RAPID COMMUNICATION

Does Apolipoprotein(a) Heterogeneity Influence Lipoprotein(a) Effects on Fibrinolysis?

By Laurence Hervio, M. John Chapman, Joëlle Thillet, Stéphane Loyau, and Eduardo Anglés-Cano

High plasma levels of lipoprotein(a) [Lp(a)] are considered to be an independent risk factor for premature cardiovascular disease and are inversely associated with apolipoprotein(a) [apo(a)] isoform sizes. The contribution of apo(a) polymorphism to the inhibition of fibrinolysis, a mechanism that may favor thrombus development, was therefore evaluated by measuring the ability of Lp(a) particles of distinct apo(a) isoform content to compete with plasminogen for fibrin binding during plasmin activation by fibrin-bound tissue-type plasminogen activator. The rate of plasmin generation was most efficiently inhibited by an isoform with a molecular weight (Mr) of ~540 Kd. An isoform with Mr ~590 Kd produced a less pronounced effect, whereas the isoform with Mr ~610 Kd failed to inhibit plasminogen activation. These effects were directly proportional to the amount of Lp(a) bound to the carboxy-terminal lysine residues of degraded fibrin. The relative affinity of the binding (kd range, 16 to 180 nmol/L) reflected the ability of individual Lp(a) isoforms to inhibit the binding of plasminogen (kd, 600 nmol/L). The question of the influence of kringle sequence variability on the binding to fibrin was not addressed by the present work. These data suggest that apo(a) isoform types with high affinity for fibrin may influence the ability of Lp(a) to interfere with fibrinolysis and contribute thereby to the association of elevated levels of Lp(a) with atherosclerotic and thrombotic risks.

Lipoprotein(a) [Lp(a)] is now recognized as an independent risk factor linking the pathophysiological processes of atherosclerosis and thrombosis in coronary artery disease. Lp(a) is distinct from low-density lipoprotein (LDL) particles in its content of a unique apolipoprotein, apo(a). The cDNA-derived sequence of apo(a) shows exceptional homology with plasminogen, the zymogen of the fibrinolytic enzyme plasmin. Indeed, multiple triple-loop, disulfide-bridged structures exhibiting up to 75% homology with that of plasminogen kringle 4 are present in apo(a). The number of such kringles may vary from approximately 15 to 40, giving rise to a series of apo(a) isoforms of variable size. In addition to the possession of kringle 4-like repeats, apo(a) also contains a single copy of plasminogen kringle 5 and a protease region that shares 94% homology with the corresponding domain of plasminogen, including the catalytic triad His-Asp-Ser. However, an Arg→Ser mutation in apo(a) at the peptide bond corresponding to the activation cleavage site of plasminogen (Arg$_{561}$→Val$_{562}$) impairs the generation of plasmin-like activity by activators. Cleavage of the Arg$_{561}$→Val$_{562}$ bond in plasminogen by the tissue-type plasminogen activator (t-PA) leads to the generation of plasmin at the surface of blood clots upon binding of plasminogen to fibrin. Such binding is mediated by lysine-fibrin binding subsites that have been localized to kringles 1 and 4 of plasminogen. Because apo(a) and plasminogen share kringle structure homology, Lp(a) may compete with plasminogen for fibrin binding. Indeed, Harpe et al. have shown that Lp(a) inhibits the binding of Glu-plasminogen to plasmin-modified immobilized fibrinogen. In a recent study we established that Lp(a) binds to the fibrin surface and thereby competes with plasminogen so as to inhibit its activation. Similar results have been reported by other groups. Such unique behavior was recently shown to rely on the fibrin-binding properties conferred by the kringle 4 repeats of apo(a). The number of such kringles and thereby the apo(a) isoform size are inversely related with Lp(a) levels. However, a relationship with their effect on fibrinolysis has not as yet been established. To gain insight into this question, we evaluated the interaction of Lp(a) particles of distinct apo(a) isoform content with plasminogen at the fibrin surface. We presently demonstrate that Lp(a) particles containing a single, distinct isoform of apo(a) may have different functional properties with regard to their affinity for fibrin. These findings prompt the hypothesis that determination not only of the absolute circulating concentration of Lp(a), but also of the functional heterogeneity of the apo(a) isoforms are primordial for the evaluation of thrombotic risk.

