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Research Article

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Apolipoprotein(a) Kringle 4–Containing Fragments in Human Urine

Relationship to Plasma Levels of Lipoprotein(a)

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Abstract

Apo(a) is a large glycoprotein of unknown function that circulates in plasma as part of lipoprotein(a). Apo(a) is structurally related to plasminogen and contains at least 10 kringle (K)4 repeats (type 1-10), a K5 repeat and sequences similar to the protease domain of plasminogen. Plasminogen generates two biologically active peptides: plasmin and angiostatin, a kringle-containing peptide. As a first step in determining if apo(a) generates a similar kringle-containing peptide, human urine was immunologically examined. Fragments ranging in size from 85 to 215 kD were immunodetected using antibodies directed against epitopes in the K4-type 2 repeat, but not the K4-type 9 repeat or protease domain. NH_2-terminal sequence analysis revealed sequences specific for the K4-type 1 repeat, confirming that the fragments are from the NH_2 terminus of the K4 array. The amount of urinary apo(a) rose in proportion to the plasma lipoprotein(a) concentration. Even individuals with trace to no apo(a) in plasma had immunodetectable apo(a) fragments in their urine. Intravenous administration of the human urinary apo(a) into mice resulted in the rapid appearance of the same sized fragments in the urine. These findings suggest that the apo(a) fragments found in urine are formed extrarenally and then excreted by the kidney. (*J. Clin. Invest*. 1996. 97:858–864.) Key words: apolipoprotein(a) • lipoprotein(a) • angiostatin • kringles • plasminogen

Introduction

Apo(a) is a highly polymorphic glycoprotein that circulates in plasma covalently linked to the apolipoprotein B-100 (apo B) of LDL in a particle called lipoprotein(a) [Lp(a)][1]. High plasma levels of Lp(a) are associated with coronary as well as peripheral atherosclerosis (1). Apo(a) is present in the plasma of hedgehogs, old world monkeys, great apes, and humans, but not in other species (2, 3). In humans, the level of plasma Lp(a) varies from < 0.1 mg/dl to over 100 mg/dl (4). Individuals who have little or no apo(a) detectable in their plasma appear to be normal and healthy, which has raised the question as to whether apo(a) has a physiological function (5).

The apo(a) gene resides within 50 kb of the plasminogen gene on chromosome 6 and shares a high degree of sequence identity with its neighbor (6, 7). The plasminogen gene encodes a protein with five cysteine-rich domains called kringles (K1–K5), followed by a protease domain. Apo(a) does not contain sequences homologous to K1–K3 of plasminogen but it has 12–51 copies of a sequence that resembles K4 (8). Not all of the K4 repeats in apo(a) are identical in sequence; there are 10 different types of K4 repeats (K4–type 1 to K4–type 10), which are each present in a single copy, except for the K4–type 2 repeat, which varies in number from 3 to 42 between apo(a) alleles (8, 9). The tandem array of K4 repeats is followed by a single K5 repeat and then a region that shares 94% sequence identity with the protease domain of plasminogen (7). Plasminogen circulates in blood as a zymogen and undergoes proteolytic cleavage by tissue plasminogen activator to release the protease domain as plasmin, which plays a key role in thrombolysis. Though apo(a) is structurally related to plasminogen, it is not cleaved by tissue plasminogen activator and the protease domain has not been convincingly shown to have serine protease activity. Recently, Folkman and colleagues have demonstrated that not only the protease, but also the kringle domain of plasminogen, generates a biologically active peptide (10). They purified a 38-kD protein from the urine of mice implanted with a Lewis lung carcinoma and showed that this polypeptide, angiostatin, which contains sequences extending from K1 through K4 of plasminogen, inhibited capillary proliferation in vitro and the growth of metastases in vivo (10).

As a first step in determining if similar kringle-containing fragments are generated from apo(a), we used apo(a) K4–specific antibodies to examine human urine for the presence of apo(a)–immunoreactive fragments. In 1992, Oida and colleagues had identified a series of apo(a) immunoreactive fragments in human urine (11). The sizes of these fragments ranged from 44- to 200-kD, but the region of apo(a) from which these fragments were generated was not determined, since the epitope of the detecting antibody was not known. The amount of urinary apo(a) fragments was reduced in individuals with renal insufficiency but was not related to the plasma level of Lp(a).

In this paper, we have characterized the sequence composition of these urinary fragments and demonstrated their pres-

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1. *Abbreviations used in this paper:* K, kringle; Lp, lipoprotein.

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ence in the urine of all individuals, including those with no detectable apo(a) in plasma. Unlike Oida, we have found that the amount of the fragments in urine was directly related to the plasma Lp(a) levels. When the partially purified urinary apo(a) fragments were injected into mice, they were rapidly excreted by the kidney, suggesting that these fragments are present in plasma and then excreted by the kidneys into the urine.

Methods

Immunoblot analysis of plasma and urinary apo(a). Urine was collected from a healthy Caucasian individual with a plasma Lp(a) level of 58 mg/dl and an apo(a) genotype of *apo(a)* $K4_{16}$/*apo(a)* $K4_{19}$ (Subject 1). The urine was subjected to centrifugation at 1,300 *g* for 15 min, concentrated 10-fold using a Centriprep-30 filter (Amicon Corp., Beverly, MA), respun for 5 min at 10,000 *g* and then subjected to an additional 20-fold concentration in a Centricon-30 filter. 10 μl of concentrated urine was added to 10 μl of sample buffer (20% [vol/vol] glycerol, 4.6% [wt/vol] SDS, 150 μg/ml bromophenol blue, 10% [vol/vol] β-mercaptoethanol, and 0.125 M TrisCl, pH 6.8) and 18 μl were loaded onto a 5% SDS-polyacrylamide gel (SDS-PAGE). Immunoblot analysis of the urine and plasma samples was performed exactly as previously described (8) using the following antibodies: (*a*) IgG-$1A^2$, a mouse monoclonal anti–apo(a) K4–type 2 antibody (kindly provided by Dr G. Utermann, University of Innsbruck, Innsbruck, Austria) (12), (*b*) IgG–a-6, an anti–apo(a) mAbs that also recognizes the K4–type 2 repeat (13), (*c*) IgG–a-5, an mAb whose epitope is in the K4–type 1 and type 2 repeat, (*d*) IgG–a-40, an anti–apo(a) mAb whose epitope is located in the apo(a) K4–type 9 repeat (13), (*e*) IgG-10C5, an mAb developed against a synthetic peptide specific for the apo(a) protease domain (14), and (*f*) IgG-MB3, an anti–human apo B–100 mAb (15) (kindly provided by S. Young, Gladstone Institute, San Francisco, CA). A horseradish peroxidase–conjugated sheep anti–mouse secondary antibody (Amersham Corp., Arlington Heights, IL) was used for detection, except in the case of $1A^2$, which was conjugated directly to horseradish peroxidase (16). ECL chemiluminescence kit (Amersham Corp.) was used for signal detection, and the filters were exposed to NEF-496 films (Dupont, Wilmington, DE) for the times indicated.

