Molecular Analysis of Apo(a) Fragmentation in Polygenic Hypercholesterolemia
Characterization of a New Plasma Fragment Pattern

Sophie Gonbert, Bruno Saint-Jore, Philippe Giral, Chantal Doucet, John Chapman, Joëlle Thillet

Abstract—Hypercholesterolemia is frequently associated with elevated Lp(a) levels, an independent risk factor for coronary, cerebrovascular, and peripheral vascular disease. A portion of apolipoprotein(a) [apo(a)] circulates as a series of fragments derived from the N-terminal region of apo(a). The relationship of elevated lipoprotein(a) [Lp(a)] levels to those of circulating apo(a) fragments in polygenic hypercholesterolemia is indeterminate. Therefore, plasma Lp(a) and plasma and urinary apo(a) fragment levels were measured by ELISA in 82 patients with polygenic type IIa hypercholesterolemia (low density lipoprotein cholesterol ≥4.13 mmol/L and triglycerides <2.24 mmol/L) and in 90 normolipidemic subjects. Lp(a) levels were significantly elevated in patients compared with control subjects (0.35±0.4 and 0.24±0.31 mg/mL, respectively; median 0.13 and 0.11 mg/mL, respectively; P=0.039), although apo(a) isoform distribution did not differ. Patients displayed significantly higher plasma and urinary apo(a) fragment levels than did control subjects (respective values were as follows: 4.97±5.51 and 2.15±2.57 [median 2.85 and 1.17] μg/mL in plasma, P<0.0001; 75±86 and 40±57 [median 38 and 17] ng/mg urinary creatinine in urine, P<0.0001). The ratio of plasma apo(a) fragments to Lp(a) levels was also significantly higher in patients than in control subjects (1.93±1.5% and 1.75±2.36%, respectively; P<0.0001). We conclude that increased plasma Lp(a) levels in polygenic hypercholesterolemia are associated with elevated circulating levels of apo(a) fragments but that this increase is not due to decreased renal clearance of apo(a) fragments. Furthermore, we identified a new pattern of apo(a) fragmentation characterized by the predominance of a fragment band whose size was related to that of the parent apo(a) isoform and that was superimposed on the series of fragments described previously by Mooser et al. This new pattern was associated with small apo(a) isoforms and did not discriminate between hypercholesterolemic and normal subjects. However, this new apo(a) fragment pattern may constitute a novel marker for cardiovascular risk.

Key Words: hypercholesterolemia ■ apo(a) ■ vascular disease ■ Lp(a)

Lipoprotein(a), an LDL-like lipoprotein, is characterized by the presence of apo(a), a highly glycosylated apolipoprotein that is linked to the apoB-100 moiety of the LDL particle by a disulfide bond. Plasma levels of Lp(a) represent an independent risk factor for coronary, cerebrovascular, and peripheral vascular disease (see review). Lp(a) levels differ widely among individuals and are mainly genetically determined by the apo(a) gene locus. The apo(a) gene is highly polymorphic in size because of a variable number of tandem repeats encoding a protein motif called kringle 4. Plasma Lp(a) levels are inversely correlated to apo(a) size, and from studies in normolipidemic subjects, it has been estimated that between 41% and 69% of Lp(a) levels are determined by the apo(a) size polymorphism. However, wide variation in Lp(a) levels is seen among subjects with the same apo(a) isoform. Thus, although the apo(a) genotype exhibits a major influence on Lp(a) levels, other determinants, which may be metabolic and/or environmental in nature, are operative.

Mooser et al have reported that a portion of circulating “free” apo(a) consists of a series of apo(a) fragments that are derived from the N-terminal region of apo(a) and contain a variable number of kringle-4 type-2 repeats. In normal subjects, the circulating concentration of apo(a) fragments is correlated with Lp(a) plasma levels. Historically, apo(a) fragments have been detected first in urine, although the urinary fragments are typically smaller in size than their counterparts in plasma. The concentration of urinary apo(a) fragments is also proportional to that in plasma. From these data, it has been concluded that urinary apo(a) fragments are generated by proteinolysis of circulating apo(a) or Lp(a) and that apo(a) fragmentation may contribute to Lp(a) catabolism. Apo(a) fragments with patterns similar to those ob-
served in plasma and urine have been identified in atherosclerotic plaques; this finding has raised the possibility that apo(a) fragmentation may be implicated in the atherogenicity of Lp(a). Consequently, interest has grown regarding the mechanisms and site(s) of the generation of apo(a) fragments. However, most studies in which the concentration of apo(a) fragments in plasma and urine have been evaluated are restricted to patients with various forms of nephropathy,6,10,11 thereby reflecting the potential role of the kidney in Lp(a) catabolism.12

