Lipoprotein(a), Fibrin Binding, and Plasminogen Activation

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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 (Kₐ = 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 obtained6-7-8 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 kringle 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.15 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 KabiVitrum, Stockholm, Sweden. Matrex Gel 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-aminocaproic 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. Na¹²¹I 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 normal individuals by sequential ultracentrifugation as previously described.16 Poly-
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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% NaN₃, 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 described previously. 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 at 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 M₀ 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 (M₀=24 800), one kringle-5 domain (M₀=11 300), and 13 kringle-4 domains (M₀=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 of 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 pre-incubated with 0.5 to 1.0 mCl 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 previously described. Lp(a) and LDL preparations were radioiodinated by the iodine monochloride method of McFarlane with modifications. Lp(a) and LDL-bound radiodine 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-Comin, 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

Polyacrylamide Gel Electrophoresis and the apparent molecular weights (Mr) and 5% acetic acid and were destained by diffusion. The method of Lowry and colleagues.28

Osborne26 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 (Mr) were estimated by interpolation.

Protein Determinations

Protein concentrations were determined by the method of Lowry and colleagues.28
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).

Figure 3. The effect of Lp(a) on plasminogen activator (t-PA) activity enhanced by soluble fibrin monomer (SFM). A. In these coupled assays, 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/Δt versus Δt. B. The x-intercepts (1/Km app) of the double reciprocal plots of A are plotted as a function of Lp(a) concentration. The Km is derived from the x-intercept (=-Km). Concentration (Figure 3B) permits the estimation of Km, yielding a value of 15 nM.

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, 46 nM of t-PA was incubated with a range of concentrations of glu-plasminogen (0 to 3.4 μM) and 0.8 mM S-2251 in the presence of 3.3 μg/ml SFM in 10 mM Tris, pH 7.4, 0.15 M NaCl. The reactions were then monitored over a range of concentrations (0 to 165 nM) of Lp(a). Initial rates (derived from plots of change in absorbance/Δt versus Δt) were determined in this coupled assay and were plotted inversely against the reciprocal of plasminogen concentration (Figure 3A). These double reciprocal plots effectively define a series of parallel lines as a function of increasing Lp(a) concentration. A plot of 1/Km app against the Lp(a) concentration (Figure 3B) permits the estimation of Km, yielding a value of 15 nM.

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. Plasminogen contains five kringle regions, of which the first and fourth bind to lysine and fibrin(ogen), while the third are important for antiplasmin binding. The second kringle of t-PA is also involved in lysine and fibrin(ogen) binding.

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).

The 2.5-fold difference in apparent estimated $K_i$ 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:

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

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 atherogenicty 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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References

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