Effect of Individual Plasma Lipoprotein(a) Variations In Vivo on Its Competition With Plasminogen for Fibrin and Cell Binding

An In Vitro Study Using Plasma From Children With Idiopathic Nephrotic Syndrome

Thierry Soulat, Stéphane Loyau, Véronique Baudouin, Lydia Maisonneuve, Marie-France Hurtaud-Roux, Nicole Schlegel, Chantal Loirat, Eduardo Anglés-Cano

Abstract—Simultaneous natural changes in lipoprotein(a) [Lp(a)] and plasminogen occur in the nephrotic syndrome and offer a unique opportunity to investigate their effects on plasminogen activation under conditions fashioned in vivo. Plasminogen, Lp(a), and apolipoprotein(a) in plasma were characterized, and their competitive binding to carboxy-terminal lysine residues of fibrin and cell membrane proteins was determined in nephrotic children during a flare-up of the disease (61 cases) and after 6 weeks (33 cases) and 6 months (42 cases) of remission. Low plasminogen concentrations (median 1.34 μmol/L, range 0.39 to 1.96 μmol/L) and high Lp(a) levels (median 0.27 g/L, range 0.07 to 2.57 g/L) were detected at flare-up. These changes were associated with an increased Lp(a) binding ratio onto fibrin (3.13 ± 0.48) and cells (1.53 ± 0.24) compared with binding ratios of control children (1.31 ± 0.19 and 1.05 ± 0.07, respectively) with normal plasminogen and low Lp(a) (median 0.071 g/L). After 6 weeks and 6 months of remission, the values for net decrease in Lp(a) binding to fibrin were 1.7 ± 0.22 (after 6 weeks) and 1.88 ± 0.38 (after 6 months) and were correlated with low Lp(a) concentrations (median 0.2 g/L, range 0.07 to 0.8 g/L; and median 0.12 g/L, range 0.07 to 1.34 g/L) and inversely associated with increased plasminogen levels (median 1.82 μmol/L, range 1.4 to 2.1 μmol/L; and median 1.58 μmol/L, range 1.1 to 2.1 μmol/L). The studies provide the first quantitative evidence that binding of Lp(a) to lysine residues of fibrin and cell surfaces is directly related to circulating levels of both plasminogen and Lp(a) and that these glycoproteins may interact as competitive ligands for these biological surfaces in vivo. This mechanism may be of relevance to the atherothrombotic role of Lp(a), particularly in nephrotic patients. (Arterioscler Thromb Vasc Biol. 2000;20:575-584.)

Key Words: lipoprotein(a) ■ apolipoprotein(a) isoforms ■ binding, competitive ■ plasminogen inhibition ■ lysine binding site

Thromboembolic events represent some of the most serious complications in patients with nephrotic syndrome.1–3 Several alterations, potentially related to these events, have been identified in blood coagulation factors (increased concentrations of factors V and VIII and fibrinogen) and inhibitors (decreased concentrations of antithrombin III and free protein S).4 Modifications of the fibrinolytic system are less well defined.5,6 For instance, modifications in the levels of plasminogen may be related to age, in view of the fact that low levels have been found in nephrotic children7,8 and normal or increased levels have been found in nephrotic adults.9,10 In contrast, increased concentrations of Lp(a), the lipoprotein particle containing apo(a), a glycoprotein genetically related and structurally homologous to plasminogen, have been reported in nephrotic adults9,11–13 and in children.14,15 Because high plasma levels of Lp(a) are now recognized as a risk factor in cerebrovascular and cardiovascular diseases,16–18 increased concentrations of Lp(a) may represent an added thrombotic risk in nephrotic subjects.19,20

Plasminogen and apo(a) contain triple-loop disulfide-linked structures called “kringles” and a serine-proteinase domain.21,22 Among the 5 kringles of plasminogen, kringles 1 and 4 contain a lysine-binding site, which allows binding of plasminogen to carboxy-terminal lysine residues of fibrin and cell membrane proteins. Plasminogen thus bound is transformed into plasin, the enzyme responsible for fibrinolysis and pericellular proteolysis, after cleavage of the Arg561-Val562 peptide bond by activators.23

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Apo(a) contains multiple tandem repeats of plasminogen-like kringle 4 (61% to 75% homology) followed by a single copy of kringle 5 and of the protease domain (≈94% homology).24 The variable number of plasminogen-like kringle 4 copies in apo(a) gives rise to isoforms of different size, which have been identified by analysis of the protein25 and the cDNA.26 The size of these isoforms (300 to >800 kDa) is inversely correlated with the plasma level of Lp(a).27 Data concerning the distribution of these apo(a) isoforms in children with renal diseases are scarce.14

