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Effects of lipoprotein(a) on the binding of plasminogen to fibrin and its activation by fibrin-bound tissue-type plasminogen activator

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Abstract

Molecular assembly of plasminogen and tissue-type plasminogen activator (t-PA) at the surface of fibrin results in the generation of fibrin-bound plasmin and thereby in the dissolution of a clot. This mechanism is triggered by specific interactions of intra-chain surface lysine residues in fibrin with the kringle domains of plasminogen, and is further amplified via the interaction of plasminogen kringles with the carboxy-terminal lysine residues of fibrin that are exposed by plasmin cleavage. By virtue of its marked homology with plasminogen, apo(a), the specific apolipoprotein component of Lp(a), may bind to the lysine sites available for plasminogen on the surface of fibrin and thereby interfere with the fibrinolytic process. A sensitive solid-phase fibrin system, which allows the study of plasminogen activation at the plasma fibrin interface and makes feasible the analysis of products bound to fibrin, has been used to investigate the effects of Lp(a) on the binding of plasminogen and its activation by fibrin-bound t-PA. Plasma samples from human subjects with high levels of Lp(a) were studied. We have established that Lp(a) binds to the fibrin surface and thereby competes with plasminogen ($K_i = 44$ nM) so as to inhibit its activation. We have further shown that Lp(a) blocks specifically carboxy-terminal lysine residues on the surface of fibrin. To further explore the role of apo(a) on the Lp(a) fibrin interactions, we have performed ligand-binding studies using a recombinant form of apo(a) that contains 17 kringle 4-like units. We have shown that recombinant apo(a) binds specifically to fibrin ($K_d = 26 \pm 8$ nM, $B_{max} = 26 \pm 2$ fmol/well) and that this binding increases upon treatment of the fibrin surface with plasmin ($K_d = 8 \pm 4$ nM, $B_{max} = 115 \pm 14$ fmol/well). Altogether, our results indicate clearly that binding of native Lp(a) through this mechanism may impair clot lysis and may favor the accumulation of cholesterol in thrombi at sites of vascular injury.

Key words: Lipoprotein(a); Apolipoprotein(a); Plasminogen activation; Fibrinolysis; Kringle domain; Lysine-binding sites; Lysine-fibrin carboxy-terminal residues

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1. Introduction

The formation of a clot at sites of vascular injury triggers specific fibrin plasma protein interactions leading to the generation of fibrin-bound Glu-plasmin and thereby to the lysis of the thrombus (Schafer, 1987). Both coagulation and fibrinolysis involve surface-dependent reactions that offer catalytic advantage for enzyme substrate complex formation (Wiman and Collen, 1978; Mann et al., 1990). Indeed, the catalytic rates of fibrinolytic enzymes in solution are extremely low; in contrast, the assembly of the t-PA • plasminogen fibrin-bound enzyme substrate complex leads to an important enhancement ($\sim$1000-fold) of the reaction rate (Hoylaerts et al., 1982; Petersen et al., 1989). Furthermore, early fibrin degradation accelerates fibrinolysis via the interaction of lysine-binding sites in plasminogen kringle 1 and 4 with carboxy-terminal lysine residues in fibrin which are exposed following plasmin cleavage (Lerch et al., 1980; Lucas et al., 1983; Vali and Patthy, 1984; Suenson et al., 1984; Wu et al., 1990).

The adsorption of plasminogen to fibrin and the surface dynamics of transformation of both fibrin and plasminogen during fibrinolysis have been well studied by radioisotopic dilution methods and immunological means (Holvoet et al., 1985; Rouy and Anglés-Cano, 1990). In a plasma milieu, the progression of such a process is markedly influenced by α_2-antiplasmin, the specific plasmin inhibitor that limits the number of carboxy-terminal lysine residues and thereby the amount of bound plasminogen (Anglés-Cano et al., 1992). On the other hand, the blockade of such residues by isolated plasminogen kringle 4 has been shown to interfere competitively with clot lysis (Sugiyama et al., 1987) by a mechanism involving binding to lysine-fibrin residues. Since the kringle domains behave as autonomous functional structures (Patthy et al., 1984), the presence in proteins of kringle modules structurally related to those of plasminogen may result in analogous interactions with fibrin. Such a protein is present in human plasma as a part of lipoprotein(a), Lp(a), a complex particle discovered by Berg (1963), which contains the plasminogen-like apolipoprotein(a), apo(a). Besides this unique feature, Lp(a) shares with low-density lipoproteins (LDL) its lipid composition and apolipoprotein B100; apo(a) is linked to apo B100 by a disulfide group (Fless et al., 1984).

By amino acid sequence analysis and cDNA cloning (McLean et al., 1987; Eaton et al., 1987) it has been established that a variable number of kringle domains that share 61–75% homology with kringle 4 of plasminogen is present in apo(a). The kringle 4-like repeats of apo(a) are followed by a single copy of plasminogen kringle 5 and a protease domain that shares 94% homology with the corresponding domain of plasminogen. However, the arginine residue of the activation cleavage site (Arg_{561}-Val_{562}) in plasminogen has been replaced by serine in apo(a), a substitution that impairs the generation of plasmin-like activity by activators. Since the kringle 4 domain of plasminogen contains subsites with high affinity for lysine, which function as binding sites for exposed surface lysine residues in fibrin (Wu et al., 1991), apo(a) may bind to the lysine sites available for plasminogen on the fibrin surface, thereby interfering with the fibrinolytic process. The existence of such a potential pathophysiological mechanism and its relevance to the development of thrombosis are strengthened by the fact that high levels of the Lp(a) particle in human plasma are considered as an independent risk factor for atherosclerotic cardiovascular disease (see Scanu and Fless, 1990, for a review). The effect of Lp(a) on the fibrinolytic system has therefore been studied by several groups (Hajjar et al., 1989; Gonzalez-Gronow et al., 1989; Loscalzo et al., 1990; Leerinck et al., 1991; Rouy et al., 1991, 1992b; Aznar et al., 1992). Although binding of Lp(a) to immobilized fibrinogen has been demonstrated (Harpel et al. 1989), the existence of an antifibrinolytic effect of Lp(a) and the specific mechanism underlying such an effect have been questioned (Alessi et al., 1990; Garcia Frade et al., 1991; Oshima et al., 1991; Smith and Crosbie, 1991).

