Multiple Binding with Identical Linkage: A Mechanism That Explains the Effect of Lipoprotein(a) on Fibrinolysis†

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ABSTRACT: We have previously shown that both recombinant apo(a) and native Lp(a) inhibit the binding of Glu-plasminogen to fibrin surfaces [Fleury & Anglés-Cano (1991) *Biochemistry 30*, 7630−7638; Rouy et al. (1992) *Biochemistry 31*, 6332−6339]. The aim of the present study was to characterize the mechanism of this inhibition and to define the parameters governing binding when two different Lp(a) species compete with plasminogen for fibrin, a situation that may be found *in vivo* in subjects heterozygous for the apo(a) trait. The K_d for the binding of plasminogen to fibrin was 660 nM whereas the affinity of Lp(a) was inversely related to apo(a) size (K_d range: 50 to >500 nM). To determine the effect of plasminogen on Lp(a) binding and reciprocally, competition experiments were performed. The K_d of either Lp(a) or plasminogen for fibrin remained unchanged in the presence of the other competitor whereas B_{max}, the maximal amount bound, was importantly decreased. In a similar fashion, competition for fibrin binding among Lp(a) isoforms was shown with the use of Lp(a) density fractions containing varying proportions of isoforms B (~460 kDa) and S3 (~640 kDa); variations in K_d values (from 141 nM to 460 nM) as a function of the relative content in isoform S3 were observed. Altogether, these results are indicative of multiple binding by ligands that bind with different affinities to equivalent but independent sites. Thus, in plasma from heterozygous subjects, the influence of each Lp(a) isoform on fibrinolysis will depend on their affinity for fibrin and on their concentration relative to each other and to plasminogen.

High plasmas levels of lipoprotein(a), Lp(a),[1] are now recognized as a risk factor in cerebro and cardiovascular diseases (Dahlen et al., 1986; Rhoads et al., 1986; Durrington et al., 1988; Sandkamp et al., 1990; Schreiner et al., 1993). However, the mechanism by which Lp(a) may favor the atherogenic and/or thrombogenic processes is not clearly understood. Its particular structure, an inactive serine proteinase, apo(a), which is disulfide linked to the apo B-100 of an atherogenic LDL-like particule (Utermann et al., 1987), may explain in part this phenomenon. Apo(a) is a highly glycosylated protein which shares a high degree of homology (75−94%) with plasminogen, the precursor of the fibrinolytic enzyme plasmin (Mc Lean et al., 1987; Eaton et al., 1987). Both proteins are constituted by specific domains, called kringles, containing approximately 80 amino acid residues organized in a triple-loop structure stabilized by 3 disulfide bridges. Among the five different kringle domains of plasminogen, kringles 1 and 4 contain lysine-binding subsites which show high affinity for lysine residues on fibrin (Vali & Patthy, 1982; Wu et al., 1991). Apo(a) contains 10−40 copies of plasminogen kringle 4 and 1 copy of kringle 5. These kringle structures are followed by a serine proteinase domain which in spite of its important homology (~95%) with that of plasminogen shows, however, an important difference—an arginine by serine substitution located at the site where plasminogen is cleaved by its activators (Mc Lean et al., 1987), which prevents activation of apo(a).

Because of the multiple copies of kringle 4 of plasminogen contained in apo(a), Lp(a) may compete with plasminogen for binding to lysine residues of cell membrane proteins or fibrin surfaces (Miles et al., 1989; Hajjar et al., 1989; Harpel et al., 1989; Rouy et al., 1991). Indeed, kringle in position 37 of the first apo(a) cloned by Mc Lean et al. (1987) [kringle 4 type 10 according to Guevara et al. (1992)] contains a lysine-binding site similar to that of plasminogen kringle 4 (Mc Lean et al., 1987; Scanu et al., 1993). Furthermore, it has been proposed (Guevara et al., 1993; Ernst et al., 1995) that kringles 32−35 (types 5−8) may also have lysine-binding sites with differences influencing their specificity and affinity for lysine residues. High concentrations of Lp(a) in plasma may therefore represent a potential source of antifibrinolytic activity.