Some of the plasminogen-like kringle 4 copies endow apo(a) with the ability to compete with plasminogen for binding to cells,28,29 and fibrin.30,31 However, the substitution of the Arg-Val plasminogen cleavage site by Ser-Ile in apo(a) impairs the generation of plasmin-like activity by activators. Thus, the competitive binding of Lp(a) for lysine residues of fibrin and cell membrane proteins results in decreased plasmin formation and may favor the deposit of fibrin and lipids within the vascular wall.32

Because a decreased plasminogen activation may be associated with thrombosis,33,34 we thought it interesting to explore the interactions of Lp(a) with this enzyme system in nephrotic children. We hypothesized that the low levels of plasminogen and the high levels of Lp(a) induced by the nephrotic syndrome may favor binding of Lp(a) to fibrin and to human monocytes, thus inducing a decrease in fibrinolysis and pericellular proteolysis, which may contribute to the development of atherothrombosis. We undertook to explore this hypothesis in 61 children with idiopathic nephrotic syndrome. Blood samples were collected during a flare-up of the disease (n = 61) and after remission at 6 weeks (n = 33) and at 6 months (n = 42). Taking into account the plasma concentrations of Lp(a), apo(a), and plasminogen and the functional characteristics of the apo(a) isoforms, we evaluated the pathogenicity of Lp(a) under conditions fashioned in vivo.

Methods

Subjects and Blood Samples

Sixty-one children with idiopathic nephrotic syndrome were studied during a flare-up of the disease (stage 1), defined by heavy proteinuria and plasma albumin levels ≤25 g/L. Thirty-three of these children were also studied after 6 weeks of remission (stage 2), and 42 were studied after 6 months of remission (stage 3). Children aged <1 year were excluded from the study. All children were treated in the Pediatric Nephrology Department of Hôpital Robert Debré, Paris, France. The present study was approved by the ethical committee of Bichat-Claude Bernard Medical Faculty and by the Délegation à la Recherche Clinique, Assistance Publique-Hôpitaux de Paris. Written informed consent was obtained from parents and, eventually, from patients. Control samples for Lp(a) determination were obtained from the Centre Hospitalo-Universitaire of Rheims, Rheims, France. For 200 age-matched children (109 boys, 91 girls) who exhibited a normal renal function, routine biological sampling over 36 months. Venous blood was separated from blood by centrifugation at 2000g for 20 minutes at 4°C, supplemented with D-valyl-L-phenylalanine-L-lysine chloromethyl ketone (VPL, 1 μmol/L), and stored at −80°C.

Reagents and Buffers

Materials were purchased from the following sources: BSA was from Eurobio; VPL and l-phenylalanine-l-lysine chloromethyl ketone (PPACK), from France-Biochem; aprotinin (Trasylol), from Bayer Pharma; AEBFS, from Interchim; peroxidase-labeled sheep immunoglobulins, from Dako; and nitrocellulose sheets, from Sar-torius. Others products were obtained as previously described.31,35 Buffer A consisted of 0.05 mol/L sodium phosphate, pH 7.4, containing 0.08 mol/L NaCl, 0.01% Tween 20, and 0.01% NaN3. Assay buffer consisted of buffer A containing 2 mg BSA/mL. Mass buffer, a buffer that provided the mass action effects on the competitive nonspecific adsorption of plasminogen and Lp(a) to fibrin and cell surfaces, consisted of buffer A containing 40 mg of BSA/mL. PBS buffer consisted of 0.015 mol/L Na2HPO4 and 0.06 mol/L KH2PO4, pH 7.4, containing 0.14 mol/L NaCl.

Determination of Lp(a) and Plasminogen Concentrations

The concentrations of plasma Lp(a) and plasminogen were determined by immunoelectrophoresis according to the method of Laurell.36 An interassay coefficient of variation of <8% was determined by using plasmas with a known concentration of plasminogen and Lp(a). A commercial kit (ImmunoFrance) was used for Lp(a), and the concentration is given in grams per liter by reference to the kit standard. The lower limit of detectability was 0.067 g/L. For the detection of plasminogen, an assay was developed, and the concentration was expressed in moles per liter with the use of a home standard prepared by adding varying concentrations of purified plasminogen to plasminogen-depleted plasma. Electrophoresis was performed on 1% agarose gels containing a sheep antiserum directed against human plasminogen. The antiserum was prepared at the Institut National de la Recherche Agronomique (Centre de Clermont-Ferrand-Theix) by immunizing the animal with 3 subcutaneous injections of purified human plasminogen. The agarose (Indubiose A37, Sepacor) was dissolved in a buffer consisting of 50.8 mmol/L Tris, 27.2 mmol/L H3BO3, 1.0 mmol/L Na2-EDTA, and 0.01% NaN3, and was mixed at 60°C with 1% and 0.4% (final concentrations) of polyethylene glycol 6000 and the sheep antiserum, respectively. A 2-mm-thick gel layer was poured on a polyester film (GelBond, FMC Bioproducts), and 5-μL samples were loaded and electrophoresed at 70 V for 2 hours in the same buffer. After electrophoresis, the gel was washed (0.15 mol/L NaCl), dried, and stained with an amido-black solution dye in 45% methanol and 10% acetic acid.