Concentrated urine from Subject 1 was treated by serial glycosidases exactly as previously described (17). A 15-μl aliquot from each reaction was subjected to immunoblotting as described above.

Purification of plasma Lp(a) and urinary apo(a). To purify Lp(a) from plasma, a total of 1 ml of plasma from Subject 1 was diluted 10-fold with buffer A (0.15 M NaCl, 1 mM Na_2 EDTA, and 1 mM $NaPO_4$ at pH 7.4) and run on an affinity chromatography column made by coupling a-6 to activated agarose using the Aminolink kit® (Pierce Chemical Co., Rockford, IL). The column was washed with 10 mM Tris, pH 7.2, and the proteins were eluted using 100 mM triethylamine, pH 11.5. 1-ml fractions were collected in 50 μl of 1 M $NaPO_4$, pH 6.8. To purify the urinary apo(a), a liter of urine from Subject 1 was dialyzed for 20 h against distilled water at 4°C. Disodium EDTA was added to a final concentration of 0.2 mM and the proteins were precipitated using ammonium sulfate. The 35–80% ammonium sulfate cut containing ~ 95% of apo(a) was resuspended in 15 ml of 0.15 M NaCl, dialyzed against 0.15 M NaCl, and passed over the affinity column as described above.

Partial digestion of plasma Lp(a) and urinary apo(a) with thermolysin. Purified Lp(a) and urinary apo(a) were dialyzed against PBS and subjected to digestion with thermolysin (Boehringer Mannheim Biochemicals, Indianapolis, IN) in a buffer containing 125 mM TrisCl, 150 mM NaCl, 4 mM $CaCl_2$, pH 7.8, at an enzyme to substrate ratio of 1:1,000, 1:100, and 1:10. The reaction was carried out at 37°C for 30 min and was terminated by the addition of disodium EDTA to a final concentration of 10 mM. The digested products were analyzed by immunoblotting as described above.

NH_2-terminal sequencing of urinary apo(a). Apo(a) fragments were purified from 1 liter of urine of Subject 1 by affinity chromatography as described above and dialyzed against PBS. A total of 80 μg of purified apo(a) fragments were spotted onto an Immobilon PSQ filter (Millipore Corp., Bedford, MA). NH_2-terminal sequencing was performed by subjecting the sample to automated Edmon degradation using a model 477A sequence analyzer from Perkin Elmer (Foster City, CA) with the programs and chemicals recommended by the manufacturer.

Analysis of the relationship between the plasma levels and urinary excretion of apo(a). Fasting blood samples and 24-h urine samples were obtained from 34 healthy Caucasian individuals, including 25 males and 9 females, aged 25–41 yr. Plasma was isolated and stored at 4 and −80°C. The plasma and urinary albumin and creatinine levels were measured at Damon Clinical Laboratories (Dallas, TX) within 3 wk. The creatinine clearance ranged from 80 to 166 ml/min per 1.73 m^2 (mean 117). All the subjects had a urinary protein excretion of < 150 mg per 24 h. The urine volume was quantitated and a 15-ml aliquot was subjected to centrifugation at 1,300 *g* for 15 min and stored at −80°C. The amount of apo(a) in plasma and urine was quantitated within 1 mo using an ELISA. To measure apo(a) in plasma, a-6 was used as the capture antibody and a-40 as the detecting antibody, exactly as described (13). To quantitate urinary apo(a), a-6 was used as the capture antibody and a-5 was used for detection. The levels are provided in milligram of apo(a) per deciliter. To convert from plasma Lp(a) protein to Lp(a) mass, the concentration should be multiplied by 2.76.

Apo(a) genotyping and phenotyping. The size of the apo(a) gene was determined using pulsed-field gel electrophoresis of high molecular weight genomic DNA and genomic blotting (8), and the relative sizes of the apo(a) isoforms were determined by size-fractionating plasma proteins on a 2% SDS-agarose gel and immunoblotting using horseradish peroxidase–coupled $1A^2$ (8).

Infusion of Lp(a) and partially purified urinary apo(a) into mice. A total of 30 ml of plasma from Subject 1 was adjusted to a density of 1.215 g/ml and subjected to ultracentrifugation for 24 h at 412,000 *g*. The top fraction was dialyzed for 20 h against 0.15 M NaCl, 0.001% (wt/vol) disodium EDTA and diluted to 50 ml with buffer A. The sample was passed twice over a 5-ml wheat germ agglutinin column (lectin from Triticum Vulgaris, Sigma Chemical Co., St Louis, MO) (18). The column was washed with 20 column volumes of buffer A and the proteins were eluted using 100 mM *N*-acetyl glucosamine (Sigma Chemical Co.). The eluted proteins were dialyzed for 20 h against 0.15 M NaCl before injection into mice. An aliquot of Lp(a) was run on a 5% SDS-PAGE and the proteins were stained using Ponceau dye to check for purity (data not shown). Urinary proteins from 1 liter of urine from Subject 1 were precipitated as described above, and the 35–80% ammonium sulfate cut was dialyzed against 150 mM NaCl and concentrated to a final volume of 1 ml with Centriprep-30 before injection into mice. A total of 0.35 mg of Lp(a) or 27 μg of urinary apo(a) (4.5 mg of total protein) in a total volume of 300 μl were injected into the left external jugular vein of (C57BL/6 × SJL) F_1 female mice that were anesthetized using ether. Blood was drawn by retro-orbital puncture 1 min after injection. Mice were then housed individually in metabolic cages and given 10% (wt/vol) sucrose to drink. Urine was collected in chilled containers over the following time intervals: 0–6, 6–12, and 12–24 h and blood samples were collected at 6, 12, and 24 h. Aliquots were stored at −80°C and the apo(a) was quantitated by ELISA within 1 wk. The amount of urine was quantitated in each sample and then the urine was spun at 1,300 *g* for 15 min and concentrated as described above. Aliquots of plasma and urine were subjected to immunoblot analysis using an apo(a)–specific antibody as described above. The proteins from 12% of each 6 h collection period were loaded onto SDS-PAGE gels.