The antifibrinolytic potential of Lp(a) in plasma has been studied by several groups; conflicting results have been reported, and the subject still remains a matter of debate (Alessi et al., 1990; Rouy et al., 1991; Aznar et al., 1992). Recently, we have shown that distinct Lp(a) species purified from homozygous subjects display different antifibrinolytic

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[1] Abbreviations: Lp(a), lipoprotein(a); apo(a), apolipoprotein(a); t-PA, tissue-type plasminogen activator; Glu-plasminogen, native human plasminogen with Glu, residue 1, at the amino terminus; 6-Ahx, 6-aminohexanoic acid; PheProArgCH_2Cl, D-phenylalanyl-L-prolyl-L-arginine chloromethane; ValPheLysCH_2Cl, D-valyl-L-phenylalanyl-L-lysine chloromethane; CBS1065, chromogenic substrate (methylmalonyl)-hydroxyprolylarginine *p*-nitroanilide.

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effects and that this heterogeneity is related to apo(a) size polymorphism (Hervio et al., 1993). Actually, up to 34 apo(a) isoforms with variable molecular masses (range 300−800 kDa) linked to their number of kringle 4 domains have been described (Kamboh et al., 1991; Lackner et al., 1993; Marcovina et al., 1993). These apo(a) isoforms may have variable affinities for fibrin that will influence their effect on fibrinolysis. In heterozygous subjects, representing 94% of the population (Boerwinkle et al., 1992), this phenomenon is complicated by the effect of two distinct apo(a) isoforms present at a different concentration. Under these conditions, the net antifibrinolytic effect of Lp(a) will depend on the respectives affinities and concentrations of plasminogen and each of the Lp(a) species. The aim of the present study was to characterize the mechanism of competition between ligands, plasminogen and one or two Lp(a) isoforms, with similar specificity but different affinity for lysine residues on fibrin.

MATERIALS AND METHODS

Chemicals and Reagents. Materials were purchased from the following sources: D-phenylalanyl-L-prolyl-L-arginine chloromethane (PheProArgCH_2Cl) and D-valyl-L-phenylalanyl-L-lysine chloromethane (ValPheLysCH_2Cl) from France Biochem (Meudon, France); chromogenic substrate (methylmalonyl)hydroxyprolylarginine *p*-nitroanilide (CBS1065) from Diagnostica Stago (Asnières, France); aprotinin (Trasylol) from Bayer Pharma (Puteaux, France); immunoelectrophoresis plates for quantitation of Lp(a) from Immuno AG (Vienna, Austria); peroxidase-labeled sheep immunoglobulins from Dako (Glostrup, Denmark); nitrocellulose sheets from Sartorius (Palaiseau, France). Other products were obtained as previously described (Hervio et al., 1993).

Buffers. Buffer A was 0.05 M sodium phosphate, pH 7.4, 0.08 M NaCl, 0.01% Tween 20, and 0.01% NaN_3; assay buffer was buffer A containing 2 mg of bovine serum albumin/mL; mass buffer, a buffer that provided the mass action effects on the competitive nonspecific adsorption of plasminogen and lipoprotein(a) to the fibrin surfaces, was buffer A containing 40 mg of bovine serum albumin/mL.

Proteins. Native human plasminogen and fibrinogen were separated from fresh-frozen human plasma under conditions that avoid proteolysis as previously described (Kazal et al., 1963; Fleury & Anglés-Cano, 1991; Grailhe et al., 1993). Elastase-derived plasminogen fragments were prepared according to Sottrup-Jensen et al. (1978) as previously described (Rouy et al., 1992).

Lipoprotein(a) Purification and Characterization. Donors were selected among the in-patients and the medical staff of the Metabolic Department of Hôpital Robert Debré (Université de Reims, France) for their high levels of Lp(a) (≥0.7 g/L). Venous blood was drawn on 3 mM EDTA final concentration. Plasma was separated by centrifugation at 2000*g* for 15 min, supplemented with 1 mM EDTA, 0.01% NaN_3, inhibitors of proteolysis (aprotinin 100 KIU/mL; PheProArgCH_2Cl, 1 μM; ValPheLysCH_2Cl, 1 μM; and *p*-nitrophenyl *p'*-guanidinobenzoate, 10 μM; final concentrations), and an antioxidant (butylhydroxytoluene 0.05 mg/mL).

