Lipoprotein(a), Fibrin Binding, and Plasminogen Activation
Joseph Loscalzo, Mark Weinfeld, Gunther M. Fless, and Angelo M. Scanu

Lipoprotein(a) (Lp[a]) is a complex plasma lipoprotein in which apolipoprotein (apo) B-100 is covalently linked by a disulfide bridge to a unique apolipoprotein, apo(a). The cDNA of apo(a) has recently been isolated and sequenced, and a remarkable homology to human plasminogen has been noted. In this report, we demonstrate that, like plasminogen, Lp(a) binds to fibrin. In addition, Lp(a) competes with plasminogen and tissue-type plasminogen activator for fibrin binding. As a functional consequence of these binding properties, we show that Lp(a) attenuates the fibrin-dependent enhancement of tissue-type plasminogen activator activity against the native substrate, and does so as an uncompetitive inhibitor (Kᵢ = 15 nM). Finally, we show that in a plasma milieu, Lp(a) attenuates clot lysis induced by tissue-type plasminogen activator. None of these effects was noted with low density lipoprotein free of apo(a). These data suggest that Lp(a) influences the fibrinolytic system and probably does so by virtue of the fibrin binding properties conferred by the kringle repeats of apo(a). (Arteriosclerosis 10:240–245, March/April 1990)

Lipoprotein(a) (Lp[a]) is a plasma lipoprotein first described by Berg.1 When present in high concentrations in plasma, Lp(a) correlates strongly with an increased risk for coronary artery disease.2–5 Lp(a) is comprised of low density lipoprotein in which apolipoprotein (apo) B-100 is covalently linked through a disulfide bridge to a unique apolipoprotein, apo(a). While much information on the structure of Lp(a) has been obtained6–8 and the cDNA sequence of apo(a) has been determined,9 the actual function of Lp(a) and the mechanism of its atherogenicity remain to be defined.

The primary sequence of apo(a) has a striking similarity to plasminogen9; it contains: a serine protease domain that is 94% homologous with that of plasminogen, one copy of the kringle-5 region, and 37 copies of the kringle-4 domain. The structural homology to the serine protease active site of plasminogen notwithstanding, no latent enzymatic activity can be generated because of a crucial substitution of serine for arginine at the homologous activation site domain.9 In the coagulation and fibrinolytic molecules in which they were first described, the kringle domains were identified as lysine-dependent fibrin binding regions. Plasminogen contains five kringles of which the first has the greatest binding affinity,10 the second and third provide weaker affinity sites, and the fourth contains a site of intermediate affinity.11,12,13

There has been much speculation in the literature about the possibility that Lp(a) can interfere with the fibrinolytic system because of the structural similarities with plasminogen9,14; however, to date little has been published to support this hypothesis.16 In this report, we demonstrate that Lp(a) binds to fibrin, competes with both plasminogen and tissue-type plasminogen activator (t-PA) for fibrin binding sites, and attenuates the fibrin-dependent enhancement of the plasminogen activator activity of t-PA in buffer and plasma.

Methods

Materials
Human fibrinogen and S-2251 were purchased from Kabl Vitrum, Stockholm, Sweden. Matrex Pel 102 beads were obtained from Amicon, Danvers, MA. Plasminogen-free bovine thrombin was purchased from Miles Pharmaceuticals, Naperville, IL. Iodine monochloride was obtained from Kodak Chemical, Rochester, NY. t-PA was obtained from Genentech, South San Francisco, CA. L-Glycyl-L-prolyl-L-arginyl-L-proline (GPRP), epsilon-aminocaprolc acid, aprotinin, and tranexamic acid were obtained from Sigma Chemical, St Louis, MO. Lysine-Sepharose and Sephadex G-25 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Iodo-beads were obtained from Pierce Chemical, Rockford, IL. Na¹²⁵I and Na¹³¹I were obtained from Amersham, Arlington Heights, IL. All other chemicals were reagent grade or better. Deionized water was used throughout.