In the present work the role of Lp(a) on the binding and activation of plasminogen at the surface of fibrin has been investigated in plasma samples from subjects with high Lp(a) levels; parallel experiments were performed with the Lp(a) purified from such plasmas, and with a

recombinant form of apo(a). Our results indicate that, in a plasma milieu, some Lp(a) isoforms, but not LDL, impair the generation of fibrin-bound plasmin by a mechanism involving binding of Lp(a) to fibrin. Furthermore, it is shown that the binding of Lp(a) is mediated by interactions between apo(a) kringle 4-like domains and carboxy-terminal lysine residues of degraded fibrin. We conclude that the plasminogen-like behavior of apo(a) may explain the perturbation of fibrinolysis caused by the Lp(a) particle.

2. Material and methods

2.1. Reagents and buffers

All chemicals and reagents were of analytical grade and were obtained from sources previously reported (Rouy et al., 1991, 1992a; Fleury and Anglés-Cano, 1991).

Buffer A was 0.05 M sodium phosphate buffer, pH 7.4, containing 0.08 M NaCl and 0.01% sodium azide. Buffer B was 0.05 M sodium phosphate buffer, pH 6.8, containing 0.08 M NaCl and 0.01% sodium azide. Assay-buffer was buffer A containing 2 mg of bovine serum albumin/ml and 0.01% (v/v) Tween 20. Binding buffer was buffer B containing 4 mg of bovine serum albumin/ml, 0.01% (v/v) Tween 20 and 2 mM EDTA. Mass buffer, a buffer that provided the mass action effects on the competitive non-specific adsorption of plasminogen or r-apo(a) to the fibrin surfaces, was binding buffer containing 40 mg of bovine serum albumin/ml. All other buffers were prepared as described in the text.

2.2. Blood samples

Subjects consisted of healthy, normolipidemic volunteers ($n = 10$, total plasma cholesterol: 140–220 mg/dl; triglycerides: 40–110 mg/dl; Lp(a): 12 ± 7.7 mg/dl) and individuals with high levels of Lp(a) (>30 mg/dl) attending the Lipid Clinic of the Endocrinology-Metabolism Service (Hôpital de la Pitié, Paris) for the diagnosis and treatment of lipoprotein disorders. Venous blood (up to 20 ml) was drawn from the forearm into sterile polypropylene tubes containing EDTA (final concentration 3 mM). Samples were immediately centrifuged at 2000 × g for 15 min at 4°C, and plasma removed by aspiration. Plasma samples were tested in our in vitro experimental system within 2 h of isolation. The concentrations of Lp(a) in plasma and in euglobulin fractions were determined by an immunonephelometric method as detailed earlier (Chapman et al., 1988).

2.3. Proteins and lipoproteins

2.3.1. LDL and Lp(a) purification

LDL were isolated from normolipidemic human plasma in the density interval 1.024–1.050 g/ml by sequential preparative ultracentrifugation, and its purity was confirmed as described elsewhere (Chapman et al., 1988). Lp(a) was isolated from the plasmas of patients containing 80 mg/dl or more of Lp(a). Initially, a lipoprotein fraction enriched in Lp(a) was isolated in the density range 1.050–1.100 g/ml by sequential ultracentrifugation. Subsequently, Lp(a) was isolated by gel filtration chromatography on a BioGel A5m column under the conditions of Krempler et al. (1983) with minor modifications (Rouy et al., 1991). The presence of Lp(a) on the preparations was identified by its specific reactivity upon immunoblots with monoclonal antibodies to human apo(a) (Guo et al., 1989). The protein content of lipoprotein fractions was determined by the procedure of Lowry et al. (1951), using bovine serum albumin (Sigma, La Verpillière, France) as standard.

2.3.2. Recombinant apolipoprotein(a)

A recombinant form of apo(a) was purified from stably transfected human embryonic kidney cells by affinity chromatography on lysine-Sepharose 4B; the protein concentration was initially determined by amino acid analysis and then by measuring the absorbance at 280 nm using the extinction coefficient $E^{(1\%)}_{1\,cm} = 1.94$, as reported by Koschinsky et al. (1991). This recombinant protein has been shown to mimic the behavior of plasma-derived lipoprotein (a) (Koschinsky et al., 1991).

2.3.3. Glu-plasminogen and fibrinogen

Glu-plasminogen and fibrinogen were purified as previously described (Fleury and Anglés-Cano,

1991). The purified plasminogen was free of Lp(a). The purified fibrinogen was free of plasminogen, von Willebrand factor, fibronectin and factor XIII, as determined by an enzyme-linked immunosorbent assay specific for these proteins. The plasminogen fragments, mini-plasminogen (M_r 39 000), kringle 1–3 (M_r 33 000) and plasminogen kringle 4 (M_r 10 000) were prepared by digestion of plasminogen with porcine pancreatic elastase according to Sottrup-Jensen et al. (1978) with minor modifications (Rouy et al., 1992a).

2.3.4. Radioiodination of proteins

Radioiodination of Glu-plasminogen, r-apo(a) and the purified IgG against apo(a) were performed with Na[^{125}I] using the Iodogen™ method of Fraker and Speck (1978) with minor modifications (Rouy et al., 1991).

2.4. Preparation and analysis of the fibrin surfaces

Intact fibrin and plasmin-degraded fibrin surfaces were prepared and characterized as previously described (Anglés-Cano, 1986; Fleury and Anglés-Cano, 1991) with the following modifications. The intact fibrin surface was treated with 50 μg/ml of DFP-treated carboxypeptidase B in 0.05 M HEPES, pH 7.5, containing 0.5 M NaCl and 10 mM $ZnCl_2$, for 2 h at 37°C, to eliminate the possible existence of carboxy-terminal lysines. Plasmin-degraded fibrin was prepared by incubating 25 nM plasmin in assay buffer for 30 min at 37°C. Fibrin-bound plasmin was eluted by three 8-h washes with assay buffer containing 0.2 M *trans*-4-(aminomethyl)-cyclohexane carboxylic acid, 1 mM benzamidine and 10 μM dansyl-valyl-L-phenylalanyl-L-lysine chloromethylketone. The plate was sealed and stored in this state at 4°C until further use.