(A) Purification by Sequential Ultracentrifugation. Lp(a) was isolated in the density interval 1.050−1.100 g/mL KBr followed by gel filtration on a Biogel A5M column. The peak fractions containing Lp(a) were pooled, concentrated, and dialyzed in buffer A containing 2 mM EDTA. The final product was conserved at 4 °C in this state until use (usually less than 24 h). The concentration of the purified Lp(a) was determined by immunoelectrophoresis.

(B) Isolation of Lp(a) Fractions. Lp(a) fractions were obtained by a modification of a two-step gradient ultracentrifugation procedure (Swaney et al., 1987). Briefly, total lipoproteins obtained at solvent density 1.21 g/mL (48 h at 150000*g*) were separated on a concave KBr gradient between densities 1.180 and 1.040 g/mL (24 h at 38000*g* in a SW41 rotor; Beckman France, Gagny). The fractions in the density range of each of the Lp(a) isoforms in the corresponding serum were pooled, and subjected to a second gradient ultracentrifugation. Lp(a)-containing fractions were individually dialyzed against 0.15 M NaCl, 0.05 M phosphate, pH 7.4. Nondenaturing 2−16% polyacrylamide gels were used to evaluate particle size by comparison with HMW standards (Pharmacia-France, Guyancourt).

(C) Determination of Isoform Size. The isoform proportions in the various preparations were determined under reducing conditions using 3.75% polyacrylamide/0.8% agarose gels (Hervio et al., 1993). Protein bands were electroblotted to nitrocellulose according to Khyse-Andersen (1984). Apo(a) bands were localized using a sheep antibody to human apo(a), followed by a peroxidase-conjugated rabbit antibody to sheep IgG revealed with 4-chloro-1-naphthol. To quantify the proportion of each isoform from a given preparation, immunoblots were incubated overnight with the apo(a)-specific antibody radiolabeled with ^{125}I, and autoradiographed on Kodak XS-films for 24−48 h at −70 °C. The apo(a) bands were cut and counted in a γ-radiation counter. Apo(a) isoforms were identified using reference plasmas containing isoforms F, B, S1, S2, S3, and S4 generously provided by Dr. G. Utermann (University of Innsbrück, Austria). The sheep antibody to human apo(a) was shown to react equivalently with the different isoforms.

Binding of Isolated Lp(a) Density Fractions to Fibrin Surfaces. Fibrin surfaces and plasmin-degraded fibrin surfaces were prepared as described elsewhere (Fleury & Anglés-Cano, 1991). Varying concentrations of the isolated Lp(a) fractions, containing different proportions of apo(a) isoforms B/S3 in mass buffer, were incubated with partially degraded fibrin surfaces. After 18 h at 4 °C, unbound Lp(a) was removed by washing, and the surface was probed with a known concentration of a ^{125}I-labeled polyclonal antibody directed against apo(a). This antibody was previously immunodepleted using Sepharose-immobilized apoB-100 and plasminogen, and did not cross-react with these proteins. After overnight incubation at 4 °C, excess antibody was removed by washing, and the radioactivity in the wells was counted in a γ-radiation counter and transformed into the mass of antibody bound using the specific radioactivity (dpm/mol of IgG) of the labeled antibody. Bound antibody was converted to Lp(a) protein mass as indicated below (Analysis of Binding Data section). Binding of Lp(a) to the fibrin surfaces in the presence of 0.2 M 6-aminohexanoic acid (6-Ahx), considered as unrelated to interactions with lysine residues, represented less than 10% of total binding.