Determination of Size of Plasma Apo(a) Isoforms

The apo(a) isoforms were identified as follows: plasma was electrophoresed under reducing conditions with 3.75% polyacrylamide/0.8% agarose gels, and protein bands were electroblotted to a nitrocellulose membrane according to Khyse-Andersen.37 Apo(a) bands were localized by using a sheep antibody to human apo(a) prepared as described below, followed by a peroxidase-conjugated rabbit antibody to sheep IgG (Dako A/S) revealed with 4-chloro-1-naphthol. To quantify the proportion of each isoform from a given plasma, immunoblots were incubated overnight with the apo(a)-specific antibody, radiolabeled with 125I, and autoradiographed on X-ray film. Apo(a) isoforms were identified by using a recombinant apo(a) standard prepared in our laboratory (INSERM U.143) and composed of isoforms containing 10, 14, 18, 26, and 34 kringle.38 The molar concentrations of the apo(a) isoforms were calculated by relating their molecular mass and relative proportion in plasma with the concentration of Lp(a). Molecular mass estimation was based on sequence24 and by assuming a constant carbohydrate content of 23 wt%.

Purification of Proteins and Lp(a)

Human plasminogen and fibrinogen were purified from fresh-frozen human plasma under conditions that avoid proteolysis, as previously described.40,41 with modifications.42,43 Lp(a) was isolated from human plasma under conditions that avoid proteolysis, as previously described.40,41 Lp(a) was isolated from human plasma under conditions that avoid proteolysis, as previously described.
Cell Culture and Preparation of Cell Surfaces

The monocytic cell line THP-1 was obtained from the American Type Culture Collection. The cells were plated in 25-and 80-cm² Falcon plastic flasks containing Dulbecco's-Iscove supplemented with 10% FCS, 0.5% sodium pyruvate, and 1% antibiotics (streptomycin and penicillin) and cultured at 37°C in 5% CO₂. The cells were recovered after 3 or 4 days of culture, washed with PBS buffer, resuspended in the same buffer at a concentration of 1.5×10⁶ cells per milliliter, and immobilized on microtitration plates by using an adaptation of the method of Stocker and Heusser. We introduced the following modifications: flexible polyvinyl chloride plates were first treated with polyglutaraldehyde during 2 hours at 22°C, excess glutaraldehyde was eliminated by washing the plates with distilled water, and 80,000 cells were added into each well. After centrifugation at 233g during 10 minutes, the cells were incubated in the plates for 1 hour at 22°C, followed by an incubation of 10 minutes in a bath of 0.25% polygluteraldehyde; after 2 final washes with PBS, 100 μL per well of PBS containing 4 mg/mL BSA and 0.1% thimerosal was added, and the plates were sealed and stored at 4°C until further use.

Purification and Radiiodination of Immunoglobulins Directed Against Human Apo(a)

The IgG fraction of a sheep antiserum against human apo(a) was obtained as described and was separated by ammonium sulfate precipitation, ion-exchange chromatography on DEAE, and affinity chromatography on Protein-A Sepharose (Pharmacia). The purified IgG was further immunodepleted by using Sepharose-imobilized apoB-100 and plasminogen; the final antibody preparation did not cross-react with these proteins and was shown to react equivalently with different apo(a) isoforms. The IgG was labeled with sodium iodide 125I by using the Iodogen method of Fraker and Speck, an iodination time of 4 minutes at 22°C, and removal of free 125I by molecular sieving on a PD-10 Sephadex column (Pharmacia). The specific radioactivity obtained was 3 nCi/μg of anti-apo(a) IgG.

Competitive Binding of Lp(a) and Plasminogen at Plasma/Fibrin Interface

The experiments were performed with fibrin surfaces prepared and characterized as previously described and with plasminogen/Lp(a)-depleted plasma prepared as follows: Human venous blood obtained from 2 healthy volunteers was drawn into polypropylene tubes containing 4 mmol/L EDTA, 0.01% NaN₃, and inhibitors of proteolysis (10 KIU/mL aprotinin, 1 μmol/L PPACK, 1 μmol/L VPL, 10 μmol/L p-Nitrophenyl-p’-guani dino-benzozate, and 1 mmol/L AEBSF). Plasma was then separated by centrifugation at 2000g during 2 hours at 22°C, excess glutaraldehyde was eliminated by washing the plates with distilled water, and 80,000 cells were added into each well. After centrifugation at 233g during 10 minutes, the cells were incubated in the plates for 1 hour at 22°C, followed by an incubation of 10 minutes in a bath of 0.25% polygluteraldehyde; after 2 final washes with PBS, 100 μL per well of PBS containing 4 mg/mL BSA and 0.1% thimerosal was added, and the plates were sealed and stored at 4°C until further use.

