoblot of unreduced and reduced samples in 4% SDS-PAGE. Lane 1, control Lp[a]; lane 2, purified LDL used for the incubation with F2; lane 3, R-miniLp[a] purified by lysine-Sepharose affinity chromatography; lane 4, miniLp[a] purified from Lp[a] previously digested by pancreatic elastase.
**Fig. 7.** Electron micrographs of human Lp[a], miniLp[a], and R-miniLp[a]. All samples were at a concentration of 50 µg/ml in 10 mM NH₄HCO₃. Top panel: control Lp[a], miniLp[a] purified from Lp[a] previously digested by pancreatic elastase, and R-miniLp[a] purified by lysine-Sepharose affinity chromatography, respectively. The electron micrographs were taken at an instrumental magnification of 45,000. The bar indicates 100 nm. Bottom panel: bar graph of the size distribution of particles for each corresponding sample.

**Studies in human plasma and urine**

All subjects studied exhibited similar results in terms of electrophoretic band patterns irrespective of plasma Lp[a] protein levels and apo[a] size isoform. In terms of plasma, a representative fresh sample is shown on an anti-apo[a] immunostained reduced gel in Fig. 10A, lane Hu. In addition to intact apo[a], there are at least three other bands that were later recovered in the d 1.21 g/ml bottom fraction. By ELISA, this free apo[a] represented about 5% of the total plasma Lp[a] fraction.

In terms of urine, the pattern of apo[a] fragments (Fig. 10B, lane Hu) was essentially the same as that observed in mice injected with apo[a] (Fig. 10B, lane 3). As in the mouse, the apo[a] fragments in human urine failed to react against an anti-KV antibody.

**DISCUSSION**

Our studies have shown that limited proteolysis by pancreatic elastase cleaves human apo[a] at the Ile3520-Leu3521 bond located in the linker between kringles IV-4 and IV-5. As the same cleavage pattern was obtained with both apo[a] and Lp[a], it is apparent that the elastase-sensitive site is not hindered by the linkage of apo[a] to LDL, suggesting that enzyme site accessibility may depend on the intrinsic properties of the linker between kringles IV-4 and IV-5. A structural model of this linker is shown in Fig. 11. It is interesting to note that the cleavage by elastase occurs at the end of the β-structure region where the neutral amino acid Ile appears more exposed and available. The Ile-Leu cutting site is consonant with the known specificity for neutral amino acids by the enzymes of the elastase family (20).

In the case of apo[a], the cleavage by pancreatic elastase at the Ile3520-Leu3521 bond generated two discrete fragments which we called F1 and F2. F1 comprised kringles IV-1, IV-2 repeats, IV-3, and IV-4 and corresponded to the N-terminal domain of apo[a]. Consistent with the chemical data was the observation that by electrophoretic criteria the size of F1 varied according to apo[a] isoform size which is dependent on the
number of kringles IV-2 repeats (12, 16). F2 comprised kringles IV-5 to IV-10, kringle V, and the protease region and represented the C-terminal domain of apo[a].

As in the case of apo[a], elastase cleavage of Lp[a] produced F1 and F2. However, F2 was linked to LDL in the form of an LDL/F2 complex which we called miniLp[a] because it was smaller than the parent Lp[a] by having only one apo[a] fragment and also by electrophoretic criteria (see Fig. 6). Huby et al. (19) reported the generation of a miniLp[a] particle by subjecting Lp[a] to limited digestion by thermolysin. This enzyme also cleaved apo[a] in the linker between kringles IV-4 and IV-5 but at the Ala3513-Phe3514 bond, which is seven amino acids upstream of the elastase cleavage site. Thus, the miniLp[a] generated by the thermolysin digestion method contains a truncated apo[a] that is seven amino acids longer than our F2. Unfortunately, as shown by Huby et al. (19) and our current studies, neither thermolysin- nor elastase-generated miniLp[a] particles are ideal end-products because both enzymes cause a partial proteolysis of apoB-100 with retention of the fragments on the lipoprotein particle. According to the results of our current studies, a better way to produce miniLp[a] is by the reassembly approach exploiting the capacity of F2 to covalently associate with LDL. The resulting R-miniLp[a] resembles elastase-derived miniLp[a] but it has the important advantage of containing intact apoB-100. For these reasons, R-miniLp[a] was preferentially used in our work.

In the course of our studies we became attracted by the observation that a discrete cleavage of either Lp[a] or apo[a] could be produced by two naturally occurring and physiologically relevant enzymes of the elastase family and asked whether their activity on Lp[a]/apo[a] might be of general relevance to Lp[a] biology. As one of the approaches to answer this question we tested Lp[a] and its various derivatives (see Fig. 5) for their fibrinogen-binding capacity. Only F2 and the F2-containing R-miniLp[a] bound to fibrinogen whereas F1 was inactive. The activity of F2 was anticipated as F2 contains the domain between kringles IV-5 to IV-8 which, as we previously reported (5), is involved in the binding of apo[a] to fibrinogen and is partially masked when it is linked to apoB-100 (4, 5). In the latter respect, our current studies determined that F2, in its free form, had a higher binding capacity for fibrinogen than when it was a member of R-miniLp[a]. Moreover, we observed a functional divergence between F2 and F1 in the fibronectin-binding studies indicating that in apo[a], F2 is the fragment that has potentially relevant biological functions.