Lp(a) levels are elevated in polygenic hypercholesterolemia (PH),13,14 but a mechanistic explanation is lacking. We designed the present study to determine whether a decreased degree of fragmentation of apo(a) could account for the elevated Lp(a) levels characteristic of PH. For this purpose, we compared a population of subjects presenting with isolated moderate PH with a population of healthy normolipidemic subjects. Concentrations of apo(a) fragments were elevated in the plasma and urine of patients compared with control subjects, whereas the rate of urinary excretion of fragments was normal in hypercholesterolemic patients. Furthermore, a new pattern of plasma apo(a) fragmentation distinct from that previously described was identified. This fragment pattern was preferentially associated with apo(a) isoforms of small size; nevertheless, it did not discriminate between hypercholesterolemic and normolipidemic subjects. Finally, our data equally confirm that even moderate hypercholesterolemia is associated with elevated Lp(a) levels.

Methods

Subjects
A population of white patients (n = 82), consisting of 55 females and 27 males (aged 16 to 75 years), attending the outpatient Lipid Clinic in the Department of Endocrinology and Metabolism at La Pitié-Salpêtrière Hospital, Paris, was studied. Inclusion criteria were the presence of PH type IIa, defined by an LDL cholesterol concentration ≥160 mg/dL (4.13 mmol/L) and triglyceride concentration <200 mg/dL (2.24 mmol/L). Patients presenting with tendinous xanthomas, a family history of hypercholesterolemia compatible with a monogenic disease, hypolipidemic treatment, secondary hyperlipidemia, thyroid disease, diabetes mellitus, hypertension, renal failure, or coronary heart disease were excluded. The normal renal function of patients and controls was assessed by creatinine clearance, calculated according to Cockroft and Gault.15 The cutoff was set at 70 mL/min. Postmenopausal women (n = 22 in hypercholesterolemic patients and n = 2 in control subjects) were receiving hormonal replacement therapy. Patients were compared with a control group of 90 normolipemic healthy subjects (53 women, 37 men), aged 36 to 69 years, from the Stanislas Cohort recruited at the Center for Preventive Medicine, Nancy, France.16

Blood and Urinary Samples
After an overnight fast, blood was drawn on EDTA (final concentration 0.1%), and centrifuged for plasma collection. Spot samples of urine were also collected on the morning of examination and centrifuged for all PH patients and control subjects (n = 74). Plasma and urine samples were stored at −20°C before analysis.

Measurement of Lipid, Lp(a), and Apo(a) Fragment Levels
Total plasma cholesterol and triglyceride levels were determined by nephelometry. HDL cholesterol was determined by use of an enzymatic commercially available kit (Biomerieux).

Lp(a) concentration was determined by ELISA, as previously described.17 Apo(a) fragments were separated from intact Lp(a) particles by fractionation of plasma samples on heparin-Sepharose according to the method of Mooser et al8 with the use of 2 μg of Lp(a) for 100 μL of resin. The concentration of N-terminal fragments of apo(a) in the nonretained fraction of heparin-Sepharose and in urine was determined by an ELISA with the use of 2 monoclonal antibodies (21D5C6 and 23G3C4), kindly provided by Dr G. Dupuy (Biomerieux, Marcy l’Etoile, France). These antibodies recognize 2 distinct epitopes in the N-terminal region of apo(a).18 Coefficients of variation for this ELISA for intra-assays and interassays were 4.6% and 11.8%, respectively. In a previous study,6 we compared this assay with another assay,3 and a close correlation was observed between measurements performed with the 2 ELISAs.

The apparent fractional urinary excretion rate (FE) of apo(a) fragments was calculated according to the following equation: FE = [urinary apo(a) fragments × plasma creatinine]/[plasma apo(a) fragments × urinary creatinine].19

Electrophoresis and Immunoblotting

Apo(a) phenotype of plasma samples was performed on SDS-agarose gels under reducing conditions as previously described.17 SDS-PAGE was performed on 4.5% acrylamide minigels. Before electrophoresis, samples were combined with glyceral, bromophenol blue, Tris (pH 6.8), SDS, and dithioerythritol to final concentrations of 2%, 0.01%, 0.05 mmol/L, 2%, and 20 mmol/L, respectively, and then boiled at 100°C for 5 minutes. Proteins were then electrophoresed, electroblotted onto nitrocellulose, and revealed by immunoblotting. Fragments of apo(a) were revealed by using a peroxidase-conjugated polyclonal anti-apo(a) antibody20 or the monoclonal antibody 21D5C6.18 Revelation was performed by enhanced chemiluminescence (ECL, Amersham).