Isolation of Low Density Lipoprotein and Lp(a)
Low density lipoprotein (LDL) was prepared from the plasma of fasting normolipidemic volunteers by sequential ultracentrifugation as previously described.16 Poly-
acylamide gel electrophoresis was performed to ensure purity of the LDL preparation. Lp(a) was prepared from blood drawn into sterile bottles that were immersed in wet ice and contained a final concentration of 0.15% ethylenediaminetetraacetic acid (EDTA), 0.01% NaNO₃, and 0.4 μM soybean trypsin inhibitor. Plasma was separated immediately by low speed centrifugation at 4°C, and disopropylfluorophosphate was added to a final concentration of 1 mM to minimize proteolysis. Total lipoproteins were then prepared by adjusting the plasma density to 1.21 g/ml with solid NaBr and centrifuging the sample in a 60 Ti rotor at 59 000 rpm for 20 hours at 15°C. Lp(a) was isolated from the total lipoprotein fraction by using a combination of rate zonal and density gradient ultracentrifugation as previously described. Lp(a) preparations were checked for purity by sodium dodecyl sulfate polyacrylamide gradient-gel electrophoresis. If necessary, further purification was conducted by high performance liquid chromatography (HPLC)-ion exchange chromatography by using a mono-Q column (Pharmacia, Uppsala, Sweden). The sample load varied from 1 to 10 mg. Lp(a) was eluted with a 0 to 1 M NaCl gradient in 0.01 M Tris buffer (pH 7.4) at a flow rate of 1 ml/min at 8°C performed over 40 minutes. Lp(a) eluted at 0.41 M NaCl. The purity of isolated Lp(a) was again checked electrophoretically as described above.

The Lp(a) preparation used in the experiments presented here has two apo(a) subunits, each having a molecular weight of 280 000 daltons, of which 200 000 daltons represents protein and 80 000 daltons represents carbohydrate. Since the molecular weight of apo B devoid of carbohydrate is 514 000 daltons, the Mₚ of the whole protein moiety of Lp(a) is 914 000 daltons. The substructure of apo(a) can be characterized from the cDNA sequence; from this analysis, each apo(a) subunit of Lp(a) contains one protease domain (Mₚ=24 800), one kringle-5 domain (Mₚ=11 300), and 13 kringle-4 domains (Mₚ=12 600 each).

**Plasminogen Preparation**

Glu-plasminogen was purified from freshly obtained plasma or fresh frozen plasma thawed at 37°C using a modification of the method of Deutsch and Mertz with slight modification. Plasma was passed over a lysine-Sepharose column, and the column was washed with 0.3 M sodium phosphate, pH 7.4, 3 mM EDTA, and 250 U/ml aprotinin. Plasminogen was eluted from the column with 0.2 M of epsilon-aminocaproic acid, 3 mM of EDTA (pH 7.4), and 250 U/ml aprotinin. The plasminogen obtained from the donors was free of contaminant Lp(a) and was dialyzed before use against 10 mM sodium phosphate, pH 7.4, 0.15 M NaCl.

**Radioiodination**

Glu-plasminogen, t-PA, and fibrinogen were radioiodinated using lodo-beads. One lodo-bead was preincubated with 0.5 to 1.0 mCi Na¹²⁵I for 10 minutes at 25°C, after which 1 ml of 0.1 mg/ml glu-plasminogen, fibrinogen, or t-PA was added. The incubation was allowed to proceed for 12 to 15 minutes with gentle rocking, after which the solution was removed from the lodo-bead to stop the iodination reaction and was passed over a Sephadex G-25 column that had been pre-equilibrated with 1 ml of 5 mg/ml bovine serum albumin in 10 mM Tris (pH 7.8) 0.15 M NaCl. Twelve 0.3-ml fractions were collected and assayed for total and 25%-trichloroacetic acid-precipitable radioactivity. Routinely, column fractions five through eight contained maximal protein-bound counts with a specific activity of approximately 0.1 to 0.2 mCi/mg. These fractions were either used immediately or stored for up to 2 weeks at 4°C without any appreciable loss of protein-bound counts. Importantly, radioiodination of t-PA by the gentle method outlined here had little effect on its activity as a plasminogen activator (specific activity was 92% of native, uniodinated control); in addition, its fibrin binding properties were unaffected by iodination, as indicated by equivalent competition between either uniodinated t-PA and ¹²⁵I-t-PA or ¹³¹I-t-PA and ¹²⁵I-t-PA for binding to fibrin monomer on polycrylonitrile beads (see Binding Assays below).

Lp(a) and LDL were radioiodinated by the iodine monochloride method of McFarlane with modifications. Lp(a) and LDL-bound radioiodine was 97% precipitable with trichloroacetic acid; Lp(a) had a specific activity of 600 to 900 cpm/ng protein, while LDL had a specific activity of 300 to 800 cpm/ng protein.