2.5. Activation of plasma plasminogen by fibrin-bound t-PA

The activation of plasminogen by t-PA on a fibrin surface was performed as indicated elsewhere (Rouy et al., 1990). Briefly, a solid-phase fibrin plate was washed three times with binding buffer, and 50 μl per well of the same buffer containing 20 I.U./ml of t-PA were incubated for 1 h at 37°C. The plate was then washed twice with binding buffer to eliminate unbound proteins, and the reaction was started by adding 50 μl per well of plasma or its respective euglobulin fraction prepared as described (Milstone, 1941). A pair of plasmas matched for their high (>40 mg/dl) and low level (<20 mg/dl) of Lp(a) was used for each experiment. At regular intervals the soluble phase was removed and the wells washed with assay buffer, and the plasmin bound was detected by adding 50 μl of 1.5 mM CBS 1065 in assay buffer per well. The change in absorbance (ΔA_{405}/min) was measured at a double-wavelength absorbance ratio (A_{405}/A_{490}) with a microtitration plate counter (MR 5000, Dynatech). Following activation, the plate was washed and the surface was probed with a ^{125}I-labelled polyclonal sheep antibody directed against apo(a).

To quantitate the amount of fibrin-bound plasmin(ogen), experiments were performed in the presence of ^{125}I-Glu-plasminogen (~6 nM, final concentration) and the reaction was stopped with assay buffer supplemented with 1 mM benzamidine and 10 μM GGACK. At the end of the activation the radioactivity in the wells was counted in a gamma-radiation counter, and the total amount of plasminogen derivatives bound to fibrin was calculated with the following formula:

$$[\text{plasmin}]_{\text{fibrin}} = \frac{\text{dpm per well}}{(\text{dpm/mol of plasminogen})}.$$

2.6. Binding of recombinant apolipoprotein(a) and plasminogen to fibrin surfaces

Solutions of either Glu-plasminogen (0–10 μM) or r-apo(a) (0–200 nM) with different specific radioactivities were prepared in mass buffer by addition of a trace amount (~6 nM Glu-plasminogen or 0.5 nM r-apo(a), final concentrations) of the ^{125}I-labelled protein. In parallel experiments, varying amounts of a competitive ligand, 6-amino hexanoic acid (6-AHA) or elastase-derived plasminogen fragments, were added. These mixtures (50 μl/well) were incubated for 18 h at 4°C with the intact or modified fibrin surfaces. The supernatant was collected, the surface washed

three times with binding buffer and the extent of binding determined by counting the radioactivity of the wells in a γ-radiation counter. The amount of protein bound to the surface of intact or degraded fibrin was calculated as indicated above. Non-specific binding was determined from the amount of radioactivity bound in the presence of an excess of unlabelled ligand (ratio 1:500) or 0.2 M 6-AHA.

3. Results

3.1. Effect of Lp(a) on the generation of plasmin at

Plasma from subjects with high Lp(a) levels (>30 mg/dl) were tested for their ability to inhibit the generation of plasmin by t-PA bound to fibrin. To isolate the effect of Lp(a) on plasminogen activation from the possible effect of plasminogen-binding proteins (α2-antiplasmin and histidine-rich glycoprotein), the activation experiments were performed with the euglobulin fraction of plasma; this preparation contains an amount of Lp(a) similar to that of the original plasma but does not contain the aforementioned α2-globulins. The model of a fibrin surface used in these experiments allows quantitation of plasmin generation by measuring the progress of hydrolysis of a chromogenic substrate selective for plasmin. The results obtained with a pair of plasmas matched for their high and low content of Lp(a) are shown in Fig. 1. In both cases, the amount of plasmin generated at the fibrin surface increased progressively as a function of time. However, the initial rate of chromogenic substrate hydrolysis was significantly decreased in the sample containing 60 mg Lp(a)/dl, indicating that less plasmin was generated. The decrease in plasmin generation in the presence of high amounts of Lp(a) was verified (Fig. 2) by quantitating the protein mass of plasmin bound to fibrin in activation experiments performed with plasmas supplemented with a constant amount (6–9 nM, final concentration) of ^{125}I-Glu-plasminogen. In experiments where the radioactive plasminogen was omitted, the fibrin surface was probed at the end of the activation with a ^{125}I-labelled polyclonal antibody specific for apo(a).

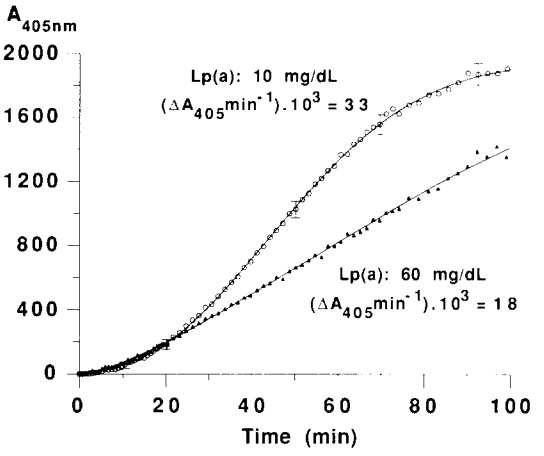

Fig. 1. Time course of the generation of plasmin by fibrin-bound t-PA with the euglobulin fraction of a plasma with high Lp(a) content (▲) and a normal plasma (○), respectively. The experiment was performed in the presence of a chromogenic substrate selective for plasmin. The curve represents the change in absorbance produced by the release of *p*-nitroaniline as a function of time. The vertical bars represent the mean ± S.D. of six determinations. Initial velocities are expressed in $(\Delta A_{405}\ \text{min}^{-1})\cdot 10^3$ of substrate degradation by plasmin generated on the fibrin surface as a function of activation time (minutes).

An increase in the binding of Lp(a) to fibrin was detected as a function of the activation time (Fig. 2, inset).

3.2. Inhibition of the binding of plasminogen to degraded fibrin by Lp(a) and recombinant apo(a)

The specific binding of plasminogen to degraded fibrin (an equivalent of the state of the fibrin surface after progressive plasminogen activation) was performed as previously reported (Fleury and Anglés-Cano, 1991). Data were fitted to the Langmuir equation (Fig. 3). B_{max} and K_d values obtained were similar to those reported previously (Fleury and Anglés-Cano, 1991). To determine the effect of Lp(a) on the binding of plasminogen similar experiments were performed in the presence of various concentrations of purified Lp(a) or r-apo(a). The binding of the Glu-plasminogen to the modified fibrin surface was efficiently inhibited by Lp(a) (Fig. 3, inset); since LDL was without effect on the binding (data not shown)