In Vitro Proteolysis of Apo(a) by Collagenase
To compare the in vivo and in vitro patterns of apo(a) fragmentation, we used collagenase, a protease known to cleave apo(a) between kringle IV-4 and IV-5 and to generate a pattern similar to that for apo(a) fragmentation in vivo.21 Limited proteolysis was performed on Lp(a) purified on heparin-Sepharose to eliminate apo(a) fragments present in vivo. Limited proteolysis with collagenase (from Clostridium histolyticum, 0.1 U/mg of lyophilized, Boehringer-Mannheim) was carried out at room temperature by incubating 100 ng Lp(a) in 125 mmol/L Tris-HCl, 150 mmol/L NaCl, 4 mmol/L CaCl2, and 2.4 mmol/L Pefabloc (Boehringer-Mannheim) at pH 7.8 at a mass ratio of enzyme to substrate of 1:5. The reaction was stopped by boiling the samples with the loading buffer of SDS-PAGE for 5 minutes. Samples were then submitted to electrophoresis and revealed as described above.

Statistical Analysis

Results are expressed as mean±SD. Median values are also given in the case of skewed distributions as seen for Lp(a), apo(a) isoforms, and apo(a) fragments. Statistical analyses were performed with the Statview TM II computer program (Abacus Concepts). A nonparametric Mann-Whitney test was applied to assess significant differences between nonnormally distributed variables in the patient and control groups. A nonparametric Spearman rank correlation test was used to assess correlations between skewed variables. A Student t test was used to compare continuous normally distributed variables. The comparison of percentages or frequencies of events in both groups was performed with a χ2 test. The statistical level of significance was set at P<0.05.

Results

Demographic and Plasma Lipid Data

Table 1 presents demographic parameters and lipid levels in patients and control subjects. Compared with the control subjects, the patients had a similar sex ratio and were slightly older. The mean body mass index in controls and patients was not significantly different. In accordance with our inclusion criteria, compared with control subjects, patients displayed

| Table 1 | Demographic and Plasma Lipid Data |
significantly higher total cholesterol and LDL cholesterol levels (Table 1). By contrast, triglyceride levels were slightly elevated in patients compared with control subjects, although patient levels were within the normal range. HDL cholesterol levels did not differ between patients and control subjects.

**Quantitative Analysis of Lp(a) and Apo(a) Fragments**

Plasma Lp(a) concentrations were significantly higher in patients than in control subjects (Table 2), although the distribution of apo(a) isoforms did not significantly differ between patients and control subjects. Consistent with previous studies, apo(a) isoform sizes were negatively correlated with plasma Lp(a) levels in both groups (Figure 1B and 1C). The apparent fractional excretion rates of urinary apo(a) fragments were calculated (see Methods) but did not differ significantly between both groups (Table 2).

**Molecular Analysis of Apo(a) Fragments**

Immunoblot analysis of plasma apo(a) fragments was performed for all patients and control subjects after purification on heparin-Sepharose. Figure 2 shows the typical patterns obtained after revelation by using a polyclonal anti-apo(a) antibody. The pattern of fragmentation of plasma from subject 1 resembles that described earlier, with apo(a) fragments present as a series of multiple bands ranging from $\approx 60$ to 200 kDa below the intact apo(a) isoform.21 The pattern of apo(a) fragments in plasma of subjects 2 to 6 differed: the same series of fragments as seen for subject 1 was still visible, but a major band was superimposed on these fragments. The molecular mass of this band (arrowed for each subject) varied from one subject to another and was positively correlated with the size of the apo(a) isoform. This major band as well as the other apo(a) fragments could be revealed with the monoclonal 21D5C6 antibody, which is specific for the N-terminal region of apo(a), thereby indicating that these bands represent N-terminal fragments of apo(a) (data not shown). The intensity of the major band did not vary under different reducing conditions (variable concentrations of di-thiothreitol, from 0 up to 100 mmol/L; data not shown).

In an attempt to determine whether the major band was inherited, we performed studies on nuclear families. Figure 3 shows a typical family. The mother (lane 1) carries the major band as do 2 children (lanes 4 and 5), who have inherited the isoform from their mother. The father (lane 2) does not carry the major band, nor does the child who has inherited the corresponding isoform (lane 3). This result indicates that the major band seems to be associated with a specific isoform.