**Soluble Fibrin Monomer and Matrex Bead Preparations**

Soluble fibrin monomer (SFM) and fibrin monomer-immobilized Matrex beads (FM-Matrex) were prepared as described previously.

**Enzymatic Activity Assays**

The t-PA activity was assayed using the native substrate glu-plasminogen in which the plasmin-specific substrate S-2251 was used to follow the reaction. The substrate hydrolysis was measured spectrophotometrically with a Gilford Response UV/Vis spectrophotometer (Ciba-Cominig, Oberlin, OH). Activity was measured at 37°C in 10 mM Tris (pH 7.8) 0.15 M NaCl. The change in optical density was followed for 5 minutes, and the initial reaction velocity was determined from plots of change in absorbance/time versus time, as described previously. Determination of kinetic inhibition constants was performed by measuring the initial reaction velocities as described above in the absence or presence of Lp(a) (53 nM, 99 nM, and 165 nM) over a range of plasminogen concentrations (0 to 3.4 μM). SFM (56 nM), S-2251 (0.8 mM), and t-PA (48 nM) were also included in the reaction solution. Reactions were carried out in 10 mM Tris (pH 7.4), 0.15 M NaCl at 37°C, and the reaction was followed by monitoring the change in absorbance at 405 nm. Numerical analysis was performed as described by Dixon.

**Binding Assays**

The binding of Lp(a) or the competitive binding of Lp(a) with glu-plasminogen or t-PA to fibrin monomer was measured using FM-Matrex prepared as described above. In direct binding assays, increasing concentra-
Protein Determinations were removed at regular intervals for the determination of for 3 hours. Aliquots of the supernatant of the suspension formed clot was measured, and t-PA was added to final extent of clot lysis.

Polyacrylamide Gel Electrophoresis and the apparent molecular weights \( M_r \) and 5% acetic acid and were destained by diffusion. The method of Lowry and colleagues. Osborne \(^{26}\) and modified by Laemmli. \(^{27}\) The gels were stained with Coomassie brilliant blue in 50% methanol and 5% acetic acid and were destained by diffusion. The molecular weight standards were processed similarly, and the apparent molecular weights \( M_r \) were estimated by interpolation.

Protein Determinations

Protein concentrations were determined by the method of Lowry and colleagues. \(^{28}\)

Figure 1. A. Binding of Lp(a) to fibrin monomer. Increasing concentrations of purified \(^{125}\)I-Lp(a) were incubated with 2 \( \times 10^6 \) FM-Matrex beads, on the surface of each of which were bound 4 \( \times 10^5 \) molecules of fibrin monomer, for 30 minutes at 25°C in 10 mM of sodium phosphate (pH 7.4) 0.15 M NaCl. At the end of the incubation period, 0.1 ml of the 0.3-ml total assay volume was washed three times with ice-cold incubation buffer with centrifugation at 8700g, after which the tip of the centrifuge tube was excised, and the radioactivity was counted to measure total binding. Nonspecific binding was determined by adding a 20-fold excess of unlabeled Lp(a) at each concentration examined or 20 \( \mu \)M tranexamic acid. Nonspecific binding accounted for approximately 20% of the total binding observed for Lp(a). Competitive binding assays were performed by incubating FM-Matrex with fixed concentrations of radiiodinated t-PA or glu-plasminogen (at 2.5 \( \times \) their estimated, apparent \( K_D \)) and increasing concentrations of Lp(a). The amount of radiiodinated ligand bound at any concentration of Lp(a) was compared to that bound in the absence of Lp(a) and plotted as a ratio \( \text{B} / \text{Bo} \) versus the total Lp(a) added.

Clot Lysis Assay

The effect of Lp(a) on lysis of preformed clots was analyzed by mixing known amounts of purified Lp(a) with plasma that was free of detectable endogenous Lp(a) and contained radiiodinated fibrinogen. Bovine thrombin (0.1 U/ml) was added to this plasma to induce fibrin clot formation, and the plasma was incubated at 22°C for 2 hours, after which the thrombin was inhibited with 1 U/ml of hirudin. The formed, radiolabeled clots were washed twice with 10 mM of sodium phosphate (pH 7.4) 0.15 M NaCl by centrifugation. The final, washed clot was allowed to remain firmly adherent to the bottom of the centrifuge tube, the supernatant was removed, and fresh plasma was added, to which the same concentration of purified Lp(a) was added as in the initial plasma from which the clot was formed. Total radioactivity in the formed clot was measured, and t-PA was added to final concentrations of 13, 26, and 50 nM, after which the assay tube was placed on a rocker at room temperature for 3 hours. Aliquots of the supernatant of the suspension were removed at regular intervals for the determination of extent of clot lysis.