Results

Immunoblot analysis was performed on plasma and 200-fold concentrated urine from Subject 1 (Fig. 1 *A*) using three differ-

ent anti–apo(a) mAbs whose epitopes are schematically illustrated in Fig. 1 *C*. A series of fragments with apparent molecular masses of 85, 115, 140, 170, 190, and 215 kD were visible in the urine using $1A^2$ and a-5 antibodies; $1A^2$ is specific for the highly reiterated apo(a) K4–type 2 repeat (12) and a-5 recognizes both the type 1 and type 2 repeats (13). The urinary apo(a) fragments were not visible using either a-40, an antibody directed against the penultimate K4 repeat in the tandem array (K4–type 9) (13), or by antibodies against the apo(a) protease domain or apo B (data not shown). From these studies we concluded that the apo(a)–immunoreactive material present in human urine contained the K4–type 2 repeat but not the K4–type 9 or the protease domain of apo(a).

To better delineate the composition of apo(a) fragments in urine, purified plasma Lp(a) and urinary apo(a) were subjected to partial digestion with thermolysin (Fig. 1 *B*). Previously, it was shown that there is a thermolysin-sensitive site in the interkringle region separating the type 4 and type 5 K4 repeats of apo(a) (19). Thermolysin digestion resulted in the formation of two fragments, an invariant 170-kD COOH-terminal fragment that contained the last six K4 repeats (K4–type 5 through K4–type 10), the K5 repeat, and the protease domain, and an NH_2-terminal fragment that is of variable size since it contains the type 2 K4 repeats, which vary in number between apo(a) alleles (Fig. 1 *C*). Lp(a) was purified from Subject 1 by affinity chromatography and subjected to increasing concentrations of thermolysin before immunoblot analysis using either a-5 or a-40 (Fig. 1 *B*). Two immunoreactive bands were identified using a-5, which binds to the K4–type 1 and –type 2 repeats (*top*), and a single band of 170 kD was seen when a-40 was used for immunoblot analysis (*middle*). An identical analysis was performed using purified urinary apo(a) fragments, and there was no change in the size of the immunoreactive fragments after incubation with thermolysin (Fig. 1 *B*, *bottom*). From this analysis we concluded that the urinary apo(a) fragments do not include sequences downstream of the K4–type 4 repeat.

To determine if the presence of multiple fragments of different sizes in the urine reflected variations in carbohydrate content, urinary proteins were subjected to serial glycosidase treatments (Fig. 2). There was a sequential 5–10% reduction in the apparent molecular mass of each of the apo(a) fragments upon removal of N-linked sugars using N-glycanase (lane *2*), sialic acid residues after treatment with neuraminidase (lane *3*), and O-linked sugars after subjection to O-glycanase treatment (lane *4*). Therefore, the multiple apo(a)–immunoreactive bands of different sizes in the mouse urine were due to variations in protein rather than carbohydrate content. Overall, there was an ~24% decrease in molecular mass, which is consistent with the previously reported estimates of the sugar content of apo(a) (20).

The urinary apo(a) fragments were affinity purified from one liter of urine to determine the NH_2-terminal sequence. After the urinary proteins were precipitated using ammonium sulfate, the 35–80% fraction was applied to an IgG–a-6 agarose column and eluted using 100 mM triethylamine, pH 11.5. Silver stain (Fig. *A*) and immunoblot analysis (*B*) of the starting material (lane *1*), the flow through (lanes *2* and *3*), and the 1 ml fractions of the eluted proteins (lanes *4–8*) are shown in Fig. 3. A series of bands ranging in size from 85 to 215 kD were seen both on the silver stain and the immunoblot. NH_2-terminal sequence was unambiguous in 13 of the first 15 residues. This sequence exactly matched the NH_2-terminal sequence of the apo(a) K4–type 1 repeat, but none of the other apo(a) K4 sequences (Fig. 3 *C*). Thus, most, if not all of the apo(a)–immunoreactive fragments in human urine contain the apo(a) K4–type 1, a variable number of copies of K4–type 2 repeat, and possibly the type 3 and type 4 K4 repeat.

Next, we examined the relationship between plasma levels

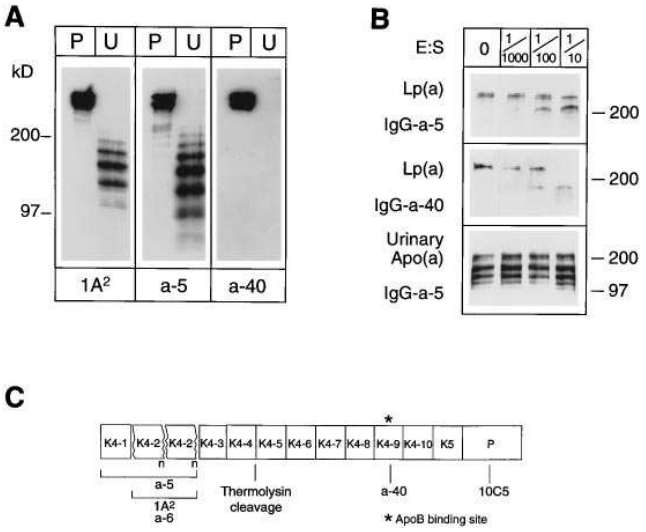


Figure 1. (*A*) Immunoblot analysis of plasma and urine apo(a) from Subject 1. A total of 0.75 μl of plasma (*P*) and 10 μl of 200-fold concentrated urine (*U*) were size-fractionated on a 5 % SDS-PAGE gel and immunoblot analysis was performed using $1A^2$, which recognize the apo(a) K4-type 2 repeat (*left*), a-5, which is directed against the apo(a) K4–types 1 and 2 (*middle*), and a-40, whose epitope is in the apo(a) K4-type 9 repeat (*right*). After washing, the filter was developed using the ECL detection system, and exposed to XAR film for 30 s with a-5 and 4 min for $1A^2$ and a-40. (*B*) Partial digestion of purified Lp(a) and urinary apo(a) using thermolysin. A total of 0.5 μg of purified Lp(a) or urinary apo(a) was incubated with thermolysin at the indicated ratios of enzyme (*E*) to substrate in a total vol of 50 μl for 30 min at 37°C. A 20-μl aliquot of the reaction product was loaded on a 4–15% SDS-PAGE gradient gel and analyzed by immunoblotting, as described above. Films were exposed for 1 min (*top*), 16 min (*middle*), and 30 s (*bottom*). (*C*) Schematic of apo(a) protein domains and sites of epitopes recognized by anti–apo(a) mAbs and the location of the thermolysin-sensitive site.