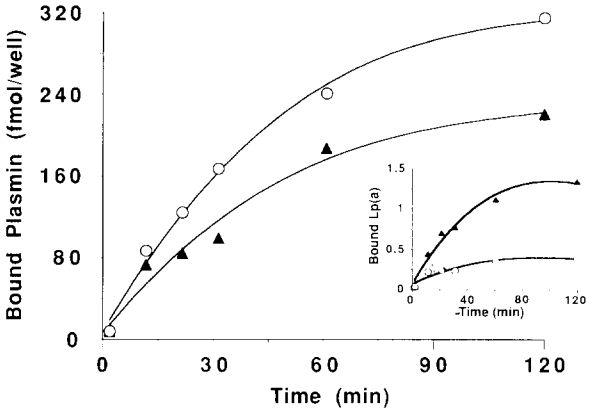

Fig. 2. Determination of the binding and activation of plasmin(ogen) (main graph) and of the binding of Lp(a) (inset). To measure plasmin (mass protein) generated at the surface of fibrin, the experiment was performed in the presence of a trace amount of ^{125}I-labelled plasminogen. The binding of Lp(a) was detected after the activation reaction shown in Fig. 1, by probing the surface with a ^{125}I-labelled polyclonal sheep antibody directed against apo(a). Bound Lp(a) is expressed in mg of antibody bound per well. (▲) Plasma with high Lp(a) content; (○) normal plasma.

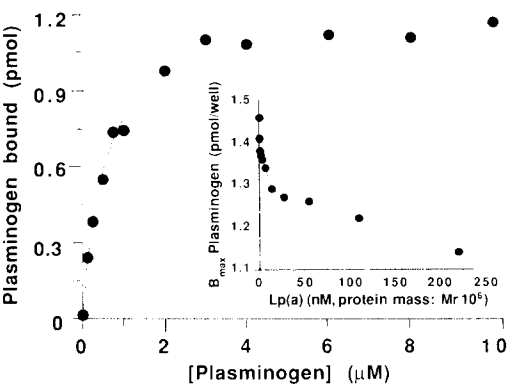

Fig. 3. Isotherm of the binding of plasminogen to plasmin-degraded fibrin and inhibition by Lp(a). The plasmin-modified fibrin surface was performed as indicated in Methods. Varying amounts of plaminogen (0–10 μM) containing trace amounts of ^{125}I-labelled plasminogen (~6 nM, final concentration) were incubated with fibrin in the presence of various concentrations of purified Lp(a) (0–225 nM). The bound radioactivity was counted and represented in mass of plasminogen bound. Data were fitted to the Langmuir equation:

$$[\mathrm{Fn} \bullet \mathrm{Pg}] = [\mathrm{Fn}_0] \frac{K[\mathrm{Pg}]}{(1 + K[\mathrm{Pg}])}$$

where $[\mathrm{Fn}_0]$ represents the total number of fibrin binding sites, $[\mathrm{Fn} \bullet \mathrm{Pg}]$ the amount of plasminogen (Pg) bound to fibrin (Fn), and K the association constant of plasminogen. The affinity was expressed by the dissociation constant, $K_d = 1/K$, and the maximum of plasminogen bound (B_{max}) was calculated from the asymptote. The main graph represents the isotherm of the binding of plasminogen in the absence of Lp(a). The inset represents the inhibition of the binding of plasminogen by increasing concentrations of Lp(a): the B_{max} of plasminogen for each experiment is plotted against the concentration of Lp(a).

and the recombinant apo(a) decreased the B_{max} of plasminogen in a concentration-dependent manner (Fig. 4), it was concluded that the inhibition of the binding of plasminogen to fibrin was mediated by the apo(a) component of Lp(a). By plotting the reciprocal of the maximum amount bound against the r-apo(a) concentration, an estimation of the inhibition was calculated, thus leading to a K_i value of 44 nM (Fig. 4, inset).

3.3. Effect of plasminogen fragments on the binding of recombinant apo(a) to fibrin

Due to its structural homology with plasminogen, r-apo(a) competed with plasminogen for the binding to fibrin. This binding was further characterized by incubating with degraded fibrin a volume of mass buffer containing varying amounts of r-apo(a) (from 0 to 200 nM) and a constant concentration (~4 nM, final concentration) of ^{125}I-r-apo(a). Binding in the presence of 0.2 M 6-AHA was substracted to yield values for specific binding. The latter was calculated from the total concentration of protein, and the radioactivity added as indicated in Methods. Data were fitted to the simple hyperbolic expression of Langmuir, and are represented in Fig. 5. The recombinant protein binds to intact fibrin (K_d = 26 ± 8 nM, B_{max} = 26 ± 2 fmol/well) and to the degraded surface of fibrin (K_d = 8 ± 4 nM, B_{max} = 115 ± 14 fmol/well) in a saturable and specific manner; values of the binding parameters were as previously reported (Rouy et al., 1992a). To identify the plasminogen kringle(s) that may be involved in the competitive interaction with fibrin, experiments were performed with a constant amount of r-apo(a) in the presence of varying concentrations of the elastase-derived plasminogen fragments kringle 1–3, kringle 4 and mini-plasminogen (kringle 5 + protease domain). Prior to examining

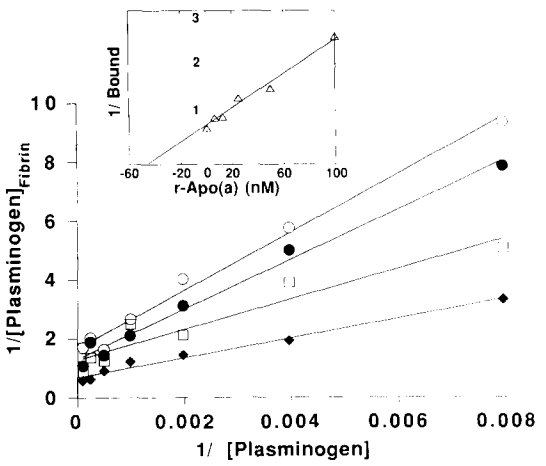

Fig. 4. Determination of the inhibition constant of r-apo(a) for the binding of plasminogen to plasmin-degraded fibrin. Plasminogen binding experiments were performed as indicated in Fig. 3. The concentrations of r-apo(a) used were 0 (◆), 25 (□), 50 (●) and 100 (○) nM. For each concentration of r-apo(a) the plasminogen binding constants were determined according to the Langmuir adsorption isotherm (see Fig. 3) as modified for the presence of r-apo(a):

$$[\mathrm{Fn} \bullet \mathrm{Pg}] = [\mathrm{Fn}_0] \frac{K[\mathrm{Pg}]}{1 + K[\mathrm{Pg}] + K'[\text{r-apo(a)}]}$$

where K' represents the association constant of r-apo(a). The main graph is a double reciprocal plot of the amount of plasminogen bound versus the plasminogen-free concentration. The inset depicts the reciprocal of the amount bound as a function of the concentration of r-apo(a). The inhibition constant, K_i = 44 nM, was calculated from the intercept of the straight line with the abscissa axis.