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Weber and Osborne\(^{28}\) and modified by Laemmli.\(^{27}\) The gels were stained with Coomassie brilliant blue in 50% methanol and 5% acetic acid and were destained by diffusion. The molecular weight standards were processed similarly, and the apparent molecular weights \( M_r \) were estimated by interpolation.

Results

Binding of Lp(a) to Fibrin

Purified Lp(a) bound to fibrin monomer covalently linked to an insoluble matrix (Matrex Pel 102), FM-Matrex (Figure 1). Binding was saturable and specific, with an estimated apparent \( K_D \) of 25 nM, and could best be described as mediated by a single class of noncooperatively sites (Figure 1B). Binding was reversible with an excess of unlabeled Lp(a), with tranexamic acid (20 \( \mu \)M), or with soluble fibrin (33 \( \mu \)g/ml). Nonspecific binding (open triangles) accounted for 24% of the total binding (closed circles) at saturation. Time course studies showed that by 20 minutes into the incubation, maximal binding occurred. With approximately 4 \( \times 10^6 \) fibrin monomers/bead and 10\(^{8}\) Matrex beads/assay, 96 ng of Lp(a) protein bound at saturation representing 5.1 \( \times 10^{10} \) molecules of Lp(a), assuming a molecular weight of the total protein moiety of Lp(a).
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Figure 2. Competitive binding of Lp(a) with plasminogen or tissue-type plasminogen activator (t-PA) to fibrin monomer. Increasing concentrations of Lp(a) were incubated with 78 nM 125I-glu-plasminogen (□) or 92 nM 125I-t-PA (△) and 2 × 10⁶ FM-Matrex beads for 30 minutes at 25°C in 10 mM of sodium phosphate (pH 7.4) 0.15 M NaCl. Assay points were processed as described in the legend to Figure 1, and the residual binding of either ligand is expressed as a fraction of the total ligand bound in the absence of Lp(a) (B/Bo). Purified LDL did not displace any significant amounts of plasminogen (•) or t-PA (△) from FM-Matrex over the same range of molar concentrations as Lp(a).

Competitive Binding of Lp(a) with Plasminogen or t-PA to Fibrin

Increasing concentrations of Lp(a) were incubated with 92 nM t-PA or with 78 nM glu-plasminogen and FM-Matrex (Figure 2). Lp(a) effectively competed with plasminogen (open circles), displacing more than 85% of the total bound plasminogen at the highest concentrations examined. The estimated apparent IC₅₀ was approximately 10 nM. Lp(a) was less effective in competing with t-PA (open triangles) than plasminogen. Complete displacement was not achieved over the range of concentrations of t-PA tested with FM-Matrex; however, approximately 40% competitive binding was noted at the highest concentration tested. LDL did not displace plasminogen or t-PA from FM-Matrex over a similar range of concentrations as Lp(a) (closed circles and closed triangles, respectively).

Effect of Lp(a) on t-PA Plasminogen Activator Activity

The effect of Lp(a) on t-PA activity against the native substrate glu-plasminogen was next examined (Figure 3). In these experiments, 48 nM t-PA was incubated with 0.8 mM S-2251, 56 nM SFM, and a range of concentrations of plasminogen (0 to 3.4 μM) in 10 mM Tris (pH 7.4) 0.15 M NaCl at 37°C. The reactions were conducted in the absence (○) or presence of Lp(a) at concentrations of 53 nM (△), 99 nM (□), or 165 nM (●) at 37°C for 5 minutes. The reactions were monitored at 405 nm, and the initial velocities were obtained from plots of absorbance/t versus t. The x-intercepts (1/Kₐapp) of the double reciprocal plots of A are plotted as a function of Lp(a) concentration. The Kₐ is derived from the x-intercept (=-K).