MATERIALS AND METHODS

Subjects and blood samples. Study participants were individuals attending the Lipid Clinic of The Endocrinology-Metabolism Service (Hôpital de la Pitié, Paris, France). Patients with high levels of Lp(a) (>50 mg/dL) were detected either during a diagnostic examination or during follow-up studies for hyperlipoproteinemia. They had no antecedents of thromboembolic disease.Venous blood was drawn from the forearm into sterile polypropylene tubes containing ethylenediaminetetraacetic acid (EDTA; 3 mmol/L, final concentration). Samples were immediately centrifuged at 392

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2,000 g for 20 minutes at 4°C. The plasma was removed by aspiration and the following components were immediately added: 0.01% NaN₃, 0.01% EDTA, and 2 mmol/L phenylmethylsulphonyl fluoride. The concentration of Lp(a) in plasma was determined by an immunonephelometric method, as detailed earlier.³ The assay was performed with a sheep antisera to Lp(a) (ImmuNo AG, Vienna, Austria) and a reference standard in which the Lp(a) mass content had been previously determined by electroimmunodiffusion (Immuno AG). This assay was linear over the range of 0 to 100 mg/dL.

Lp(a) purification. After sampling, each plasma was immediately adjusted to a solvent density of 1.050 g/mL with KBr and centrifuged at 150,000g for 24 hours at 10°C. The floating lipoproteins were removed; the infranatant was then adjusted with KBr to a density of 1.100 g/mL and recentrifuged under similar conditions for 48 hours. The 1.050 to 1.100 g/mL density fraction was removed and subjected to gel filtration on a Biogel A5M (2 x 90 cm) (BioRad, Richmond, CA) column equilibrated with 0.05 mol/L phosphate buffer at pH 7.4. The peak fractions containing Lp(a) were pooled, concentrated against Aquacide (50 IU/mL) and dialyzed against the same buffer containing 0.1 mol/L NaCl and 0.01% NaN₃. The final product was conserved at 4°C in this state until use (usually less than 24 hours). The purity of each sample was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed on 3.75% polyacryl-

amidewater level absorbance ratio (405/490 nm) with a microtitration plate counter (MR5000; Dynatech) as previously described.²⁰ After activation, the plate was washed and the surface was probed with a 125I-labeled polyclonal sheep antibody directed against apo(a) and prepared as described.⁸ The radioactivity bound in the absence of Lp(a) was extremely low, indicating that cross-reactivity of this antibody with plasminogen was negligible. The radioactivity bound was transformed into mass of protein using a standard curve prepared with varying concentrations of each Lp(a) preparation in a dot immunobinding assay and the relationship of IgG anti-apo(a) = HF[125I](Na), as published elsewhere.⁸

Binding of Lp(a) to fibrin surfaces. Varying concentrations of the purified Lp(a) isoforms in assay buffer containing 4% bovine serum albumin were incubated under equilibrium conditions with intact or degraded fibrin surfaces. After 18 hours at 4°C, unbound Lp(a) was removed by three washes with assay buffer. A constant amount of 125I-labeled sheep antibody directed against apo(a) was then added and incubated for 2 hours at 37°C, excess antibody was removed by washing, and the bound radioactivity counted in a gamma-radiation counter. Bound radioactivity was transformed into the mass of protein as indicated above. Binding of Lp(a) to the fibrin surfaces in the presence of 0.2 mol/L 6-aminoheptanonic acid was considered as unrelated to interactions with lysine residues and represented less than 10% of the total binding.