Effect of Lp(a) on Plasminogen Activation. The effect of Lp(a) on plasminogen activation was determined in a system that measures the amount of plasmin generated by t-PA at the fibrin surface in the presence of plasminogen (Anglés-

Cano, 1986). In this system, t-PA is first bound to fibrin by incubating a constant amount of the activator (50 IU/mL) for 1 h at 37 °C. Unbound protein is removed by washing and the reaction started by adding in triplicate 50 μL per well of assay buffer containing 200 nM plasminogen, varying concentrations of Lp(a), and 1.5 mM of the plasmin-selective chromogenic substrate CBS1065. The generation of plasmin was detected by measuring the change in absorbance ($\Delta A_{405/min}$) at a double-wavelength absorbance ratio (405/490 nm) with a microtitration plate counter (MR5000, Dynatech). Following activation, the plate was washed, and the surface was probed with the ^{125}I-labeled polyclonal sheep antibody directed against apo(a).

Competition between Lp(a) and Plasminogen for Fibrin Binding. Two types of experiments were performed. To evaluate the effect of Lp(a) on plasminogen binding, solutions containing varying amounts of plasminogen (0−8 μM) and a constant trace amount of ^{125}I-plasminogen (5 nM final concentration) were prepared in mass buffer alone or containing different concentrations of Lp(a). These mixtures (50 μL/well) were incubated overnight at 4 °C with a degraded fibrin surface. After elimination of the supernatant, the plate was washed and the wells were counted in a γ-radiation counter. The amount of plasminogen bound to the degraded fibrin surface was calculated by dividing the radioactivity in each well by the molar radioactivity (dpm/mol) of plasminogen in each of the solutions. Nonspecific binding was determined from the amount of radioactivity bound in the presence of 0.2 M 6-Ahx. The second type of experiment was aimed at determining the influence of constant amounts of plasminogen on the binding of Lp(a). For this purpose, solutions containing either varying amounts of Lp(a) (0−1000 μg/mL) and constant amounts of Glu-plasminogen (1 and 10 μM) or a fixed amount of Lp(a) (200 μg/mL) and varying concentrations of plasminogen or its elastase-derived fragments (0−4 μM) were prepared in mass buffer. These mixtures (50 μL/well) were incubated overnight at 4 °C with a degraded fibrin surface. After elimination of the supernatant, the plate was washed, and Lp(a) bound to the surface was detected with an ^{125}I-labeled polyclonal antibody directed against apo(a). Then, the plate was washed with assay buffer and incubated 2 h at 37 °C with the ^{125}I-IgG directed against apo(a). After the wells were washed, the bound radioactivity was counted in a γ-radiation counter and transformed into mass of Lp(a) bound as indicated below. In some experiments, a trace amount of ^{125}I-Glu-plasminogen (5 nM, final concentration) was added to the Lp(a)/plasminogen mixtures, and the amount of plasminogen bound was quantitated by counting the bound radioactivity as described above.

Analysis of Binding Data. The equilibrium binding data for the Lp(a)/fibrin and plasminogen/fibrin interactions fitted the simple Langmuir equation for adsorption at interfaces (Adamson, 1992):

$$[\mathrm{Fn{\cdot}X}] = [\mathrm{Fn_0}]\frac{K[\mathrm{X}]}{1 + K[\mathrm{X}]} \quad (1)$$

where $[\mathrm{Fn_0}]$ represents the total number of fibrin binding sites, [Fn·X] the number of molecules of ligand X adsorbed onto fibrin, K the association constant, and X either plasminogen or Lp(a). The equilibrium binding of two or more ligands (plasminogen and one or two distinct Lp[a] isoforms)

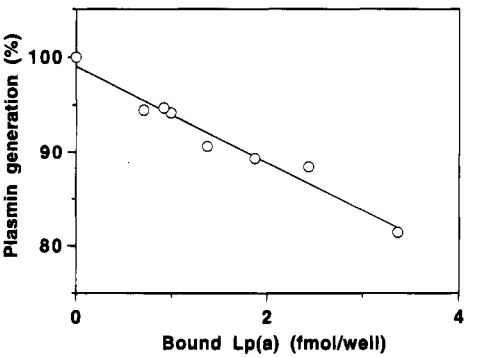

FIGURE 1: Inhibition of plasmin generation by Lp(a). Varying concentrations of Lp(a) (isoform S1) were incubated with a constant amount of Glu-plasminogen (200 nM final concentration) on fibrin-bound t-PA. Plasminogen activation was followed at 37 °C by measuring the amidolytic activity of plasmin with the synthetic substrate CBS10-65. At the end of the activation, the plate was washed, and bound Lp(a) was detected with a radiolabeled antibody directed against apo(a); bound radioactivity was transformed into femtomoles of Lp(a) as indicated under Materials and Methods. Plasminogen activation, expressed as a percentage relative to the maximum of plasmin generated in the absence of Lp(a), is plotted against the amount of bound Lp(a) per well. Data represent the mean of three determinations; the intra-assay variation was 6 ± 4%. A linear correlation (r = 0.985) was found between the inhibition of plasmin generation and the amount of Lp(a) bound.