An important difference between F1 and F2 was also revealed by the studies in which mice were injected intravenously with these two fragments. Based on ELISA and gel analyses, the injected F1 had a short residence time in the plasma and was also rapidly excreted in the urine in the form of several fragments. The remarkable aspect of this study was that the relatively homogeneous F1, after injection into the tail vein of the mouse, appeared in the plasma as four or five distinct electrophoretic bands in the size range of 220–135 kDa and as markedly smaller ones in the urine (size range 100–33

![Graph A](data:image/png;base64,iVBORw0KGgoAAAANSUhEUgAAAgAAAAAHCAMAAABQ/DxgAAAABGdBTUEAALGPC/xhBQAAAABJRU5ErkJggg==)

![Graph B](data:image/png;base64,iVBORw0KGgoAAAANSUhEUgAAAgAAAAAHCAMAAABQ/DxgAAAABGdBTUEAALGPC/xhBQAAAABJRU5ErkJggg==)

**Fig. 8.** Binding of R-miniLp[a] and apo[a] fragments to fibrinogen and fibronectin. R-miniLp[a] (○), F2 (●), and F1 (■), at concentrations in the range of 0 and 125 nM, were incubated for 2 h at 22°C with either immobilized fibrinogen (panel A) or fibronectin (panel B) as described in Materials and Methods. The binding to fibrinogen (panel A) was expressed as a specific lysine-mediated binding obtained by subtracting the binding in the presence of 200 mM EACA from the total binding. The binding to fibronectin (panel B) is represented by the total binding due to the absence of an inhibitory effect of EACA. The data are the means of two determinations for a representative experiment.

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### TABLE 2. Binding of Lp[a], apo[a], and the derived fragments to fibrinogen and fibronectin

<table>
<thead>
<tr>
<th>Sample</th>
<th>Binding to Fibrinogen</th>
<th>Binding to Fibronectin</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$K_d$ $^c$ $^nM$</td>
<td>$B_{max}$ $^c$ $fmol$</td>
</tr>
<tr>
<td>Lp[a]</td>
<td>5.8 ± 0.8</td>
<td>1.3 ± 0.8</td>
</tr>
<tr>
<td>R-miniLp[a]</td>
<td>13.3 ± 3.6</td>
<td>6.1 ± 1.3</td>
</tr>
<tr>
<td>Apo[a]</td>
<td>3.8 ± 2.9</td>
<td>36.6 ± 2.9</td>
</tr>
<tr>
<td>F2</td>
<td>10.3 ± 5.1</td>
<td>56.2 ± 32.2</td>
</tr>
<tr>
<td>F1</td>
<td>N/A $^d$</td>
<td>N/A $^d$</td>
</tr>
</tbody>
</table>

$^a$EACA-inhibitable, or lysine-mediated binding, obtained by subtracting the binding in the presence of 0.2 m EACA from the total binding.

$^b$Total binding, as in the presence of 0.2 m EACA the binding to fibronectin was not significantly affected.

$^c$Total binding, calculated as described in Material and Methods. The values are expressed as mean ± SD from at least three independent experiments in duplicate.

$^d$N/A, not available, as F1 exhibited no binding to either fibrinogen or fibronectin.

kDa). These data indicate a precursor–product relationship between injected F1, plasma fragments, and urine fragments and also suggest an enzymatic basis for these conversions. This progressive conversion can be accounted for by an elastase action as our in vitro studies showed that prolonged digestion with elastase can reduce apo[a] to fragments comparable in size to those in the urine, suggesting, although not proving, that an elastase-like system may be active in the mouse. The relatively large size of apo[a] fragments in the urine also suggests that the mouse kidney plays an active role in their excretion, in accord with the earlier proposal by Mooser et al. (21).

Contrary to F1, injected F2 had a comparatively longer plasma residence time and was excreted in rather minute amounts in the form of fragments which could only be detected in 15- to 30-fold urine concentrates. The metabolic divergence between F1 and F2 was also documented by the experiments in which the whole unfraccionated elastase digest of apo[a] was injected into the mouse, showing again that most of the rapidly excreted apo[a] urinary fragments were of F1 derivation.

Very small amounts of apo[a] fragments of the F1 type were also detected in 3- to 10-fold urine concentrates of mice injected intravenously with preparations of Lp[a] or apo[a] that had not been previously digested with elastase. Contrary to the relative homogeneity of these materials prior to injection, several bands were also present in the plasma of the injected mice. Taken together, these data appear to provide reasonable evidence that, although in a minor way, the normal mouse...