Binding of Plasma Lp(a) to Fibrin and Cell Surfaces

The procedure for the binding of plasma Lp(a) to fibrin and cell surfaces was based on previous studies and the above competitive binding experiments. Plasma (1:2 to 1:16 in mass buffer, pH 6.8, supplemented with 2 mmol/L EDTA, 1 μmol/L VPL, and 1 μmol/L dansyl-Dansyl-L-glutamyl-glycyl-L-arginine chloromethyl ketone) was incubated with fibrin or cell surfaces. After 18 hours at 4°C, unbound proteins were removed by washing, and the surfaces were probed with a known concentration of the 125I-labeled polyclonal antibody directed against human apo(a). Excess antibody was removed by washing, and the radioactivity in the wells was counted in a gamma-radiation counter and transformed into an Lp(a) binding ratio by relating the signal obtained with the plasma from patients to the signal of plasma from a healthy control with undetectable levels of Lp(a). For each plasma sample, the value used to calculate the binding ratio was the highest signal obtained as a function of the plasma dilutions tested. Specificity of the binding of Lp(a) to carboxy-terminal lysine residues of fibrin and membrane proteins of THP-1 cells was assessed as previously described. Briefly, binding experiments were performed either with surfaces previously treated with carboxypeptidase B or with plasma supplemented with 0.2 μmol/L 6-aminohexanoic acid before incubation with untreated surfaces.

Statistical Analysis

Differences between groups were calculated by using a 2-tailed paired Student t test for statistical significance. The possible effect of various hemostasis and lipid parameters on the competitive binding of Lp(a) and plasminogen to fibrin and cell surfaces was analyzed by using a linear regression model. The variability of Lp(a) and plasminogen concentrations during the course of the nephrotic syndrome was expressed by the percentiles of the distributions. In all cases, significance was established at P<0.05.

Results

Demographic Data

Sixty-one children (37 boys, 24 girls) with idiopathic nephrotic syndrome were included in the study during a flare-up of the disease (stage 1). Median age was 4 years, ranging from 1 to 16 years. Among these 61 patients, 51 had steroid-responsive and 10 had steroid-resistant idiopathic nephrotic syndrome. Renal biopsy, performed in 16 children, showed minimal change in glomerular lesions in 10 of the children, focal segmental glomerular sclerosis in 5, and mesangial proliferation in 1.

At the time of flare-up, 17 children had received no treatment, 40 had received prednisone, and 4 had received prednisone and cyclosporine. Three children had thromboembolic complications: 2 children exhibited pulmonary thromboembolism at the time of the study, and 1 child had experienced thrombosis of the external iliac vein 15 months before the study. Thirty-five children received anticoagulant therapy with either heparin (2 children), anti–vitamin K (30 children), or a combination of both (5 children). Mean serum creatinine at flare-up was 46±40 μmol/L. Five children had transient renal insufficiency: serum creatinine levels were between 74 and 106 μmol/L in 3 children and between 208 and 284 μmol/L in 2 children. At stage 2 (6 weeks of remission, 33 children), all patients received prednisone (which was associated with cyclosporine treatment in 3 children). One child received anti–vitamin K treatment. At stage 3 (6 months of remission, 42 children), 17 children received no treatment, 11 received prednisone alone, 9 received prednisone and cyclosporine, 1 received prednisone...
and cyclophosphamide, and 4 received cyclosporine alone. Only 1 child received anti–vitamin K treatment.

**Albumin, Lipids, and Fibrinolytic Parameters**

As indicated in Table 1, all patients showed heavy proteinuria, hypoalbuminemia, hypercholesterolemia, and hypertriglyceridemia during flare-up. Results of the measurement of proteins involved in fibrinolysis are depicted in Table 2; fibrinogen and plasminogen activator inhibitor type 1 (PAI-1) antigen were markedly elevated at flare-up.

**Plasminogen and Apo(a)/Lp(a) Quantification**

The plasma concentrations of plasminogen and apo(a)/Lp(a) are indicated in Table 3. The molar concentration of apo(a) was calculated by using the proportion of each isoform in plasma and concentrations of Lp(a) >0.067 g/L, the detection limit of the immunoelectrophoretic assay. An Lp(a) concentration <0.067 g/L was detected in 29% of patients at diagnosis (n=18 of 61), in 39% at 6 weeks of remission (n=13 of 33), and 45% at 6 months of remission (n=19 of 42). Opposite modifications in the levels of plasminogen and apo(a)/Lp(a) were observed. The concentration of plasminogen (1.34 μmol/L) at flare-up was significantly lower (P<0.0001) than the levels at 6 weeks (1.82 μmol/L) and at 6 months (1.58 μmol/L) of remission. In contrast, at flare-up the levels of apo(a) (median 255 μmol/L) and Lp(a) (median 0.27 g/L) were markedly elevated compared with levels at 6 weeks (0.167 μmol/L and 0.2 g/L, respectively) and at 6 months (0.234 μmol/L and 0.121 g/L, respectively) of remission. In 28 of these patients, a plasminogen concentration <1.3 μmol/L was detected. Although plasminogen levels were normal at remission, the concentrations of apo(a)/Lp(a) were still higher at 6 months of remission compared with levels previously reported for children14,15 and by reference to an age-matched control population of children composed of 109 boys and 91 girls.