for identical binding sites is typical of competitive binding and obeys modifications of the basic Langmuir equation:

$$[\mathrm{Fn{\cdot}X}] = [\mathrm{Fn_0}]\frac{K[\mathrm{X}]}{1 + K[\mathrm{X}] + \sum K_i[\mathrm{Y}_i]} \quad (2)$$

where Y represents a second or third ligand and K' its association constant.

The amount of Lp(a) adsorbed onto fibrin was calculated from the mass of anti-apo(a) antibody bound to the fibrin·Lp-(a) complex using a simplified form of eq 1 that describes the linear relationship of antibody binding at low concentrations:

$$\text{antibody bound} = \mathrm{Fn{\cdot}Lp(a){\cdot}}K\mathrm{{\cdot}[antibody]} \quad (3)$$

and by rearrangement

$$\mathrm{Fn{\cdot}Lp(a)} = K_\mathrm{d}\left(\frac{[\text{antibody bound}]}{[\text{antibody}]}\right) \quad (4)$$

where Fn·Lp(a) represents the amount of Lp(a) bound to fibrin and $K = 1/K_\mathrm{d}$ the affinity (K_d = 3 nM) of the anti-apo(a) antibody for fibrin-bound apo(a).

RESULTS

Competition between Lp(a) and Plasminogen for Fibrin Surfaces. The competition between Lp(a) and plasminogen was characterized with several methodologies using either a chromogenic substrate or ^{125}I-labeled proteins.

(A) Effect of Lp(a) on Plasmin Generation. Mixtures containing varying concentrations of purified Lp(a) (isoform S1), 200 nM plasminogen, and a chromogenic substrate selective for plasmin were incubated with fibrin-bound t-PA. Plasminogen activation was monitored by quantification of the amidolytic activity of the plasmin generated. As shown in Figure 1, Lp(a) may inhibit up to 20% of plasmin

Table 1: Inhibition of the Binding of Glu-Plasminogen to Fibrin by Lp(a) (Isoform S1)[a]

Lp(a) added (μg/mL)	plasminogen binding constants	
	K_d (μM)	B_{max} (pmol/well)
0	1.13	3.71
50	1.19	2.40
100	0.6	1.32
200	0.7	0.95

[a] The plasminogen binding parameters are reported for experiments that were conducted in the presence of various concentrations of purified Lp(a) (isoform S1) and a trace amount of radiolabeled plasminogen (5 nM; 650 000 dpm/well). B_{max} and K_d for plasminogen at each Lp(a) concentration were derived from the linearized expression of the Langmuir equation, eq 1. By plotting the reciprocal of the modified B_{max} versus the concentration of added Lp(a), an inhibition constant, K_i = 32 nM, was determined at the intersect of the linear regression with the abscissa axis.

generation at the concentrations used (0−200 μg/mL); inhibition was directly correlated ($r = 0.985$) with the binding of Lp(a).

(B) Effect of Lp(a) on Plasminogen Binding. The specific binding of plasminogen to degraded fibrin surfaces was characterized previously (Fleury & Anglés-Cano, 1991), and the effect of various concentrations of purified Lp(a) was determined as described under Materials and Methods. Parameters of the binding constants B_{max} (the maximal amount of plasminogen bound) and K_d (the dissociation constant) obtained in the presence of various concentrations of Lp(a) (isoform S1) are summarized in Table 1. B_{max} decreased importantly as a function of Lp(a) concentrations whereas modifications in the K_d were within the normal range (0.6−1.2 μM). These results are indicative of competitive inhibition between plasminogen and Lp(a) for the same type of binding sites. By plotting the reciprocal of the maximum amount bound against the concentration of added Lp(a), an estimation of the inhibition was calculated: K_i = 32 nM.