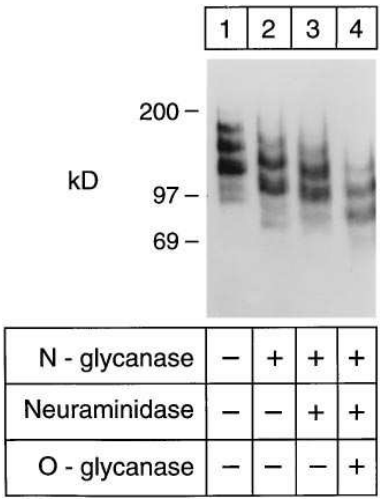
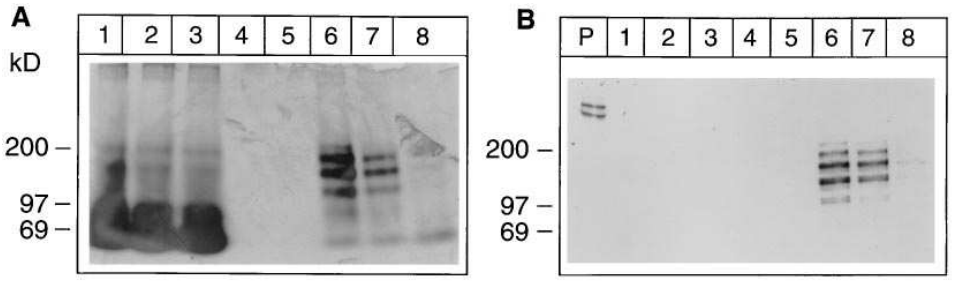


Figure 2. Immunoblot analysis of glycosidase-treated urinary proteins. An aliquot of fresh urine from Subject 1 that had been concentrated 75-fold was subjected to serial digestion with N-glycanase, neuraminidase and O-glycanase exactly as described in the Methods. The urinary proteins were immunoblotted using $1A^2$ as described in Fig. 1. The filter was exposed for 3 min.



C

Residue Number			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
N-terminus sequence			E	Q	S	H	V	V	Q	D	G?	Y	H	G	?	G	Q
K4- type 1			E	Q	S	H	V	V	Q	D	C	Y	H	G	D	G	Q
K4-type 2	P	T	-	-	R	P	G	-	-	E	-	-	-	-	N	-	-
K4-type 3	-	-	-	-	R	P	G	-	-	E	-	-	-	-	N	-	-
K4-type 4	-	-	-	-	R	P	G	-	-	E	-	-	-	-	N	-	-
K4-type 5	L	-	-	E	T	P	G	-	-	E	-	-	Y	H	Y	-	-
K4-type 6	-	-	-	-	-	P	G	-	-	-					-	-	-
K4-type 7	-	-	-	-	-	P	T	-	-	-	-	-	-	-	-	-	-
K4-type 8	-	-	-	N	-	T	G	-	-	-	-	-	R	-	-	-	-
K4-type 9	-	P	-	K	-	P	-	-	-	-	-	-	-	-	-	-	R
K4-type 10	-	-	-	-	T	-	-	-	R	Q	-	-	-	-	N	-	-

Figure 3. Purification of urinary apo(a) fragments by affinity chromatography. The proteins from one liter of urine of Subject 1 were precipitated using ammonium sulfate. The 30–80% fraction was resuspended in 20 ml of buffer A and passed twice over an affinity chromatography column, which was made by cross-linking a-6 to activated agarose. A total of 17 μl of the starting material, the first flow-through and the second flow-through were loaded on a 4–15% gradient gel (lanes *1*, *2*, and *3*, respectively). After washing, proteins were eluted with 100 mM triethylamine, pH 11.5, and 1-ml fractions were collected. 1 μl of a 30-fold concentrated fractions 1 to 5 was loaded on lanes *4–8.* An aliquot of 0.75 μl of plasma from Subject 1 (*P*) was used as a control. Samples were analyzed by silver staining (*A*) and immunoblotting (*B*). (*C*) NH_2-terminal sequence of urinary apo(a) fragments. A total of 80 μg of purified apo(a) fragments were subjected to NH_2-terminal sequence analysis as described in the Methods. The sequence is compared to the published amino acid sequence of the apo(a) K4–type 1 to apo(a) K4–type 10 (7).

of Lp(a) and the 24 h excretion of apo(a) fragments in 34 healthy Caucasian individuals. Aliquots of plasma (Fig. 4, *top*) and urine (equivalent to 0.02% of the 24-h urine output) (*bottom*) from eight representative individuals were subjected to immunoblot analysis using $1A^2$ (Fig. 4 *A*). All subjects, with the exception of one individual (lane *1*), had the same pattern of fragments in their urine, irrespective of the size of their apo(a) alleles or the amount of plasma Lp(a). There was no relationship between the sizes and amount of the apo(a) fragment in the urine and the sizes of plasma apo(a) isoforms. Interestingly, in two individuals who had no detectable apo(a) in their plasma by immunoblotting (lanes *1* and *2*), apo(a) was clearly detectable in the urine.

To determine if the pattern of apo(a)–immunoreactive bands observed in lane *1* (Fig. 4) was specifically associated with very low plasma Lp(a) levels, immunoblot analysis was performed on urine samples from six additional individuals with a plasma Lp(a) level < 1.0 mg/dl. The banding pattern was similar to that observed in lanes *2–8* (data not shown). We also examined urine from five Chinese and five African-American individuals to determine if there were inter-ethnic differences in urinary excretion pattern of apo(a). The pattern of apo(a)–immunoreactive bands was identical to that seen in Caucasians (data not shown).