**Apo(a) Phenotyping**

To simplify the analysis and taking as a basis a mean isoform size of 22 kringles, which discriminates apo(a) isoforms expressing a greater risk for coronary heart disease,49 we classified the apo(a) isoforms of nephrotic children into 2 groups: small molecular size isoforms (range 16 to 21 kringles) and high molecular size isoforms (range 23 to 30 kringles). Apo(a) isoforms could not be identified in 23 plasma samples with Lp(a) concentrations ≥0.067 g/L. Six patients were apparently homozygous and had single isoforms (16 kringles, 1 patient; 17 kringles, 2 patients; and 18 kringles, 3 patients), whereas all others (32 patients) were heterozygous and had a low molecular mass isoform (mean size 16±1 krlangles) and a high molecular mass isoform (mean size 26±2 kringles) as shown in Figure 1. In agreement with the known inverse relationship between Lp(a) concentration and apo(a) isoform size, the mean concentration of low molecular mass isoforms (0.23±0.27 μmol/L) was higher than the mean concentration of high molecular mass isoforms (0.16±0.22 μmol/L). Individual modifications in the relative concentration of the apo(a) isoforms in heterozygous subjects were observed with the decrease in Lp(a) concentrations after remission. In some cases, the observed decrease in Lp(a) did not allow detection of the apo(a) isoform band of high molecular mass with our phenotyping system (Figure 1).

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**TABLE 1. Proteinuria, Serum Albumin, and Lipid Levels**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal Levels</th>
<th>Flare-Up (n=61)</th>
<th>6 wk (n=33)</th>
<th>6 mo (n=42)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinuria, g/L</td>
<td>...</td>
<td>13.7±14.5 (0.7–75)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Serum albumin, g/L</td>
<td>35–47</td>
<td>13.6±4.9* (3.3–25)</td>
<td>41.5±3.6 (34.3–50)</td>
<td>41.6±4.2 (33.8–49.4)</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>1.16±0.26†</td>
<td>1.6±2.4 (0.2–1.7)</td>
<td>1.3±0.3 (0.7–1.9)</td>
<td>1.26±0.3 (0.7–1.9)</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>4.29±0.67†</td>
<td>12.9±3.4* (4.9–25)</td>
<td>5.4±1.45 (3–8.7)</td>
<td>4.71±0.9 (2.9–7.08)</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.25±0.74†</td>
<td>4.3±3.0* (0.76–15.3)</td>
<td>1.7±0.95 (0.41–3.95)</td>
<td>1.3±0.9 (0.39–3.74)</td>
</tr>
</tbody>
</table>

Values are mean±SD. Numbers in parentheses indicate range.

*P<0.0001 vs remission levels.

†Normal levels for children from Reference 14.

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**TABLE 2. Fibrinolytic Data**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal Levels</th>
<th>Flare-Up (n=61)</th>
<th>6 wk (n=33)</th>
<th>6 mo (n=42)</th>
</tr>
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<tbody>
<tr>
<td>tPA Ag, ng/mL</td>
<td>5±3</td>
<td>5.2±4.9</td>
<td>4.11±2.6</td>
<td>3.82±3.9</td>
</tr>
<tr>
<td>PAI-1 Ag, ng/mL</td>
<td>16.4±12</td>
<td>67.4±33.4*</td>
<td>25.7±16.6</td>
<td>33.4±27.0</td>
</tr>
<tr>
<td>α2-AP, %</td>
<td>85–100</td>
<td>113.6±18.6</td>
<td>115.5±12.2</td>
<td>119.3±35.9</td>
</tr>
<tr>
<td>Fibrinogen, g/L</td>
<td>2–4</td>
<td>7.0±2.6</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are mean±SD. tPA Ag indicates tPA antigen; PAI-1 Ag, PAI-1 antigen; α2-AP, α2-antiplasmin; and ND, not determined. Normal levels are those of age-matched control children (n=14) with neither renal nor lipid disorders. tPA Ag and PAI-1 Ag were determined by ELISA (Diagnostica Stago). α2-AP was determined as percent activity relative to a control plasma as measured by chromogenic substrate assay.31 Fibrinogen was measured by clotting rate assay.