RESULTS AND DISCUSSION

The length polymorphism of apo(a) is due to allelic differences in the number of sequences encoding kringel 4.22 The influence of this variability on the anti-fibrinolytic effect of Lp(a) is indeterminate. Our aim was therefore to investigate if this isoform heterogeneity, as characterized by electrophoretic mobility, may be related to distinct effects of Lp(a) on fibrinolysis. Most of the plasmas (n = 12) with high Lp(a) content (>50 mg/dL) that were screened for their ability to inhibit plasminogen activation contained two apo(a) isoforms, as determined by SDS-PAGE electrophoresis and immunoblotting. Indeed, heterozygocity may be as high as 94% when detected by apo(a) gene size determination.²³ Because of the difficulty of evaluating the contributions of each of the two apo(a) isoforms per individual, we selected three distinct plasma samples [I, Lp(a) 120 mg/dL; II, Lp(a) 82 mg/dL; III, Lp(a) 72 mg/dL], each of which contained a unique isoform. Lp(a) preparations were purified from these plasmas; their chemical compositions markedly resembled each other and those described earlier.²⁴ The electrophoretic migration of these isoforms corresponded approximately to those of apo(a) isoforms B, S2, and S3, as denoted by Utermann et al.⁴ However, as we did not use either the same methodology or the same electrophoretic
Fig 1. Analysis of apo(a) isoform content in purified Lp(a) preparations isolated from single donors. (A) Immunoblot analysis. Lp(a) preparations were treated with dithiothreitol, boiled for 6 minutes, and analyzed by SDS-PAGE with the use of 3.75% polyacrylamide/0.6% agarose gels (amount of protein loaded: 3 μg for isoforms I and II, 1.5 μg for isoform III). The protein bands were transferred to nitrocellulose membranes and the isoforms localized with an MoAb directed against apo(a) followed by a peroxidase-conjugated antibody to mouse IgG. (B) Silver stain gel. Lp(a) preparations (1.5 μg of each isoform) were treated with dithiothreitol, boiled for 6 minutes, and analyzed by SDS-PAGE with the use of 3.75% polyacrylamide/0.8% agarose gels. After silver staining of the gels, molecular weight of isoforms were determined from the relative migration of the bands using apoB-100 and cross-linked phosphorylase b (Sigma) as reference markers. The apparent molecular weights of apo(a) isoforms were 540 Kd for isoform I, 590 Kd for isoform II, and 610 Kd for isoform III; the arrow indicates the migration of apoB-100 (Mr 540 Kd).

standards, and because as many as 23 different polymorphs may occur,25 these isoforms were designated with roman numerals. Their apparent size, as calculated by reference to cross-linked phosphorylase b and apo B-100, was 540 Kd for apo(a) isoform I, 590 Kd for isoform II, and 610 Kd for isoform III (Fig 1). These apparent molecular masses are indicative of their differences in size but do not allow a precise calculation of their exact number of kringle.

Relationship between apo(a) isoform and Lp(a) inhibition of plasminogen activation by fibrin-bound t-PA. In confirmation of our earlier findings8 and those of others,9-11 variation in the level of inhibition of plasmin generation was observed in the present studies with plasma samples from different subjects (data not shown). The presence of two different isoforms in the majority of these plasmas did not allow us to establish a correlation between isoform type and their effect on fibrinolysis. Therefore, to evaluate the effect of individual apo(a) isoforms on plasminogen activation, we determined the ability of purified Lp(a) preparations containing a unique isoform to compete with plasminogen for binding to a solid-phase fibrin surface to which t-PA was bound. This system constitutes a useful model for plasminogen activation experiments,21 allowing for quantitation of the nature of the adsorbed molecules during the reaction. An additional advantage is that the Lp(a) bound can be detected on the same surface at which the binding of plasminogen and the generation of plasmin were impaired. This experimental model was used to determine the generation of plasmin in the absence and in the presence of native Lp(a) particles, which have been denoted as a function of their apo(a) isoform content. In the absence of Lp(a), the rate of plasmin generation at a saturating concentration of plasminogen was proportional to the amount of fibrin-
Correlation between the binding of Lp(a) to fibrin and plasmin generation. After plasminogen activation for 60 minutes at 37°C in the presence of increasing concentrations of Lp(a) (conditions as indicated in Fig 2), the binding of this particle to fibrin was measured with a radiolabeled polyclonal antibody against apo(a). Inhibition of the initial rate of plasmin generation, \(1 - (V_1/V_0)\), relative to a control without added Lp(a) \((V_0)\) is plotted as a function of the amount of Lp(a) isoform I bound using the equation \(1 - (V_1/V_0) = f(\text{bound Lp(a)})\). A direct correlation \(r = .98\) between the amount of Lp(a) bound and the inhibition of plasmin generation at the fibrin surface was observed. The correlation for isoform II \(r = .87\) was less pronounced, whereas isoform III was without effect (data not shown).