the effect of these fragments on the binding of the recombinant apo(a), the functional integrity of plasminogen kringles 4 and 1–3 was verified by determining the ability of the corresponding plasminogen fragments to inhibit Glu-plasminogen binding to degraded fibrin surfaces (Fig. 6). As shown in the inset of Fig. 5, only the plasminogen fragment kringle 1–3 decreased the binding of r-apo(a); the concentration needed to inhibit 50% of the binding was ~10 μM. Native plasminogen produced a similar effect at a concentration of 3 μM. Mini-plasminogen had no effect on the binding of the recombinant protein (data not shown).

4. Discussion

Abnormally high concentrations of LDL-cholesterol are associated with accelerated atherosclerosis and coronary heart disease morbidity and mortality (Golstein and Brown, 1977). Lipoprotein(a), another cholesterol-rich particle, is now recognized as an independent risk factor for cardiovascular disease (Rhoads et al., 1986; Sandkamp et al., 1990). The atherogenic potential of

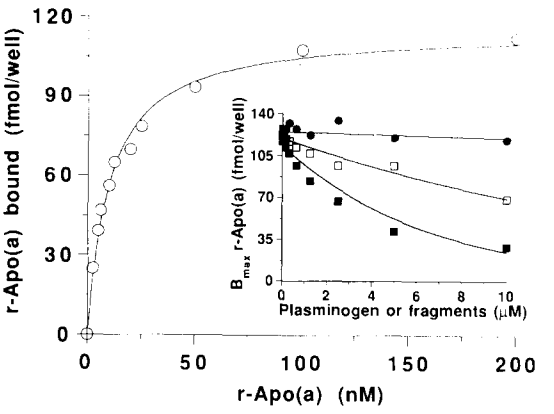

Fig. 5. Binding of recombinant apo(a) (○) to degraded fibrin (main graph) and inhibition of the binding by elastase-derived plasminogen fragments (inset). The binding experiments were performed in the presence of a trace amount of ^{125}I-labelled r-apo(a). As for plasminogen, fitting of data to the Langmuir equation (see Fig. 4) indicated single-site binding. The inset represents the inhibition of the binding of r-apo(a) by kringle 4 (●), kringle 1–3 (□) and plasminogen (■).

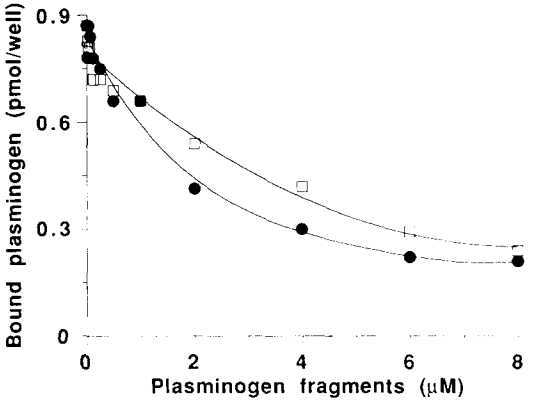

Fig. 6. Inhibition of the binding of plasminogen to degraded fibrin by elastase-derived plasminogen fragments. Varying amounts of plasminogen (0–10 nM) containing a trace amount of ^{125}I-labelled plasminogen (~6 μM, final concentration) were incubated with fibrin in the presence of various concentrations of either kringle 4 (□) or kringle 1–3 (●). B_{max} were calculated as indicated in Fig. 3. The B_{max} of the plasminogen fibrin interaction is plotted against the concentration of the plasminogen fragments.

low-density lipoproteins is mainly related to their capacity to transport ~70% of total serum cholesterol from liver to peripheral cells. Accumulation of cells loaded with oxidatively modified LDL ('foam cells') and amorphous lipid accumulation (fatty streaks) in the intima of coronary and cerebro-basilar arteries constitutes the earliest mechanism of atheroma formation. Lp(a) is similar to LDL in its lipid composition and apo B-100 content; its singularity resides in the presence of a second apolipoprotein, apo(a), linked to the first by a disulfide bridge. The presence of this specific glycoprotein in Lp(a) may explain the higher atherogenic potential of this particle at serum concentrations much lower than those of LDL. The recent demonstration of an important structural homology between plasminogen, the precursor of the fibrinolytic enzyme plasmin and apo(a) (McLean et al., 1987; Eaton et al., 1987) suggests that a direct implication of Lp(a) in the fibrinolytic process may constitute the link between atherosclerosis and thrombosis. Since apo(a) cannot be transformed in an active enzyme, binding of Lp(a) to fibrin may interfere with the evolution of fibrinolysis at the surface of both fibrin and cells. In an attempt to prove this hypothesis, a number of studies have been conducted in several laboratories (Harpel et al., 1989; Hajjar et al., 1989; Miles et al., 1989; Loscalzo et al., 1990; Leerinck et al., 1991). The initial demonstration by Harpel et al. (1989) that Lp(a) binds to immobilized fibrinogen was further developed in the present studies; we provide evidence indicating that Lp(a) competes with plasminogen for the binding to fibrin, and interferes thereby with the fibrinolytic process.

We have studied plasmas with a high content in Lp(a), native purified Lp(a) and a recombinant form of apo(a) in a system that mimics the surface of a clot, obeys the biochemical principle of heterogeneously catalyzed reactions and allows monitoring of the progression of fibrinolysis. We show that in spite of the relatively low concentrations of Lp(a) in plasma as compared to plasminogen, a decrease in the generation of plasmin and a parallel increase in the binding of Lp(a) to progressively degraded fibrin were observed (Figs. 1 and 2). Under similar conditions, LDL did not modify the activation of plasminogen and was not detected at the fibrin surface (data not shown). Strictly similar results were obtained with plasmas from the hedgehog (*Erinaceus europaeus*), a typical hibernator known to have Lp(a) as the prominent apolipoprotein B-containing lipoprotein (Laplaud et al., 1988; Rouy et al., 1992b). However, in human studies, conflicting results on the effect of Lp(a) on the fibrinolytic system or on its binding to fibrin have been reported (Eaton and Tomlison, 1989; Alessi et al., 1990; Garcia Frade et al., 1991; Oshima et al., 1991; Smith and Crosbie, 1991). In particular, no modification in the levels of the fibrin parameters have been detected in patients with high levels of Lp(a). With regard to this controversy, it should be considered that, fibrinolysis being a local event directly associated with the surface of a thrombus, the levels of the fibrinolytic parameters in the circulation may not be affected. Moreover, the pathogenic role of Lp(a) on atherosclerotic lesions is most probably a chronic phenomenon that results in growing of the atheroma by the progressive deposition of cholesterol and the accumulation of fibrin due to impaired fibrinolysis. In contrast, myocardial infarction is the acute complication of plaque rupture due to explosive activation of platelets and coagulation zymogens.