*P<0.0001 vs values at remission and control values.
Competitive Binding of Plasma Lp(a) to Fibrin and Cell Surfaces

Competition between plasminogen and Lp(a) for binding to fibrin has been previously demonstrated in purified systems. In the present study, the binding assays were performed in plasma. Therefore, we sought to rule out potential confounding plasma factors that might influence the assay. For that purpose, the competitive binding of plasminogen and Lp(a) separated from the plasma of volunteer adults was studied at the plasma/fibrin interface by using a system consisting of a fibrin surface and plasma depleted in plasminogen and Lp(a).

Table 3. Lp(a), Apo(a), and Plasminogen Concentrations

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Flare-up (n=61)</th>
<th>6 wk (n=33)</th>
<th>6 mo (n=42)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lp(a), g/L</td>
<td>0.27 (0.07–2.57)*</td>
<td>0.20 (0.07–0.83)</td>
<td>0.12 (0.07–1.34)</td>
</tr>
<tr>
<td>75th percentile</td>
<td>0.585</td>
<td>0.367</td>
<td>0.470</td>
</tr>
<tr>
<td>90th percentile</td>
<td>1.016</td>
<td>0.696</td>
<td>0.632</td>
</tr>
<tr>
<td>Apo(a), μmol/L</td>
<td>0.255 (0.03–1.26)†</td>
<td>0.167 (0.05–0.36)</td>
<td>0.234 (0.04–0.45)</td>
</tr>
<tr>
<td>75th percentile</td>
<td>0.414</td>
<td>0.335</td>
<td>0.283</td>
</tr>
<tr>
<td>90th percentile</td>
<td>0.740</td>
<td>0.356</td>
<td>0.326</td>
</tr>
<tr>
<td>Plasminogen, μmol/L</td>
<td>1.34 (0.4–1.96)§</td>
<td>1.82 (1.4–2.1)</td>
<td>1.58 (1.1–2.1)</td>
</tr>
<tr>
<td>75th percentile</td>
<td>1.54</td>
<td>1.93</td>
<td>1.76</td>
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<tr>
<td>90th percentile</td>
<td>1.65</td>
<td>2.08</td>
<td>1.86</td>
</tr>
</tbody>
</table>

Values are expressed as median, with range shown in parentheses. The frequency distribution is expressed by the 75th and 90th percentiles.

Lp(a) and plasminogen concentrations were measured by electroimmunodiffusion. Apo(a) concentrations of nephrotic children with ≥0.067 g/L Lp(a) (detection limit of the assay), were calculated for flare-up (n=43) and after 6 wk (n=20) and 6 mo (n=23) of remission as indicated in Methods.

Figure 1. Concentration of Lp(a) and distribution of apo(a) isoforms during the evolution of the nephrotic syndrome. Top, Changes in the concentration of large (≥22K, gray bars) and small (<22K, white bars) apo(a) isoforms in 4 heterozygous patients at diagnosis (left column) and at remission after 6 weeks (middle column) and 6 months (right column). Bottom, Apo(a) phenotype of plasmas shown in top panel. Apo(a) isoform size is indicated in number of kringles (29/20, 30/17, 25/17, and 26/18) by reference to a recombinant apo(a) standard.