Fig 3. Correlation between the binding of Lp(a) to fibrin and plasmin generation. After plasminogen activation for 60 minutes at 37°C in the presence of increasing concentrations of Lp(a) (conditions as indicated in Fig 2), the binding of this particle to fibrin was measured with a radiolabeled polyclonal antibody against apo(a). Inhibition of the initial rate of plasmin generation, \(1 - (V_1/V_0)\), in the presence of Lp(a) \((V1)\) relative to a control without added Lp(a) \((V0)\) is plotted as a function of the amount of Lp(a) isoform I bound using the equation \(1 - (V1/V0) = f(\text{bound Lp(a)})\). A direct correlation \(r = .98\) between the amount of Lp(a) bound and the inhibition of plasmin generation at the fibrin surface was observed. The correlation for isoform II \(r = .87\) was less pronounced, whereas isoform III was without effect (data not shown).

Characteristics of the binding of Lp(a) to fibrin surfaces. The evaluation of the direct binding of Lp(a) isoforms to the intact fibrin surface and to plasmin-degraded fibrin, an equivalent of the fibrin surface after plasminogen activation, showed that isoform III did not bind significantly to the fibrin surface. In contrast, the binding of both the I and the II isoforms (Fig 4) was specific for fibrin lysine residues (the binding was inhibited with 6-aminohexanoic acid, a lysine-analogue), approached saturation, and obeyed the Langmuir equation:

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[Fn \cdot \text{Lp(a)}] = [Fn_o][K[\text{Lp(a)}]/1 + K[\text{Lp(a)}])
\]

for adsorption at interfaces, indicating single-site binding and a single association-dissociation mechanism. The equilibrium dissociation constants \((kd)\) were therefore derived from the linearized expression of the Langmuir equation, as

Fig 4. Isotherms for the direct binding of Lp(a) isoforms I and II to fibrin surfaces. Various concentrations of each Lp(a) isoform were incubated with intact (●) and degraded fibrin surfaces (○). Bound Lp(a) was detected with a radiolabeled polyclonal antibody directed against apo(a). The specific binding was calculated by subtracting the radioactivity bound in the presence of 0.2 mol/L 6-aminohexanoic acid from the total radioactivity bound. The specific radioactivity bound was transformed into protein mass of Lp(a) as indicated in Materials and Methods. Data (triplicate assays for each isoform) were fitted to the Langmuir equation and the dissociation constant \((kd)\) and maximum bound \((B_{max})\) were determined. (A) Binding of isoform I to fibrin \((kd = 16 \text{ nmol/L}; B_{max} = 5.6 \text{ fmol/well})\) and to degraded fibrin \((kd = 24 \text{ nmol/L}; B_{max} = 43 \text{ fmol/well})\). (B) Binding of isoform II to fibrin \((kd = 180 \text{ nmol/L}; B_{max} = 3 \text{ fmol/well})\) and to degraded fibrin \((kd = 189 \text{ nmol/L}; B_{max} = 9 \text{ fmol/well})\).
previously described. The kd for isoform I was 16 nmol/L, and for isoform II was 180 nmol/L; the corresponding values for binding to degraded fibrin were similar (24 and 189 nmol/L, respectively). These results indicate that isoform I interacted with plasminogen binding sites on fibrin with higher affinity than isoform II. Indeed, the binding constant of plasminogen to intact fibrin (kd = 990 nmol/L) and to plasmin-degraded fibrin (kd = 660 nmol/L) is higher than the corresponding values for isoforms I and II (see below), indicating that some Lp(a) isoforms interact with fibrin with an affinity that is even higher than that of plasminogen. For plasminogen, an increase in the specific binding of isoforms I (~8-fold) and II (~3-fold) was observed after plasmin digestion of the intact fibrin surface; such binding was markedly decreased by treatment of the degraded surface with carboxypeptidase B, an exopeptidase that specifically releases carboxy-terminal lysine. These results indicate that the increase in the fibrin binding of isoforms I and II during plasminogen activation was due to the unveiling of new carboxy-terminal lysine residues. The interactions of such residues with the kringle domains of apo(a) has been previously shown. In the present study, isoform I showed the highest affinity for fibrin and is the most effective inhibitor of plasminogen activation. Its affinity for fibrin and its molecular mass are similar to those of a recombinant molecular form of apo(a) having 17 kringle 4 copies, which had similar kd values (26 nmol/L for fibrin and 8 nmol/L for degraded fibrin), as previously reported.