Recombinant apo(a) was used to demonstrate that the apo(a) moiety of Lp(a) is responsible for the binding of Lp(a) to fibrin. This recombinant protein has been shown to bind specifically to other apoB-100-containing lipoproteins and to mimic the behavior of apo(a) form plasma-derived lipoprotein(a) (Trieu et al., 1991; Koschinsky et al., 1991). In the present studies, recombinant apo(a) was shown to bind specifically to fibrin with higher affinity than plasminogen (K_d = 30 nM versus 900 nM for plasminogen). As a consequence, the binding of plasminogen to fibrin was competitively inhibited by r-apo(a) (K_i = 44 nM). This result is in agreement with the value of 30 nM reported by Harpel et al. (1989) for the inhibition by Lp(a) of the binding of Glu-plasminogen to immobilized fibrinogen.

Discrepancies in the dissociation constants of Glu-plasminogen and r-apo(a) for the fibrin surface may be explained by the existence of a co-

operative effect by adjacent copies of kringle 4 of r-apo(a) that may not be observed with plasminogen. The absence of binding of the LDL particle to fibrin and the specific binding of both Lp(a) and r-apo(a) indicated clearly that binding of Lp(a) to fibrin is mediated by a mechanism that involves interaction of the kringle domains of apo(a) and the lysine-fibrin binding sites for plasminogen. Attempts were made to characterize the type of kringle-fibrin interactions of r-apo(a) using elastase-derived fragments of plasminogen. The use of these fragments allowed functional isolation of the two types of plasminogen kringles that interact with lysine residues in fibrin: kringle 1 present in fragment kringle 1–3, and the isolated kringle 4. The structure of the lysine binding subsite of kringle 1 and 4 of plasminogen, a hydrophobic trough harboring an anionic/cationic dipole, has been well defined (see review by Tulinsky, 1991). In spite of the high degree of structural homology between plasminogen kringle 4 and the majority of kringles present in the r-apo(a) molecule, the isolated plasminogen kringle 4 failed to inhibit the binding of r-apo(a) to degraded fibrin. The functional integrity of isolated kringle 4 of plasminogen was demonstrated by its ability to inhibit the binding of the parent molecule (Fig. 6) by a mechanism, blockade of lysine-fibrin sites, that decreases the plasminogen B_{max}.

In contrast, the binding of r-apo(a) was efficiently inhibited by fragment kringle 1–3, suggesting that kringle 1 was functionally similar to the r-apo(a) kringle(s) interacting with fibrin; the hydrophobic trough harboring the ionic center of the lysine binding subsite in one or more of the kringles of apo(a) may be composed of amino acids which generate a binding site functionally analogous to that found in plasminogen kringle 1. Furthermore, the affinity of r-apo(a) for carboxy-terminal lysine residues may be enhanced by the

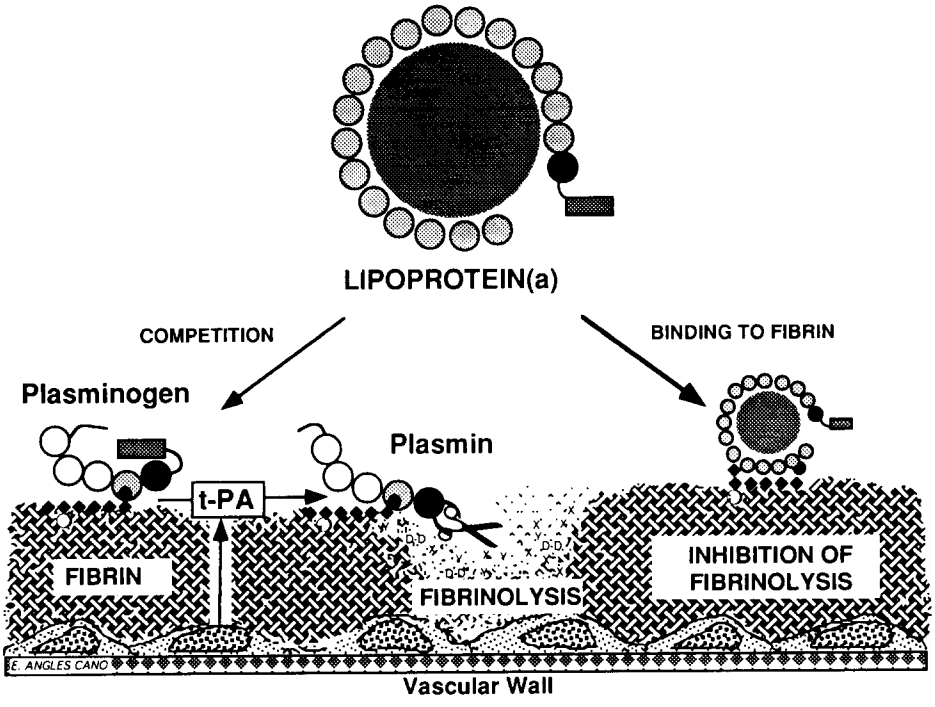

Fig. 7. Schema representing the inhibitory effect of Lp(a) on the binding of plasminogen to the surface of fibrin and on its activation by fibrin-bound t-PA.

sequence arrangement of kringles in the tertiary structure of the protein, thus explaining its higher affinity compared with plasminogen.

5. Conclusions

We have shown that the presence of the unique apo(a) glycoprotein in Lp(a), distinguishing this particle from other apoB-100-containing lipoproteins (LDL), determines the interaction of Lp(a) with fibrin and thereby its hypofibrinolytic effect. Inhibition of the binding of plasminogen by a competitive mechanism results in decreased plasmin generation and increased binding of Lp(a) to fibrin, thus favoring accumulation of cholesterol and fibrin at sites of vascular injury. These mechanisms are summarized in the scheme represented in Fig. 7. Altogether these results provide support for the hypothesis that Lp(a) is an important link between the processes of atherosclerosis and thrombosis.