Figure 2. Binding of Lp(a) and plasminogen to fibrin in a plasma milieu. Increasing concentrations of Lp(a) added to Lp(a)- and plasminogen-depleted plasma were incubated with fibrin in the absence (●) and in the presence of plasminogen at 1 (●) and 2 (●) μmol/L. Bound Lp(a) was detected with a radiolabeled sheep antibody specific for apo(a). Radioactivity bound was then transformed into femtomoles of antibody bound to fibrin. In separate wells, bound plasminogen was activated with tPA and detected with a chromogenic substrate selective for plasmin; the initial velocity of the reaction was transformed into mass of plasminogen as indicated. The amount of anti-apo(a) antibody bound (fmol/well, main graph) and of plasminogen bound (pmol/well, inset) are represented against the input concentration of Lp(a) added to plasma. To simplify, the graph data shown represent specific binding obtained by subtracting binding in the presence of 6-aminohexanoic acid, a lysine analogue, from total binding. The Lp(a) tested contained an 18-kringle apo(a) isoform with high affinity for fibrin (Kd 12 nmol/L) as calculated from the binding isotherms according to Hervio et al.
a study on the binding of plasma Lp(a) to fibrin and cells, particularly in nephrotic plasmas during a flare-up. The amount of Lp(a) bound to fibrin was a function of the input concentration added to plasma and decreased as the concentration of plasminogen was incremented from 0 to 1 and 2 μmol/L (Figure 2, main graph). In a similar fashion, the amount of plasminogen bound at these concentrations decreased as a function of the plasma Lp(a)/plasminogen molar concentrations (Figure 2, inset). Lp(a) and plasminogen thus detected were specifically bound to carboxy-terminal lysine residues of fibrin and cell surfaces, as indicated by the absence of binding to surfaces treated with carboxypeptidase B and by its inhibition with the lysine analogue 6-aminohexanoic acid. These results indicate that binding of Lp(a) and plasminogen to fibrin at the interface with plasma complies with mechanisms similar to those described in purified systems; ie, the amount bound is a function of their plasma concentration and of their affinities for fibrin. Of note, the amount of Lp(a) and plasminogen bound to fibrin was not affected by fibrinogen at concentrations similar to those found in nephrotic plasmas (data not shown). On the basis of these results, the competitive binding behavior of Lp(a) and plasminogen was explored at fibrin and cell interfaces with plasmas from nephrotic children during a flare-up (n = 61) and after 6 weeks (n = 33) and 6 months (n = 42) of remission. The plasmas were incubated with the surfaces at various dilutions to mimic different ligand/surface interactions, as indicated in Methods. The amount of Lp(a) bound that was measured with an antibody to apo(a) was chosen as the end point of the assay and was expressed by a patient/control binding ratio that relates the respective binding values. The control plasma used had no detectable Lp(a), as assessed by immunoelectrophoresis and phenotyping. The value used to calculate the binding ratio was the highest amount of fibrin-bound Lp(a) detected as a function of the plasma dilution (1:8 in most patients). The results are expressed in Table 4. Mean binding ratios obtained with both fibrin (3.13 ± 0.48) and cell (1.53 ± 0.24) surfaces at flare-up were significantly higher (P < 0.0001) than the mean values obtained at 6 weeks (1.7 ± 0.22 and 1.16 ± 0.09) and at 6 months (1.88 ± 0.38 and 1.22 ± 0.08) of remission. Values obtained at 6 months of remission approximated the mean normal values obtained with plasmas from 14 age-matched children (fibrin 1.31 ± 0.19; cells 1.05 ± 0.07) and the cut-off Lp(a) fibrin binding ratio (mean +2 SD 1.74) established in a healthy adult population (n = 108; E.A.-C. et al, unpublished data, 1999). Figure 3 clearly shows that the decrease in the Lp(a) binding ratio (Figure 3A) that evolves with the nephrotic syndrome from the clinical active phase (stage 1) to remission at 6 weeks (stage 2) and at 6 months (stage 3) was directly linked to the increase in plasma plasminogen levels and to the decrease in apo(a) concentration (Figure 3B). The relation between the increased concentration of apo(a) and the decreased concentration of plasminogen with the binding ratio is clearly shown in Figures 4 and 5. Binding ratios for each apo(a) isoform were calculated by using their relative concentrations in plasma and affinity values, ie, the dissociation constant (Kd), calculated with equivalent Lp(a) isoforms purified from the plasma of healthy adults. Typical binding isotherms used to calculate binding affinities are shown in Figure 2 for an 18-kringle Lp(a) isoform (Kd 12 nmol/L). In

![Figure 3](image-url)

**Figure 3.** Evolution of Lp(a), apo(a), and plasminogen parameters at different stages of the nephrotic syndrome. A, Binding of Lp(a) to fibrin (gray bars) and THP-1 cell surfaces (white bars), performed and quantified as indicated in Figure 4. B, Plasma concentrations of plasminogen (vertically lined bars) and apo(a) (diagonally lined bars) determined by electroimmunodiffusion according to Laurell.32 The bars indicate the standard deviation of mean values (height of columns) determined at flare-up (stage 1) and at 6 weeks (stage 2) and at 6 months (stage 3) of remission.
Figure 4, the amount of each apo(a) isoform bound to fibrin (A) and THP-1 cells (B) is plotted against the apo(a) isoform/plasminogen molar concentration ratio. An increase in the amount of apo(a) bound is observed as a function of the apo(a) isoform/plasminogen molar ratio, thus indicating that the binding of Lp(a) to the fibrin and cell surfaces was directly related to the increase in the concentration of Lp(a) and inversely related to plasminogen concentrations. Further evidence of the inverse relation between plasminogen concentrations and the amount of apo(a) bound could be clearly shown at concentrations of plasminogen, <1.3 μmol/L (Figure 5). In contrast, the Lp(a) binding data thus obtained did not correlate with any of the lipid and hemostatic variables, including serum albumin (P>0.20), cholesterol (P=0.17), triglycerides (P=0.25), tPA (P=0.13), PAI-1 (P=0.21), and fibrinogen (P=0.21) concentrations.