Figure 2 shows the effect of plasminogen (1 and 10 μM final concentrations) on the binding of Lp(a) (isoforms S2 80%/S4 20%) to degraded fibrin. The dissociation constant for the binding of Lp(a) to fibrin was not modified by plasminogen. In contrast, the amount of Lp(a) bound to degraded fibrin decreased with increasing concentrations of added plasminogen; B_{max} decreased from 5.3 fmol/well in the absence of plasminogen to 2.8 and 1.1 fmol/well in the presence of 1 and 10 μM, respectively (Figure 2A). In a similar fashion, the amount of plasminogen bound decreased as a function of the concentration of Lp(a) bound to the degraded fibrin surface (Figure 2B).

Competition between Lp(a) and Plasminogen Fragments for Fibrin Surfaces. Mixtures containing a constant concentration of purified Lp(a) (isoforms S2 80%/S4 20%) and varying concentrations of either elastase plasminogen fragments (K_{1+2+3} or K_4) or plasminogen were incubated with a degraded fibrin surface. After overnight incubation at 4 °C, the plate was washed, and the amount of Lp(a) bound to the surface was quantified with the radiolabeled sheep antibody directed against apo(a). Results in Figure 3 show that plasminogen and fragments K_{1+2+3} and K_4 inhibit the binding of Lp(a) to degraded fibrin surfaces. IC_{50} values determined were 1.1 μM for K_{1+2+3}, 0.8 μM for K_4, and 0.3 μM for plasminogen.

Competition between Two Lp(a) Isoforms for Fibrin Surfaces. The experiments were performed with fractions containing defined proportions of Lp(a)-B and Lp(a)-S3 as obtained from the plasma of a heterozygous subject. Varying concentrations of each Lp(a)-enriched fractions were diluted in mass buffer and incubated with intact and degraded fibrin

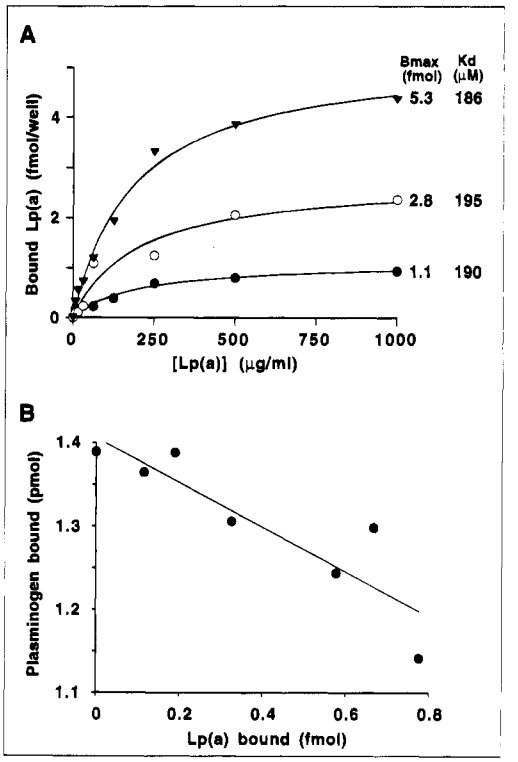

FIGURE 2: Inhibition by plasminogen of the binding of Lp(a) to fibrin surfaces. Increasing concentrations (0−1000 μg/mL) of Lp-(a) (S2/S3) were incubated overnight with fibrin in the presence of 0 (▼), 1 μM (○), and 10 μM (●) Glu-plasminogen. In parallel experiments, a trace amount of radiolabeled plasminogen (5 nM; 650 000 dpm/well) was added to the mixtures. (A) Lp(a) bound to the surface, in the absence of radiolabeled plasminogen, was quantified using an ^{125}I-labeled polyclonal antibody and converted to femtomoles of Lp(a) as described under Materials and Methods. (B) In the presence of radiolabeled plasminogen, bound radioactivity was counted in a γ-radiation counter and transformed into picomoles of plasminogen bound per well. Bound plasminogen was plotted against bound Lp(a) as determined in panel A. Data were fitted with a linear regression analysis [$r = 0.791$ for 1 μmo/L (○) and $r = 0.891$ for 10 μmo/L (●) plasminogen].