The relationship between the level of plasma Lp(a) and 24-h urine excretion of apo(a) in the 34 healthy Caucasian individuals is shown in Fig. 4 *B.* No individual was identified who did not have any apo(a) detectable in their urine. Even individuals with trace to no detectable apo(a) in plasma had measurable amounts of apo(a) in their urine. Overall, there was a curvilinear relationship between plasma and urinary apo(a) levels.

To probe the mechanism responsible for the presence of apo(a) in the urine, we injected human Lp(a) or partially purified urinary apo(a) into six (C57BL/6xSJL) F_1 female mice. Blood and urine samples were collected and examined for the presence of apo(a) by immunoblotting and ELISA (13) (Table

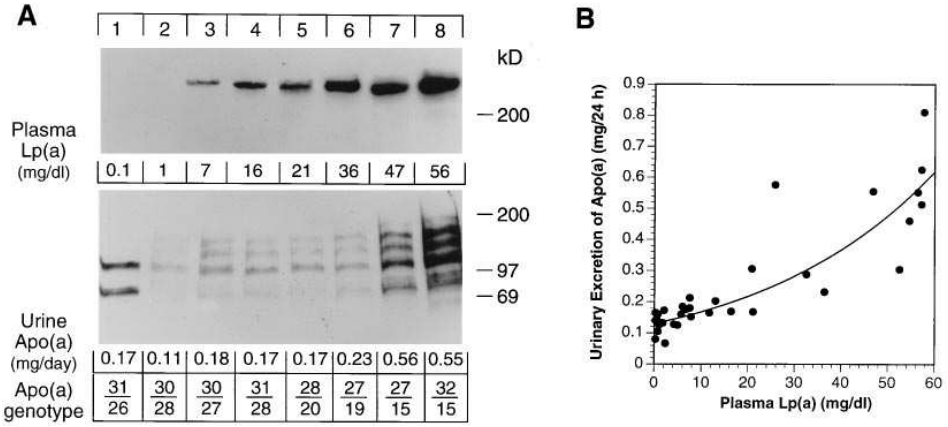


Figure 4. (*A*) Immunoblot analysis of plasma and urine apo(a) from eight healthy Caucasian individuals. A 0.75 μl aliquot of plasma (*top*) and 0.02% of a 24-h collection of urine (*bottom*) from each individual were size-fractionated on 4-15% SDS-PAGE gels and immunoblotted using $1A^2$ as described in Fig. 1. Filters were exposed for 2 min (*top*) and 3 min (*bottom*). Plasma and urine apo(a) levels were quantitated using a sandwich ELISA as described in the Methods. The apo(a) genotypes were determined by genomic blotting as described (8). (*B*) Relationship between the plasma levels of Lp(a) and 24 h urinary apo(a) levels. The apo(a) levels in the plasma and 24-h urine samples from 34 healthy Caucasian individuals were quantitated by ELISA as described in Methods. Plasma Lp(a) levels are given on the *x*-axis and the 24 h urine apo(a) levels are given on the *y*-axis.

Table I. Plasma Apo(a) Levels and Urinary Excretion of Apo(a) in Mice Injected with Partially Purified Lp(a) or Urinary Apo(a)

	Plasma Apo(a)		Urine Apo(a)	
Time	Group 1*	Group 2‡	Group 1	Group 2
h	*mg/dl*		*μg/6 h*	
0	12.2±0.7§	2.1±0.1		
6	5.7±0.4	0.3±0.1	0.53±0.18	2.09±0.36
12	2.3±0.2	1.15±0.07	0.15±0.06	0.74±0.19
24	0.9±0.1	0.09±0.06	0.09±0.02	0.25±0.13

*Group 1: Mice injected with human Lp(a), $n = 6$; ‡Group 2: Mice injected with partially purified urinary apo(a), $n = 6$; §Mean (±SEM).

I). The result of the immunoblot analysis for three mice in each group is illustrated in Fig. 5. Within 24 h of injection of 0.35 mg of purified human Lp(a), no apo(a) was detectable in the plasma (Fig. 5 *A*). Over this same time period, there was no apo(a) detected in the urine by immunoblot (Fig. 5 *B*) but there were trace amounts detectable in the urine using an ELISA (Table I). The same study was performed using 27 μg of apo(a) fragments partially purified from human urine. Again, in all three mice, the apo(a) fragments had almost completely disappeared from the plasma within 24 h (Fig. 5 *C*). A significant proportion of the apo(a) fragments of similar sizes were found in the urine within 6 h (Fig. 5 *D*), and a total of 11% of the injected immunoreactive apo(a) was recovered in the urine after 24 h. Taken together, these results suggest that the apo(a) in the urine is not derived from intact Lp(a) in the mice. This does not exclude the possibility that the fragments seen in human urine are a metabolic product of Lp(a) since mice may not have the machinery to process Lp(a). The rapid excretion of the fragments into the urine, despite their very large size and their negative charge, strongly suggests that apo(a) fragments are actively secreted by the kidney rather than being filtered.

Discussion

In this paper we describe the characterization of the apo(a)–immunoreactive fragments that are present in human urine. Immunoblot analysis with epitope-specific anti–apo(a) antibodies together with NH_2-terminal sequence analysis of purified urinary fragments are consistent with these fragments containing the apo(a) K4–type 1 repeat and a variable number of apo(a) K4–type 2 repeats, and perhaps the type 3 and type 4 K4 repeats. The fragments did not contain a thermolysin-sensitive site located between the K4–type 4 and K4–type 5 repeats and, thus, the fragments do not extend beyond the K4–type 4 repeat. With one exception, the pattern of fragments was similar in all individuals examined. Even individuals with no plasma apo(a) detectable by immunoblotting had apo(a) detectable in the urine. The amount of the apo(a) K4–containing fragments in urine from healthy Caucasian individuals rose in proportion to the amount of Lp(a) in plasma. This observation makes it unlikely that urinary excretion of apo(a) contributes significantly to the inter-individual variability in plasma Lp(a) levels and rather suggests a precursor–product relationship between plasma apo(a) and the urinary fragments. Finally, intravenous administration of partially purified human urinary apo(a), but not Lp(a), into mice resulted in the rapid appearance of immunoreactive apo(a) in the urine, suggesting that the apo(a) K4–containing peptides are formed in plasma or tissues and then excreted into the urine.