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7. References

Alessi, M.C., Parra, H.J., Joly, P., Vu-Dac, N., Bard, J.M., Fruchart, J.C., and Juhan-Vague, I., 1990, The increased plasma Lp(a):B lipoprotein particle in angina pectoris is not associated with hypofibrinolysis. Clin. Chim. Acta 188, 119–128.

Anglés-Cano, E., 1986, A spectrophotometric solid-phase fibrin-tissue plasminogen activator activity assay (SOFIA-tPA) for high-fibrin affinity tissue plasminogen activators. Anal. Biochem. 153, 201–210.

Anglés-Cano, E., Rouy, D., and Lijnen, H.R., 1992, Plasminogen binding by α2-antiplasmin and histidine-rich glycoprotein does not inhibit plasminogen activation at the surface of fibrin. Biochim. Biophys. Acta. 1156, 34–42.

Aznar, J., Estellés, A., Breto, M., Espana, F., and Alos, T., 1992, Euglobulin clot lysis induced by tissue-type plasminogen activator is reduced in subjects with increased levels of lipoprotein(a). Thromb. Res. 66, 569–582.

Berg K., 1963, A new serum type system in man: the Lp system. Acta. Pathol. Microbiol. Scand. 59, 369–382.

Chapman, M.J., Laplaud, P.M., Luc, G., Forgez, P., Bruckert, E., Goulinet, S., and Lagrange D., 1988, Further resolution of the low density lipoprotein spectrum in normal human plasma: physicochemical characteristics of discrete subspecies separated by density gradient ultracentrifugation. J. Lipid. Res. 29, 442–458.

Eaton, D., and Tomlinson, J., 1989, Does Lp(a) alter fibrinolysis? Circulation 80 (Suppl. II), II-467.

Eaton, D., Fless, G.M., Kohr, W., McLean, J., Xu, Q., Miller, C., Lawn, R., and Scanu, A.M., 1987, Partial amino acid sequence of apolipoprotein(a) shows that it is homologous to plasminogen. Proc. Natl. Acad. Sci. USA. 84, 3224–3228.

Fless, G.M., Rolih, C.A., and Scanu, A.M., 1984, Heterogeneity of human plasma lipoprotein (a). J. Biol. Chem. 259, 11470–11478.

Fleury, V., and Anglés-Cano, E., 1991, Characterization of the binding of plasminogen to fibrin surfaces: role of carboxy-terminal lysines. Biochemistry. 30, 7630–7638.

Fracker, P.M., and Speck, J.C., 1978, Protein cell menbrane iodinations with a sparingly soluble chloroamide, 1, 3, 4, 6-tetrachloro-3a,6a-diphenylglycoluril. Biochem. Biophys. Res. Comm. 80, 849–857.

Garcia Frade, L.J., Alvarez, J.J., Rayo, I., Torrado, M.C., Lasuncion, M.A., Garcia Avello, A., Hernandez, A., and Marin, E., 1991, Fibrinolytic parameters and lipoprotein(a) levels in plasma of patients with coronary artery disease. Thromb. Res. 63, 407–418.

Golstein, J.L., and Brown, M.S., 1977, The low-density lipoprotein pathway and its relation to atherosclerosis. Annu. Rev. Biochem. 46, 897–930.

Gonzalez-Gronow, M., Edelberg, J.M., and Pizzo S.V., 1989, Further characterization of the cellular plasminogen binding site: evidence that plasminogen 2 and lipoprotein (a) compete for the same site. Biochemistry 28, 2374–2377.

Guo, H.G., Armstrong, V.W., Luc, G., Billardon, C., Goulinet, S., Nutsede, R., Seidel, R., and Chapman J., 1989, Characterization of five monoclonal antibodies to apolipoprotein (a) from human Lp(a): evidence for weak plasminogen reactivity. J. Lip. Res. 30, 23–37.

Hajjar, K.A., Gavish, D., Breslow, J.L., and Nachman, R.L., 1989, Lipoprotein (a) modulation of endothelial cell surface fibrinolysis and its potential role in atherosclerosis. Nature 339, 303–305.

Harpel, P.C., Gordon, B.R., and Parker, T.S., 1989, Plasmin catalyses binding of lipoprotein(a) to immobilized fibrinogen and fibrin. Proc. Natl. Acad. Sci. USA 86, 3847–3851.

Harpel, P.C., Chang, T.S., and Verderber E., 1985, Tissue plasminogen activator and urokinase mediate the binding of Glu-plasminogen to plasma fibrin I. J. Biol. Chem. 260, 4432–4440.

Holvoet, P., Lijnen, H.R., and Collen, D., 1985, A monoclonal antibody specific for lys-plasminogen. Application to the study of the activation pathways of plasminogen in vivo. J. Biol. Chem. 260, 12106–12111.

Hoylaerts, M., Rijken,D.C., Lijnen, H.R., and Collen, D., 1982, Kinetics of the activation of plasminogen by human tissue plasminogen activator. J. Biol. Chem. 257, 2912–2919.

Koschinsky, M., Tomlinson, J., Zioncheck, T., Schartz, K., Eaton, D., and Lawn, R., 1991, Apolipoprotein(a): expression and characterization of a recombinant form of the protein in mammalian cells. Biochemistry 30, 5044–5051.

Krempler, F., Kostner, G.M., Roscher, A., Bolzano, K., and Sandhofer, F., 1983, Studies of the role of specific surface receptors in the removal of lipoprotein (a) in man. J. Clin. Invest. 71, 1433–1441.

Laplaud, M., Beaubatie, L., Rall, S.C., Luc, G., and Saboureau, M., 1988, Lipoprotein(a) is the major apoB-containing lipoprotein in the plasma of a hibernator, the hedgehog (*Erinaceus europaeus*). J. Lipid. Res. 29, 1157–1170.

Leerinck, C.B., Gimpel, J.A., Kortland, W., Bouma, B.N., and van Rijn, H.J.M., 1991, Kinetic analysis of lipoprotein(a) inhibition of plasminogen activation by tissue plasminogen activator in vitro. Fibrinolysis 5, 233–238.

Lerch, P.G., Rickli, E.E., Lergier, W., and Gillessen, D., 1980, Localization of individual lysine-binding regions in human plasminogen and investigation of their complex-forming properties. Eur. J. Biochem. 107, 7–13.