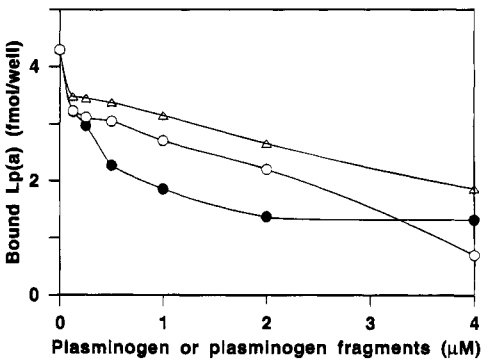

FIGURE 3: Effect of elastase-derived plasminogen fragments on the binding of Lp(a) to degraded fibrin surfaces. A constant amount of Lp(a) (isoform S2/S3) was incubated with varying concentrations of either Glu-plasminogen (●) or plasminogen fragments [K_{1+2+3} (○) or K_4 (△)]. After overnight incubation, Lp(a) bound to the surface was detected with a radiolabeled antibody directed against apo(a), and bound radioactivity was transformed into mass of antibody using its specific radioactivity. Antibody bound was converted to femtomoles of bound Lp(a) using eq 4.

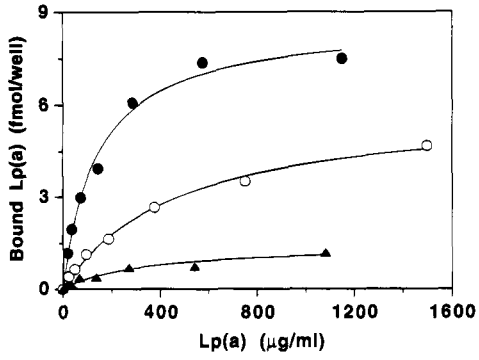

FIGURE 4: Binding of Lp(a) fractions to degraded fibrin surfaces. Fractions (n = 7) containing varying proportions of isoforms B and S3 were diluted in mass buffer and incubated overnight on degraded fibrin surfaces. Bound Lp(a) was detected with the radiolabeled antibody directed against apo(a). The radioactivity bound was transformed into mass of antibody using its specific radioactivity and then to femtomoles of Lp(a) using eq 4. Data were fitted to the Langmuir equation, eq 1. To simplify the plot, only fractions containing 86% B/14% S3 (●, K_d = 140 μM), 40% B/60% S3 (○, K_d = 475 μM), and 20% B/80% S3 (▲, K_d = ~1 μM) are shown.

surfaces. After overnight incubation, bound Lp(a) was detected with the radiolabeled sheep antibody directed against apo(a). As shown in Figure 4, fractions containing the highest proportions of Lp(a)-B display the highest affinity for fibrin surfaces. K_d values increased from ≈140 nM to ≥480 nM with the amounts of Lp(a)-S3. The fraction containing more than 80% of isoforms S3 did not reach saturation; however, an estimation of the apparent dissociation constant was obtained using the linearized expression of the Langmuir equation: apparent K_d = 1.1 μM.