**Fig. 9.** Clearance of Lp[a] and derivatives from mouse plasma. Panel A: mice were injected with 25–250 µg of R-miniLp[a] (■), Lp[a] (○), F2 (▲), apo[a] (●), unfractionated apo[a] digest (◇), and F1 (▼) in sterile PBS. Blood samples (50–100 µl) were collected from the orbital vein in heparinized haematocrit tubes and plasma apo[a] immunoreactive components were measured by ELISA as described in Materials and Methods. Panel B: anti-apo[a] immuno blot of reduced plasma samples in 4% SDS-PAGE. Plasma was collected at the indicated times after injection of 250 µg of an unfraccionated elastase digest of apo[a] and an equal volume (1 µl) of each sample was analyzed. The lane marked 0 designates the material before injection and is equivalent to 80 ng of total protein.
may have an active elastase-like system capable of cleaving Lp[a] or apo[a]. This interpretation may appear at variance with the results of the studies by Mooser et al. (21) who injected Lp[a] into normal mice via the jugular vein and found no apo[a] fragments in the urine. Based on our current observations, it is possible that those negative results may be attributable to the relation between amount of injected material and degree of urinary concentration not permitting immunodetection of the apo[a] fragments by their assay.

Our data with injected mice provide a useful basis for interpreting the results of our studies in normal human subjects. In these subjects fragments of apo[a] were spontaneously present in their plasma. By ELISA they represented about 5% of the total plasma Lp[a] protein and were significantly larger than those in the urine. Moreover, the fragments in the urine had the size and band pattern of those seen in the urine of injected mice. In the absence of sequence analyses it is difficult to make adequate comparisons between mice and human urinary fragments. However, the results invite the speculation that in both animal species these fragments might have derived from the action of elastase-like enzymes, probably from the formed elements of the blood. According to this hypothesis both mice and humans would have an enzymatic make-up able to digest a small portion of the total apo[a] mass. A priori, this notion may be difficult to accept as apo[a] is not present in the mouse.

On the other hand, mice produce plasminogen, a five kringle zymogen, that has a high degree of homology with apo[a] (16). In this context, elastase is known to cleave plasminogen in vitro in a predictable manner (6, 22); moreover, recent studies have shown that plasminogen fragments are present in mouse urine (23). The latter studies are of interest in that those fragments were found to have angiostatic action. Whether the same action applies to human apo[a] fragments remains to be established.

The fact that urinary excretion of apo[a] fragments under physiological conditions is a rather modest event from the quantitative viewpoint indicates that, in both samples were concentrated, reduced, and analyzed by anti-apo[a] immunostaining of 4–12% gradient SDS-PAGE gels. The urine samples collected from mice injected with Lp[a], apo[a], and F1 were concentrated 3.5-fold and that from mice injected with F2, 15-fold. In each case, an aliquot of 30 μl of the concentrated sample was analyzed. Lane 1, control normal uninjected mouse; lane 2, Lp[a] after injection; lane 3, apo[a] after injection; lane 4, F1 after injection; lane 5, F2 after injection; lane Hu, human urine collected from the same patient as in panel A and concentrated 200-fold.

![Western blot analysis of mouse plasma and urine before and after injection. Panel A: in these studies Lp[a] and derivatives were injected into the mice and plasma was collected 24 h by quantitative ELISA.](image-url)
humans and in mice injected with Lp[a] or derivatives, urinary excretion is unlikely to represent a major catabolic pathway and thus, cannot account for the differences in plasma residence time between Lp[a] and apo[a] injected into the mouse. At present no specific sites or receptors for Lp[a] or apo[a] have been identified (24). Thus, we can only speculate that the relatively fast residence time in mouse plasma of apo[a] compared to Lp[a] may be due to the higher potential (5) of the former to affiliate with components of cell and tissue surfaces, i.e., sulphated proteoglycans (25), glycosaminoglycans (25), fibrin (26), or components of the extracellular matrix, for instance fibronectin as shown by previous (27, 28) and our current studies.

To conclude, enzymes of the elastase family cleave apo[a] in vitro at a discrete site generating two main fragments with different chemical, functional, and metabolic behavior. From the results in mice and humans we may speculate that these enzymes are involved in the in vivo cleavage of apo[a] and its by-products. Such cleavage would be unlikely to occur in the plasma as the elastase activity in this medium under physiological conditions would be inhibited by the action of α1 antitrypsin and β2 macroglobulin (22). Instead, apo[a] proteolysis would be more likely to occur at the level of cell membranes of polymorphonuclear cells, platelets, or macrophages. According to this hypothesis, elastase-dependent apo[a] fragmentation would be expected to occur under pathological conditions, for instance at sites of inflammation involving an active recruitment of polymorphonuclear cells and macrophages. Of interest, the presence of apo[a] fragments has been reported in human atherosclerotic lesions (29). According to our hypothesis, these fragments would be of the F2 type and thus unaffected by the F1-dependent size polymorphism of apo[a]. As a corollary to this hypothesis, F2 would be more pathogenic than F1 from the cardiovascular standpoint.

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