Effect of Lp(a) on Clot Lysis In Plasma

The fraction of clot lysed in plasma containing known amounts of Lp(a) to which three different t-PA concentrations are added is shown in Figure 4. Increasing concentrations of Lp(a) attenuated the fraction of clot lysed by 3 hours from 65% to 53% (open circles, p<0.05) for 50 nM t-PA; from 56% to 42% (open triangles, p<0.02) for 26 nM t-PA; and from 46% to 34% (open squares, p<0.03) for 13 nM t-PA. In this assay, spontaneous lysis in the absence of t-PA amounted to approximately 13% at 3 hours and was not affected by the presence of Lp(a) (closed symbols).

Discussion

The kringle domains of plasminogen and t-PA are triple-loop structures that serve as regulatory sites and...
are important for activation of these serine proteases. While the general structural features of these domains are similar, relatively minor differences in the primary sequence impart significant differences in functional properties to specific kringle regions. Plasminogen contains five kringle regions, of which the first and fourth bind to lysine and fibrin(ogen)\(^{29}\), while the first three are important for antiplasmin binding. The second kringle of t-PA is also involved in lysine and fibrin(ogen) binding.\(^{30}\)

Since the Lp(a) used in this study contains 26 kringle regions with significant homology to the fourth kringle domain of plasminogen, the ability of Lp(a) to compete with plasminogen for fibrin binding is not unexpected. Lp(a) also competes with t-PA for fibrin, but less well and less completely than it does for plasminogen. Less effective competition with t-PA for fibrin is probably a reflection of the additional involvement of a nonkringle structural domain of t-PA in fibrin binding (the fibronectin finger domain).\(^{31}\)

The 2.5-fold difference in apparent estimated \(K_d\) for Lp(a) binding to fibrin in the Matrex system and the apparent estimated \(IC_{50}\) in the competitive binding assay with plasminogen (25 nM versus 10 nM) is probably a reflection of the steric constraints imparted by the large Lp(a) particle in the competitive binding assay. In addition, the multiple kringle domains of a given Lp(a) particle may also interact in a cooperative manner on fibrin binding (although such cooperativity is not apparent from the binding isotherm or its derived Scatchard plot shown here) to account for this difference between estimated direct and competitive binding constants.

Lp(a) clearly attenuates plasminogen activation by t-PA in the presence of fibrin. No such inhibitory effect was noted in the absence of fibrin, nor was any such effect noted with apo(a)-free LDL. Kinetic analysis of the data suggests that the inhibitory mechanism is uncompetitive. This must be viewed as an operational definition since there is no evidence to support the binding of Lp(a) directly to the enzyme-substrate complex. However, if we consider that the active catalytic complex is comprised of enzyme-substrate-activator (t-PA-plasminogen-fibrin), and that Lp(a) binds to the activator, thereby making it unavailable for binding to the catalytic complex, we are left with the much less active enzyme-substrate complex (t-PA-plasminogen) and, in effect, the equivalent of uncompetitive kinetics. The following equations can be used to define this system:

\[
\begin{align*}
\text{t-PA} + \text{plasminogen} + \text{fibrin} &\rightarrow (1) \\
\text{t-PA} + \text{plasminogen} + \text{fibrin} + \text{Lp(a)} &\rightarrow (2)
\end{align*}
\]

where \(k_1 > k_2\).

One group of investigators\(^{32}\) has recently demonstrated that Lp(a) can attenuate the fibrinolytic activity of plasma generated by addition of streptokinase, but the concentrations of Lp(a) required were significantly higher than noted in our experiments with t-PA (0.43 and 0.86 mg/ml). From these data and the recent data of Edelberg and colleagues\(^{33}\) it appears that streptokinase binds to Lp(a) and thereby inhibits streptokinase-mediated plasminogen activation competitively as well as uncompetitively. In contrast to these findings in which a nonphysiologic activator streptokinase was used, we were unable to detect any inhibition of basal activity of t-PA by Lp(a). We noted only uncompetitive inhibition of fibrin-dependent enhancement of basal activity of t-PA, both in a purified system and in a clot lysis assay.

The importance of these observations in regard to the atherogenicity of Lp(a) remains to be determined. Clearly, investigators have identified reduced t-PA activity\(^{34}\) or increased t-PA inhibitory activity\(^{35}\) in the plasma of young survivors of acute myocardial infarction. Inhibition by Lp(a) of t-PA or plasminogen binding to fibrin with consequent abrogation of the enhancement of fibrinolytic activity may confer the potential for vascular occlusion to individuals with elevated levels of this unusual lipoprotein particle.

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