DISCUSSION

Fibrinolysis is a highly regulated and localized process leading to dissolution of fibrin clots and to restoration of the vascular endothelium (Nachman, 1992). Adsorption of t-PA and plasminogen to the surface of fibrin allows generation of plasmin and thereby its degradation (Lijnen et al., 1994). It is now well recognized that binding of plasminogen to lysine residues in fibrin initiates plasminogen activation and amplifies the fibrinolytic process (Suenson et al., 1984; Harpel et al., 1985; Fleury et al., 1993). Lp(a) may also bind to carboxy-terminal lysine residues of fibrin (Harpel et al., 1989) and thus interfere with fibrinolysis as apo(a) in Lp(a) has no catalytic activity (Mc Lean et al., 1987). We have previously shown that, indeed, both r-apo(a) and Lp(a) inhibit the binding of Glu-plasminogen to fibrin surfaces (Rouy et al., 1992). In the present study, our aim was to characterize the mechanism of this inhibition and to define the peculiarities of binding to fibrin when two different Lp(a) species compete with plasminogen, a situation that occurs in subjects heterozygous for the apo(a) trait. We provide here quantitative evidence for reciprocal inhibition of binding to fibrin between plasminogen and Lp(a). In both cases, the K_d values of interaction with fibrin remain constant whereas B_{max} decreased as a function of the concentration of competitor added. These results are typical of competitive inhibition between two different molecules for binding to the same type of binding sites. Lysine residues in fibrin constitute the specific binding sites for both plasminogen and Lp(a), as indicated by the inhibition of binding by both lysine analogues (6-AHA) and carboxypeptidase B treatment of the degraded fibrin surface (Fleury & Anglés-Cano, 1991; Rouy et al., 1992). The specificity of the plasminogen/Lp(a) competitive interaction with fibrin was demonstrated by the effectiveness of elastase-derived plasminogen fragments (K_{1+2+3} and K_4) to inhibit the binding of Lp(a). In agreement with previous findings obtained using recombinant apo(a) (Rouy et al., 1992), the competitive effect was ranked as follows: plasminogen > K_{1+2+3} and K_4.

Inhibition of plasminogen binding to fibrin was observed with Lp(a) at concentrations that may occur in some subjects. Similarly, plasminogen at physiological concentrations (1−2 μM) might inhibit Lp(a) binding. These results suggest that *in vivo*, binding of either Lp(a) or plasminogen to fibrin is the result of interactions governed by their relative affinities and concentrations; as plasminogen concentration and affinity for fibrin are relatively constant between individuals, Lp(a) parameters should be determinant in the resulting fibrinolytic potential of a given subject. Furthermore, in heterozygous individuals, two different Lp(a) species may participate to different degrees in the competitive inhibition of plasminogen binding as illustrated by the binding of Lp(a) fractions containing varying proportions of isoforms B and S3. In these experiments, since the binding of Lp(a) to fibrin was detected with a polyclonal antibody directed against apo(a), the amount bound reflected total Lp(a) binding regardless of apo(a) isoform; an apparent occupancy-dependent dissociation constant (K_d^{app}) was thus determined. An increase in the apparent K_d was detected correlating with the increase in the average bound Lp(a) and depended upon the relative content in isoforms of high molecular mass. Fractions containing a majority of higher molecular mass isoforms Lp(a)-S3 showed the highest K_d value and therefore lowest affinity whereas those fractions with a majority of lower molecular mass isoforms showed the highest affinity as indicated by lowest K_d values. These results as well as the decrease of B_{max} with increasing relative concentrations of the high molecular mass isoform suggest that binding to fibrin was mainly due to the content in low molecular mass isoform. Indeed, a plot of the relative concentration of the lower molecular mass isoform versus the amount bound resulted in K_d values that were close to the value obtained with fractions containing the highest proportion of the small apo(a) isoform (not shown); the similarity in K_d values and in B_{max} thus obtained constitutes an indirect indication of the low contribution of the higher molecular mass isoform to fibrin binding. This type of interaction is typical of multiple binding by two (or more) ligands that bind to equivalent but independent sites (identical linkage) with different affinities (Adamson, 1992). Binding is mutually exclusive and obeys modifications of the Langmuir equation, eq 2, for the competitive adsorption of two or more components.

These results clearly indicate that differences in the equilibrium dissociation constant of single Lp(a) particules occur in the plasma of heterozygote subjects having two isoform phenotypes. In whole plasma, this competition also includes the interaction with plasminogen for fibrin; thus, the amount of Lp(a) bound in the presence of plasminogen will be a function of the affinity of each of the apo(a) isoforms and of their concentration relative to each other and to plasminogen.

In conclusion, altogether the present data constitute the first quantitative demonstration of the complex interplay among plasminogen and apo(a) isoforms having functional

heterogeneity with regard to fibrin binding. These interactions are typical of multiple binding with identical linkage. Fractions containing these isoforms were isolated from a heterozygous subject, thus suggesting that this situation may well be found *in vivo*.

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