The characteristics of the specific binding of Lp(a) isoforms I and II to the fibrin surface closely resemble those of plasminogen and are consistent with a similar mechanism of interaction. Because the main structural difference between the isoforms almost certainly reflects a variable number of kringle 4-like repeats that are known to mediate plasminogen binding to fibrin, it is reasonable to propose that the inhibition exerted by isoforms I and II arises from interaction of their kringle 4 structures with the fibrin surface. Among the multiple kringle 4-like repeats of the single isoform of apo(a) sequenced to date, only that at position 37 appears to contain a lysine-fibrin binding subsite similar to that present in plasminogen. The structure of this subsite, a hydrophobic trough harboring an anionic/cationic dipole, has been well defined. Whether this subsite is common to other apo(a) isoforms or whether heterogeneity exists in the structure of lysine-binding subsites between individual isoforms is indeterminate. Scnan et al have recently proposed that point mutations in kringle 4 at position 37 may occur in humans and that phenotypes with the same number of kringle 4 repeats may be functionally different in terms of their thrombogenic potential. The determination of sequence variability of apo(a) isoforms in a large number of samples would be necessary to clarify this point; this question was beyond the aim of the present study. Furthermore, because the structure/function relationship of a protein is governed by interactions between its different structural domains, sequence variability may not necessarily result in an altered function. For instance, cofacial kringle-kringle interaction of the binding site regions in neighboring kringle structures has been described, and may well occur in isoforms with an elevated number of kringles, thereby accounting for the inaccessibility of a specific lysine binding subsite in apo(a) for fibrin. The high-affinity fibrin binding of a recombinant apo(a) isoform containing 17 kringle 4-like repeats and the absence of binding of the 37 kringle 4-like isoform from which it derived support this hypothesis. Furthermore, efficient inhibition of the binding of recombinant apo(a) to fibrin was obtained only with the elastase-derived plasminogen fragment kringle 1 + 2 + 3 and not with the isolated plasminogen kringle 4; this finding suggests the existence of potential contributions from adjacent kringles and underscores the possible role of variations in the number of kringles with regard to fibrin binding.

The level of circulating Lp(a) has been shown to be inversely correlated with the size of apo(a) protein and gene. High levels of circulating Lp(a) (>0.3 mg/mL) and small size apo(a) phenotype have been shown to be associated with coronary artery disease. The present results suggest that inhibition of plasminogen activation, a mechanism that impairs fibrinolysis, may also be correlated with isoform heterogeneity with regard to fibrin binding. Consequently, association of high Lp(a) levels and isoforms with high affinity for fibrin could result in a significant amplification of atherosclerotic and thrombotic risks.

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