Additional experiments were performed to further characterize the predominant fragment corresponding to the major band. In vitro proteolysis of Lp(a) is known to generate different reducing conditions (variable concentrations of di-thiothreitol, from 0 up to 100 mmol/L; data not shown).

In vitro proteolysis of Lp(a) is known to generate N-terminal and C-terminal apo(a) fragments with the cleavage site situated between kringles IV-4 and IV-5.22 Among the different proteases that can be used to generate the in vitro profile of fragmentation, collagenase yields a pattern that closely resembles that observed in vivo.21 Consequently, we compared the in vivo and in vitro apo(a) fragment patterns for the same plasma sample, with the latter being generated by collagenase. A typical example of such a comparison is given in Figure 4. Collagenase generated a series of fragments (Figure 4, lane C), which were fully visible when revealed with an antibody specific for the N-terminal region of apo(a) (21D5C6). Within the series of N-terminal fragments present in the in vitro collagenase-derived pattern, a major band was visible, but this band was larger than the in vivo counterpart. Comparison of the migration of the major bands in the in vivo and in vitro patterns revealed that the major band present in

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**Table 1. Demographic Parameters and Lipid Levels in Normolipidemic Control Subjects and Hypercholesterolemic Patients**

<table>
<thead>
<tr>
<th></th>
<th>Control Subjects</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex ratio (female/male), n</td>
<td>53/37</td>
<td>55/27</td>
</tr>
<tr>
<td>Age, y</td>
<td>45.2±5.1</td>
<td>48.4±12.3*</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.9±3.7</td>
<td>23.6±3.4</td>
</tr>
<tr>
<td>Total Chol, mg/dL</td>
<td>204±24</td>
<td>292±40†</td>
</tr>
<tr>
<td>LDL Chol, mg/dL</td>
<td>121±22</td>
<td>209±38†</td>
</tr>
<tr>
<td>HDL Chol, mg/dL</td>
<td>65±18</td>
<td>63±19</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>90±38</td>
<td>103±36*</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; Chol, cholesterol. Values are mean±SD. *P<0.05 and †P<0.001 vs control subjects.

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**Table 2. Concentrations of Lp(a) and Apo(a) Fragments in Plasma and Urine of Normolipidemic Control Subjects and Hypercholesterolemic Patients**

<table>
<thead>
<tr>
<th></th>
<th>Control Subjects</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lp(a), mg/mL</td>
<td>0.24±0.1 (0.11)</td>
<td>0.35±0.4* (0.13)</td>
</tr>
<tr>
<td>Apo(a) isoform size, nb of kringles</td>
<td>26±4 (28)</td>
<td>27±5 (27)</td>
</tr>
<tr>
<td>Plasma apo(a) fragments, μg/mL</td>
<td>2.15±2.57 (1.17)</td>
<td>4.97±5.51† (2.85)</td>
</tr>
<tr>
<td>Apo(a) fragments/Lp(a), %</td>
<td>1.75±2.36 (1)</td>
<td>1.93±1.53 (1.58)</td>
</tr>
<tr>
<td>Urinary apo(a) fragments, ng/mg urinary creatinine</td>
<td>40±57 (17)</td>
<td>75±86‡ (38)</td>
</tr>
<tr>
<td>FE, %</td>
<td>0.26±0.58</td>
<td>0.18±0.13</td>
</tr>
</tbody>
</table>

nb indicates number; FE, fractional urinary excretion rate. Values are mean±SD. Median values are in parentheses.

*P<0.05, †P<0.001, and ‡P<0.01 vs control subjects.
vivo was always 2 kringle smaller than its in vitro counterpart, irrespective of the size of the apo(a) isoform. Because the in vitro collagenase-cleavage site is situated between kringle IV-4 and IV-5, this result suggests that in vivo proteolytic generation of the major fragment in the apo(a) in vivo pattern is likely to occur between kringle IV-2 and IV-3. This region represents the frontier between the repeated kringle present in variable number (IV-2) and the kringle present as unique copies (IV-3 to IV-10).