Discussion

Thromboembolic complications increase the risk of morbidity and mortality in patients with nephrotic syndrome. Vascular occlusions (pulmonary thromboembolism and arterial or venous thrombosis) have been observed in both adults and children. The reported incidence is lower in children despite more marked coagulation abnormalities. However, the possibility of clinically silent thrombosis in children cannot be ruled out. These conditions result, most probably, from the conjunction of hypoalbuminemia and hyperlipemia with several changes in blood coagulation, fibrinolysis, and platelet functions. Marked lipid abnormalities, such as elevation of lipoproteins containing apo B, ie, VLDL, LDL, and Lp(a), have been previously reported and were confirmed by results of the present studies. Nephrotic children have significantly higher plasma concentrations of Lp(a) than do healthy children; these high levels of Lp(a) are an abnormality currently considered to be an independent risk factor for the development of cardiovascular disease in the general population. The mechanism by which Lp(a) may favor the atherothrombotic process may be related to the lysine-binding properties of apo(a). It has been proposed that Lp(a) may inhibit the binding of plasminogen to cells and fibrin by a competitive mechanism and, thereby, interfere with the fibrinolytic process. Furthermore, we have previously shown that in subjects heterozygous for the apo(a) trait, the influence of the various apo(a) isoforms on fibrinolysis depends on their affinity for fibrin and cell surfaces directly related to the increase in the concentration of Lp(a) and inversely related to plasminogen concentrations. Further evidence of the inverse relation between plasminogen concentrations and the amount of apo(a) bound could be clearly shown at concentrations of plasminogen <1.3 μmol/L (Figure 5). In contrast, the Lp(a) binding data thus obtained did not correlate with any of the lipid and hemostatic variables, including serum albumin (P=0.20), cholesterol (P=0.17), triglycerides (P=0.25), tPA (P=0.13), PAI-1 (P=0.21), and fibrinogen (P=0.21) concentrations.

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umented at a flare-up of the nephrotic syndrome and their normalization with remission constitute a model for the study of common interactions between these proteins and biological surfaces. This natural model offers a unique opportunity to disclose the potential atherothrombotic role of Lp(a) under exceptionally large changes in the concentrations of 2 genetically linked homologous glycoproteins: apo(a) and plasminogen. The present study was therefore restricted to experimental procedures performed with native plasma to preserve the in vivo molecular interactions between plasminogen and Lp(a) induced by the nephrotic syndrome and to determine their effects on fibrin and cell binding in vitro. The experimental setup was designed to functionally isolate the Lp(a)/plasminogen competition for carboxy-terminal lysine residues of fibrin and cell membrane proteins and to preserve the original plasma environment.

One of the most important features of the present study was the evolution of Lp(a) binding to fibrin and cells with disease activity. The increased binding of Lp(a) to fibrin and cell surfaces observed during a flare-up of the nephrotic syndrome was significantly correlated with the apo(a)/plasminogen molar concentration ratio, thus indicating that it was directly related to the concentration of Lp(a) and inversely proportional to the level of plasminogen. Of note, other hemostatic and lipid parameters that also showed large variations were not correlated with the binding of Lp(a). Indeed, high fibrinogen in plasma from nephrotic adults has been shown to have no influence on plasminogen activation. Furthermore, the current in vitro experiments that used whole plasma failed to show any effect of plasma proteins, including high fibrinogen concentrations, on the competitive binding of Lp(a) and plasminogen. This binding was mainly due to the effect of apo(a) isoforms of low molecular mass and was accentuated at low plasminogen concentrations. Of note, 2 of the nephrotic children in the present study and 2 of 20 nephrotic children reported by Garnot et al developed thrombosis and had high levels of apo(a) isoforms of low molecular mass. The decrease in the binding of Lp(a) to fibrin and cell surfaces at 6 weeks and at 6 months of remission, which was simultaneous with the normalization of plasminogen and the decrease in Lp(a) levels, confirmed this hypothesis and indicated clearly that the binding was a consequence of the important modifications in the plasma levels of Lp(a) and plasminogen. On the other hand, the increased levels of PAI-1 observed during flare-up may efficiently neutralize tPA available for fibrinolysis and thereby accentuate the antifibrinolytic effect of Lp(a).

Another important feature of the present study was the observed differences in the proportion of each isoform with disease activity. The decrease in the concentration of Lp(a) from the acute phase to remission produced a concomitant decrease in the relative proportion of the apo(a) isoforms, which in some cases could not be detected with the phenotyping system used. This phenomenon was more frequently observed with isoforms of high molecular mass, which also presented the lowest initial concentration, thus suggesting a difference in protein synthesis rather than a selective decrease in one of the apo(a) isoforms. However, the underlying mechanism responsible for the increased Lp(a) concentrations in nephrotic patients remains obscure. Lp(a) may behave as an acute phase reactant, and its plasma concentra-

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Effect of Individual Plasma Lipoprotein(a) Variations In Vivo on Its Competition With Plasminogen for Fibrin and Cell Binding: An In Vitro Study Using Plasma From Children With Idiopathic Nephrotic Syndrome

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