Multiple observations are consistent with the urinary apo(a) fragments not being synthesized de novo by the human kidney. First, no apo(a) mRNA was detectable on Northern blot analysis of renal tissue from the rhesus or cynomolgus monkey (21, 22). This is despite the fact that apo(a) fragments of similar size were detected in the urine of cynomolgus monkeys (data not shown; samples kindly provided by R. Ramharack and R. Newton, Parke-Davis, Ann Arbor, MI). We performed reverse-transcriptase PCR on human kidney mRNA using oligonucleotides that hybridize specifically with apo(a) K4–type 2 encoding exons (8, 23). No amplification was found using human renal mRNA but a fragment of the expected size was seen using hepatic mRNA isolated from an apo(a)–transgenic mouse (16). To verify the integrity of the renal mRNA, we successfully amplified a region of the LDL-receptor–associated protein from the same samples (data not shown). This analysis, along with the monkey data, leads us to conclude that the apo(a) fragments detectable in urine are not synthesized in the kidney but are derived from circulating Lp(a) or apo(a) fragments.

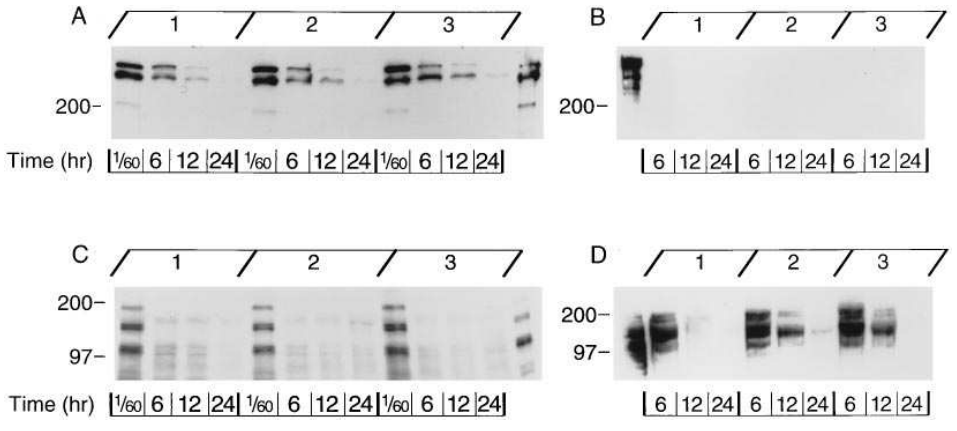


Figure 5. Immunoblot analysis of mouse plasma and urine after injection of partially-purified human Lp(a) or apo(a) fragments from human urine. A solution containing 0.35 mg of Lp(a) (*A* and *B*) or 27 μg of urinary apo(a) (*C* and *D*) in 300 μl was injected intravenously into each of three mice. A 0.5-μl aliquot of the injected material is shown in the last lane (*A* and *C*). Blood was collected by retro-orbital puncture at the indicated time points (*A* and *C*). The plasma was isolated and 0.75 μl was immunoblotted using IgG-1A[2] as described in Fig. 1. Urine was collected after 6, 12, and 24 h, concentrated, and 12% of each collection was loaded on a 4–15% SDS-PAGE gel, and immunoblotted using IgG-1A[2]. Filters were exposed for 8 min. A 0.5-μl aliquot of the injected material is shown in the first lane (*B* and *D*).

The origin of the K4-containing urinary apo(a) fragments has not been fully elucidated. We do not know if the apo(a) fragments are formed from free apo(a) or from apo(a) that is associated with lipoproteins. We have not excluded the possibility that the apo(a)–K4 fragments are produced by differential splicing of the apo(a) mRNA, although Northern blot analysis of hepatic mRNA using an apo(a) K4 specific probe has repeatedly failed to reveal mRNA species of smaller size (21, 22, 24, 25). In humans, there is an apo(a)-like gene that resides next to the apo(a) gene (6). The mRNA for this gene has been identified in human liver, as well as in selected human tumors (26). Although it remains to be shown that a protein is produced from this transcript, this gene cannot be the source of the apo(a) fragments we have identified since it is predicted to produce a protein that contains only a single K4 repeat.

The apo(a) that circulates in plasma as part of Lp(a) is synthesized by hepatocytes and secreted independently of apo B–100 (27). It is possible that apo(a)-K4 containing fragments are generated from newly secreted apo(a) molecules that fail to properly complex with LDL, thus making the apo(a) susceptible to proteolytic cleavage. Consistent with this scenario is the observation that individuals with very low plasma Lp(a) levels still have apo(a) fragments in their urine.

Alternatively, the urinary apo(a) may be a proteolytic cleavage product of Lp(a), rather than from the apo(a) that circulates in plasma free of lipoproteins. Electron microscopic and hydrodynamic studies of the Lp(a) particle are consistent with apo(a) having only a single point of attachment to LDL. The bulk of the molecule, including most of the K4 repeats, extends away from the LDL particle into the aqueous solution (28), which would make the K4 region of apo(a) accessible to proteolytic cleavage. The mechanism by which the apo(a) that circulates as part of Lp(a) is removed from the plasma is not known. Initially, it was thought that the LDL portion of Lp(a) may mediate clearance of the Lp(a) particle by the LDL receptor, but recent metabolic studies are not consistent with this scenario and suggest that apo(a) and apo B–100 may have independent fates (29–31). If the apo(a) fragments found in urine are generated in the liver, peripheral tissues, or in the circulation, they would be expected to also be found in plasma. We have detected apo(a) fragments in human plasma and are in the process of quantifying their amount and characterizing their sequence composition (Mooser, V., et al., manuscript in preparation).

Multiple pathological studies have revealed that atherosclerotic lesions are selectively enriched in apo(a). Some of the immunoreactive apo(a) found in atherosclerotic lesions may be due to the presence of the same K4-containing fragments we have identified in human urine. Morrisett and colleagues showed that the ratio of plasma concentration of apo B–100 to apo(a) is around 9 to 1, whereas in venous graft lesions this ratio is only 3 to 1 (32). Approximately 50% of the apo(a) extracted from atherosclerotic lesions is not associated with lipids (33). Even individuals with little to no Lp(a) measurable in plasma have detectable apo(a) in arterial lesions (34). Moreover, Hoff et al. have identified a series of apo(a)–immunoreactive fragments in the $d > 1.21$ g/ml fraction of human atherosclerotic lesions, that were similar in size to those we have characterized (35). More detailed immunocytochemical studies need to be performed to determine if these fragments are similar in size and sequence composition to those found in human urine.