Subsequently, we classified controls and patients into 2 groups according to the presence or absence of the major fragment band of apo(a) in plasma. The frequency of this band did not significantly differ between patients and controls (28% and 26% in patients and controls, respectively). We also examined the distribution of the major fragment band in relation to apo(a) isoform size. The frequency of null alleles and of single and double isoforms did not differ between controls and patients (8.9% and 7.4%, 23.3% and 14.6%, and 67.8% and 78%, respectively). The frequency of the major band did not differ between carriers of single or double apo(a) isoforms. By contrast, the presence of a major band was associated with short apo(a) isoforms (25.6 ± 4 versus 28 ± 4 kringles, $P = 0.0001$). By use of the median apo(a) isoform size as a cutoff, the frequency of the major band was significantly different below and above this cutoff (46.8% and 6.3%, respectively). Nonetheless, Lp(a) and apo(a) fragments levels did not differ with respect to the presence or absence of this band.
The patterns of urinary apo(a) fragments were also determined for all subjects. In contrast to patterns for apo(a) fragments in plasma, those for urinary apo(a) fragments were similar in all subjects and resembled those described earlier (data not shown).8

Discussion

In the present study, we demonstrate for the first time that elevated Lp(a) levels found in hypercholesterolemic patients, compared with those found in normolipemic subjects, are associated with high levels of apo(a) fragments in plasma and urine, although the fractional excretion rate of apo(a) fragments was similar in both groups. In addition, we have identified a new pattern of degradation of apo(a) in plasma, which is preferentially associated with small apo(a) isoforms.

In keeping with previous studies,13,14 we found elevated Lp(a) levels in our population of PH patients. We speculate that the major genetic factors known to influence Lp(a) levels can be excluded. Our population consisted of PH patients; therefore, the influence of an LDL receptor defect is unlikely. It should be noticed that in the case of FH patients, a role of protease(s) has been suggested; therefore, the involvement of metalloproteases such as MMP-2 is possible. However, it has been shown that elevated amounts of circulating elastase released in acute situations, such as cardiopulmonary bypass, are not associated with increased apo(a) fragmentation.29 It should be pointed out that in the former study, Lp(a) concentrations were not increased, in contrast to our observations in the present study. Nevertheless, the involvement of elastase in apo(a) fragmentation cannot be completely ruled out, because it can act at the cell surface rather than in plasma. For example, matrix metalloproteinase-12 is secreted by monocytes/macrophages and could be active at the surface of these cells.30 Another possibility would be the presence of proteases in the kidney, which could produce apo(a) fragments, as suggested by Kronenberg et al.12

We also characterized for the first time a new pattern of apo(a) fragmentation in plasma. The typical pattern of apo(a) fragmentation in plasma consists of a series of multiple bands whose size ranged from 60 to 200 kDa less than that of the intact apo(a) isofrom.3 The new pattern consisted of a similar series of bands on which a major band was superimposed. This band was variable in size from one individual to the other and was related to apo(a) isoform size. Compared with subjects with the typical fragmentation pattern, subjects with an apo(a) pattern characterized by a major band possessed smaller apo(a) isoforms but did not have different Lp(a) levels. The frequency of the fragmentation pattern displaying a predominant band did not differ between patients and control subjects (28% and 26%, respectively), indicating that the presence of the major band is not associated with hypercholesterolemia. On the basis of the comparison between the migration of the major band in the in vivo and in vitro patterns, we suggest that the site of preferential cleavage generating this major band may be located between kringle IV-2 and IV-3. This region is situated between kringle IV-2 present in variable number and the other kringle (IV-3 to 10) present as unique copies. One possible explanation for the generation of the major band would be the presence of a polymorphism at the genomic level, creating a site sensitive to proteolytic cleavage. This hypothesis was reinforced by the fact that family studies (Figure 3) have shown that the major apo(a) band was inherited. We tried to verify this hypothesis, but this region is highly repeated, and despite intensive efforts, we were not able to determine whether a poly-
morphism at the genomic level could explain the occurrence of the major band. Therefore, the mechanisms underlying the presence of the predominant apo(a) fragment remain to be determined.

Interestingly, the major band was never observed in urine, suggesting 2 different hypotheses: (1) Plasma apo(a) fragments are processed in the kidney by proteolytic enzymes erasing the plasma pattern of fragments; indeed the patterns of fragments in urine are constant, with urinary fragments always being smaller than their counterparts in plasma (whose size ranged from ~40 to 200 kDa). (2) Apo(a) urinary fragments may be produced by the kidney from intact Lp(a), independent of apo(a) fragments in plasma. However, the relative clearance of fragments in urine is highly correlated with plasma Lp(a) and Lp(a) levels, thereby indicating a possible relationship between plasma and urine concentrations of apo(a) fragments.

In summary, we demonstrate for the first time that increased plasma Lp(a) levels in PH patients are associated with elevated levels of apo(a) fragments in plasma and urine. The increase of apo(a) fragments in plasma is not due to a decreased renal clearance of apo(a) fragments. Furthermore, a new pattern of apo(a) fragmentation that is associated with small apo(a) isoforms has been identified. Further studies are needed to determine whether this pattern could constitute a new marker for cardiovascular risk.

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References

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