The large size and highly glycosylated nature of the apo(a) fragments make it unlikely that they enter the urine via filtration and suggest that they are excreted by an active process. Consistent with this scenario is the observation that some renal patients with a very low creatinine clearance have considerable amounts of apo(a) in their urine (11). Finally, if apo(a) fragments were filtered, one would expect higher amounts of apo(a) in patients with nephrotic syndrome. Oida et al. found no relationship between the 24-h urinary excretion of apo(a) and albumin (11).

It is of interest that mice, which do not have apo(a) in their plasma, have the capability to excrete these apo(a) fragments into their urine. Based on this finding, we speculate that mice may have a plasma protein containing sequences similar to the K4 repeats of apo(a). apo(a) is a member of a large family of kringle-containing proteins (36), and the kringle domains of at least two of the family members have independent biological functions. As alluded to previously, plasminogen generates a kringle-containing peptide, angiostatin, that has in vitro effects on capillary proliferation and in vivo effects on the growth of tumor metastases (10). Another protein that shares striking resemblance to apo(a) in structure is hepatocyte growth factor (37). This protein is a 92-kD disulfide-linked heterodimer consisting of a 62-kD heavy chain and a 32-kD light chain. The heavy chain contains 4 kringle domains and the light chain has a serine protease-like sequence that has no known protease activity, which is similar to the protease domain of apo(a). A truncated variant of hepatocyte growth factor that has two kringle repeats is produced by a human lung carcinoma cell line (38). This variant binds the hepatocyte growth factor receptor (the c-*met* proto-oncogene) and mediates some of the same effects as the full-length growth factor (37, 38). Efforts are now underway to determine whether the kringle 4–containing fragments of apo(a) antagonize or mimic the effects of angiostatin or some other kringle-containing proteins.

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References

1. Utermann, G. 1995. Lipoprotein(a). *In* Metabolic Basis of Inherited Disease. C. Scriver, A. Beaudet, W. Sly, and D. Valle, editors. New York. 1887–1912.

2. Laplaud, P.M., L. Beaubatie, S.C. Rall, Jr., G. Luc, and M. Saboureau. 1988. Lipoprotein(a) is the major apoB-containing lipoprotein in the plasma of a hibernator, the hedgehog (*erinaceus europaeus*). *J. Lipid Res.* 29:1157–1170.

3. Makino, K., A. Abe, S. Maeda, A. Noma, M. Kawade, and O. Takenaka. 1989. Lipoprotein(a) in nonhuman primates: presence and characteristics of Lp(a) immunoreactive materials using anti-human Lp(a) serum. *Atherosclerosis.* 78:81–85.

4. Albers, J.J., S.M. Marcovina, and M.S. Lodge. 1990. The unique lipoprotein(a): properties and immunochemical measurement. *Clin. Chem.* 36:2019–2026.

5. Gaw, A., and H.H. Hobbs. 1994. Molecular genetics of lipoprotein (a): new pieces to the puzzle. *Curr. Opin. Lipid.* 5:149–155.

6. Magnaghi, P., E. Citterio, N. Malgaretti, F. Acquati, S. Ottolenghi, and

R. Taramelli. 1994. Molecular characterisation of the human apo(a)-plasminogen gene family clustered on the telomeric region of chromosome 6 (6q26-27). *Hum. Mol. Genet.* 3:437–442.

7. McLean, J.W., J.E. Tomlinson, W.-J. Kuang, D.L. Eaton, E.Y. Chen, G.M. Fless, A.M. Scanu, and R.M. Lawn. 1987. cDNA sequence of human apolipoprotein(a) is homologous to plasminogen. *Nature (Lond.).* 330:132–137.

8. Lackner, C., J.C. Cohen, and H.H. Hobbs. 1993. Molecular definition of the extreme size polymorphism in apolipoprotein(a). *Hum. Mol. Genet.* 2:933–940.

9. Morrisett, J.D., J. Gaubatz, R. Knapp, and J.G. Guevara. 1990. Structural properties of apo(a): a major apoprotein of human lipoprotein(a). Academic Press, Inc. Orlando, FL. 53–74.

10. O'Reilly, M.S., L. Holmgren, Y. Shing, C. Chen, A. Rosenthal, M. Moses, W.S. Lane, Y. Cao, E.H. Sage, and J. Folkman. 1994. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell.* 79:315–328.

11. Oida, K., H. Takai, H. Maeda, S. Takahashi, A. Shimada, J. Suzuki, T. Tamai, T. Nakai, and S. Miyabo. 1992. Apolipoprotein(a) is present in urine and its excretion is decreased in patients with renal failure. *Clin. Chem.* 38: 2244–2248.

12. Dieplinger, H., G. Gruber, K. Krasznai, S. Reschauer, C. Seidel, G. Burns, H.-J. Müller, A. Császár, W. Vogel, H. Robenek, and G. Utermann. 1995. Kringle 4 of human apolipoprotein[a] shares a linear antigenic site with human catalase. *J. Lipid Res.* 36:813–822.

13. Marcovina, S.M., J.J. Albers, B. Gabel, M.L. Koschinsky, and V.P. Gaur. 1995. Effect of the number of apolipoprotein(a) kringle 4 domains on immunochemical measurements of lipoprotein(a). *Clin. Chem.* 41:246–255.

14. Gaw, A., E. Boerwinkle, J. Cohen, and H.H. Hobbs. 1994. Comparative analysis of the apo(a) gene, apo(a) glycoprotein and plasma concentrations of Lp(a) in three ethnic groups: evidence for no common null allele at the apo(a) locus. *J. Clin. Invest.* 93:2526–2534.

15. Curtiss, L.K., and T.S. Edgington. 1982. Immunochemical heterogeneity of human plasma apolipoprotein B. *J. Biol. Chem.* 257:15213–15221.

16. Chiesa, G., H.H. Hobbs, M.L. Koschinsky, R.M. Lawn, S.D. Maika, and R.E. Hammer. 1992. Reconstitution of lipoprotein(a) by infusion of human low density lipoprotein into transgenic mice expressing human apolipoprotein(a). *J. Biol. Chem.* 267:24369–24374.

17. Jokinen, E.V., K.T. Landschulz, K.L. Wyne, Y.K. Ho, P.K. Frykman, and H.H. Hobbs. 1994. Regulation of the very low density lipoprotein receptor by thyroid hormone in rat skeletal muscle. *J. Biol. Chem.* 42:26411–26418.

18. Seman, L.J., J.L. Jenner, J.R. McNamara, and E.J. Schaefer. 1994. Quantification of lipoprotein(a) in plasma by assaying cholesterol in lectin-bound plasma fraction. *Clin. Chem.* 40:400–403.

19. Huby, T., C. Doucet, H. Dieplinger, J. Chapman, and J. Thillet. 1994. Structural domains of apolipoprotein(a) and its interaction with apolipoprotein B-100 in the lipoprotein(a) particle. *Biochemistry.* 33:3335–3341.

20. Fless, G.M., M.E. Zum Mallen, and A.M. Scanu. 1986. Physicochemical properties of apolipoprotein(a) and lipoprotein(a-) derived from the dissociation of human plasma lipoprotein(a). *J. Biol. Chem.* 261:8712–8718.

21. Tomlinson, J.E., J.W. McLean, and R.M. Lawn. 1989. Rhesus monkey apolipoprotein(a). *J. Biol. Chem.* 10:5957–5965.

22. Azrolan, N., D. Gavish, and J.L. Breslow. 1991. Plasma lipoprotein(a) concentration is controlled by apolipoprotein(a) (apo(a)) protein size and the abundance of hepatic apo(a) mRNA in a cynomolgus monkey model. *J. Biol. Chem.* 266:13866–13872.

23. Sarkar, G., J. Cassady, C.D.K. Bottema, and S.S. Sommer. 1990. Characterization of polymerase chain reaction amplification of specific alleles. *Anal. Biochem.* 186:64–68.

24. Koschinsky, M.L., U. Beisiegel, D. Henne-Bruns, D.L. Eaton, and R.M. Lawn. 1990. Apolipoprotein(a) size heterogeneity is related to variable number of repeat sequences in its mRNA. *Biochemistry.* 29:640–644.

25. Wade, D.P., B.K. Knight, K. Harders-Spengel, and A.K. Soutar. 1991. Detection and quantitation of apolipoprotein(a) mRNA in human liver and its relationship with plasma lipoprotein(a) concentration. *Atherosclerosis.* 91:63–72.

26. Byrne, C.D., K. Schwartz, and R.M. Lawn. 1994. Loss of splice donor site at a 'skipped exon' in a gene homologous to apolipoprotein(a) leads to an mRNA encoding a protein consisting of a single kringle domain. *Arterioscler. Thromb. Vasc. Biol.* 15:65–70.

27. White, A.L., D.L Rainwater, and R.E. Lanford. 1993. Intracellular maturation of apolipoprotein(a) and assembly of lipoprotein(a) in primary baboon hepatocytes. *J. Lipid Res.* 34:509–517.

28. Phillips, M.L., A.V. Lembertas, V.N. Schumaker, R.M. Lawn, S.J. Shire, and T.F. Zioncheck. 1993. Physical properties of recombinant apolipoprotein(a) and its association with LDL to form an Lp(a)-like complex. *Biochemistry.* 32:3722–3728.

29. Rader, D.J., W.A. Mann, W. Cain, H. Kraft, D. Usher, L.A. Zech, J.M. Hoeg, J. Davignon, P. Lupien, M. Grossman, J.M. Wilson, and H.B. Brewer, Jr. 1995. The low density lipoprotein receptor is not required for normal catabolism of Lp(a) in humans. *J. Clin. Invest.* 95:1403–1408.

30. Knight, B.L., Y.F.N. Perombelon, A.K. Soutar, D.P. Wade, and M. Seed. 1991. Catabolism of lipoprotein(a) in familial hypercholesterolaemic subjects. *Atherosclerosis.* 87:227–237.

31. Knight, B.L. 1994. Lp(a) catabolism in hypercholesterolaemic individuals. *Chem. Phys. Lipids.* 67/68:233–239.

32. Cushing, G.L., J.W. Gaubatz, M.L. Nava, B.J. Burdick, T.M.A. Bocan, J.R. Guyton, D. Weilbaecher, M.E. DeBakey, G.M. Lawrie, and J.D. Morrisett. 1989. Quantitation and localization of apolipoproteins(a) and B in coronary artery bypass vein grafts resected at re-operation. *Arteriosclerosis.* 9:593–603.

33. Reblin, T., N. Meyer, C. Labeur, D. Henne-Bruns, and U. Beisiegel. 1995. Extraction of lipoprotein(a), apoB, and apoE from fresh human arterial wall and atherosclerotic plaques. *Atherosclerosis.* 113:179–188.

34. Rath, M., A. Niendorf, T. Reblin, M. Dietel, H. Krebber, and U. Beisiegel. 1989. Detection and quantification of lipoprotein(a) in the arterial wall of 107 coronary bypass patients. *Arteriosclerosis.* 9:579–592.

35. Hoff, H.F., J. O'Neil, G.B. Smejkal, and A. Yashiro. 1994. Immunochemically detectable lipid-free apo(a) in plasma and in human atherosclerotic lesions. *Chem. Phys. Lipids* 67/68:271–280.

36. Lawn, R.M. 1992. Lipoprotein(a) in heart disease. *Sci. Am.* June:54–60.

37. Hartmann, G., L. Naldini, K.M. Weidner, M. Sachs, E. Vigna, P.M. Comoglio, and W. Birchmeier. 1992. A functional domain in the heavy chain of scatter factor/hepatocyte growth factor binds the c-Met receptor and induces cell dissociation but not mitogenesis. *Proc. Natl. Acad. Sci. USA.* 89:11574–11578.

38. Itakura, Y., T. Yamamoto, K. Matsumoto, and T. Nakamura. 1994. Autocrine stimulation of motility in SBC-5 human lung carcinoma cells by a two-kringle variant of HGF. *Cancer Lett.* 83:235–243.