Loscalzo, J., Weinfield, M., Fless, G., and Scanu, A.M., 1990, Lipoprotein(a), fibrin binding, and plasminogen activation. Atherosclerosis 10, 240–245.

Lucas, M.A., Fretto, L.J., and McKee P.A., 1983, The binding of human plasminogen to fibrin and fibrinogen. J. Biol. Chem. 258, 4249–4256.

Mann, K.G., Nesheim, N.E., Church, W.R., Haley, P., Krishnaswamy, S., 1990, Surface-dependent reactions of the vitamin K-dependent enzyme complexes. Blood 76, 1–16.

Masson, C., and Anglés-Cano, E., 1988, Kinetic analysis of the interaction between plasminogen activator inhibitor-1 and tissue-type plasminogen activator. Biochem. J. 256, 237–244.

McLean, J., Tomlinson, J., Kuang, W., Eaton, D., Chen, E., Fless, G., Scanu, A.M., and Lawn, R., 1987, cDNA sequence of human apolipoprotein(a) is homologous to plasminogen. Nature 330, 132–137.

Miles, L.A., Fless, G.M., Levin, E.G., Scanu, A.M., and Plow E.F., 1989, A potential basis for the thrombotic risks associated with lipoprotein(a). Nature 339, 301–302.

Milstone, H., 1941, A factor in normal blood which participates in streptococcal fibrinolysis. J. Immunol. 42, 109–116.

Oshima, S., Uchida, K., Yasu, T., Uno, K., Nonogi, H., and Haze, K., 1991, Transient increase of plasma lipoprotein(a) in patients with unstable angina pectoris: does lipoprotein(a) alter fibrinolysis? Arterioscler. Thromb. 11, 1772–1777.

Patthy, L., Trexler, M., Vali, Z., Banayi, L., and Varadi, A., 1984, Kringles: modules specialized for protein binding. FEBS Lett. 171, 131–136.

Petersen, L.C., Johannessen, M., Foster, D., Kumar, A., and Mulvihill, E., 1989, The effect of polymerized fibrin on the catalytic activities of one-chain tissue-type plasminogen activator as revealed by an analogue resistant to plasmin cleavage. Biochim. Biophys. Acta 952, 245–254.

Rhoads, G.G., Dahlén, G., Berg, K., Morton, N.E., and Dannenberg, A.L., 1986, Lp(a) lipoprotein as a risk factor for myocardial infarction. J. Am. Med. Assoc. 256, 2540–2544.

Rouy, D., and Anglés-Cano, E., 1990, The mechanism of activation of plasminogen at the fibrin surface by t-PA in a plasma milieu in vitro: role of alpha-2 antiplasmin. Biochem. J. 271, 51–57.

Rouy, D., Grailhe, P., Nigon, F., Chapman, J., and Anglés-Cano, E., 1991, Lipoprotein (a) impairs generation of plasmin by fibrin-bound tissue-type plasminogen activator. Arterioscler. Thromb. 11, 629–638.

Rouy, D., Koschinsky, M.L., Fleury, V., Chapman, M.J., and Anglés-Cano, E., 1992a, Apolipoprotein(a) and plasminogen interactions with fibrin: a study with recombinant apolipoprotein(a) and isolated plasminogen fragments. Biochemistry 31, 6333–6339.

Rouy, D., Laplaud, M., Saboureau, M., and Anglés-Cano, E., 1992b, Hedgehog lipoprotein (a) is a modulator of the activation of plasminogen at the fibrin surface: an in vitro study. Arterioscler. Thromb. 12, 146–154.

Sandkamp, M., Funke, H., Schulte, H., Kohler, E., and Assmann, G., 1990, Lipoprotein(a) is an independent risk factor for myocardial infarction at a young age. Clin. Chem. 36, 20–23.

Scanu, A.M., and Fless, G.M., 1990, Lipoprotein(a) heterogeneity and biological relevance. J. Clin. Invest. 85, 1709–1715.

Schafer, A.I., 1987, Focussing on the clot: normal and pathologic mechanisms. Ann. Rev. Med. 38, 211–220.

Smith, E.B., and Crosbie, L., 1991, Does lipoprotein(a) (Lp(a)) compete with plasminogen in human atherosclerotic lesions and thrombi? Arteriosclerosis 89, 127–136.

Sottrup-Jensen, L., Claeys, H., Zaydel, M., Petersen, T.E., and Magnusson, S., 1978, The primary structure of human plasminogen: isolation of a two lysine-binding fragments and one 'mini'-plasminogen (MW 38000) by elastase-catalysed-specific limited proteolysis, in: Davidson et al. (Eds.), Progress in Chemical Fibrinolysis and Thrombosis, Raven, New York, 3, 191–209.

Suenson, E., Lutzen, O., and Thorsen, S., 1984, Initial plasmin-degradation of fibrin a basis of a positive feed-back mechanism in fibrinolysis. Eur. J. Biochem. 140, 513–522.

Sugiyama, N., Iwamoto, M., Abiko, Y., 1987, Effects of kringles derived from human plasminogen on fibrinolysis in vitro. Thromb. Res. 47, 459–468.

Tran-Thang, C., Kruithof, E.K.O., Atkinson, J., and Bachman, F., 1986, High-affinity binding sites for human Glu-plasminogen unveiled by limited plasmic degradation of human fibrin. Eur. J. Biochem. 160, 599–604.

Tulinsky, A., 1991, The structures of domains of blood proteins. Thromb. Haemost. 66, 16–31.

Trieu, V.N., Zioncheck, T.F., Lawn, R.M., and McConathy, W.J., 1991, Interaction of apolipoprotein (a) with apolipoprotein B-containing lipoproteins. J. Biol. Chem. 266, 5480–5485.

Vali Z., and Patthy L., 1984, The fibrin-binding site of human

plasminogen: arginines 32 and 34 are essential for fibrin affinity of the kringle 1 domain. J. Biol. Chem. 259, 13690–13694.

Wiman, B., and Collen, D., 1978, Molecular mechanisms of physiological fibrinolysis. Nature 272, 349–350.

Wu, T.P., Kaillathe, P., Tulinsky, A., and Mulichak, A.M., 1991, The refined structure of the ε-aminocaproic acid complex of human plasminogen kringle 4. Biochemistry 30, 10589–10594.

Wu, H.L., Chang, B.I., Wu, D.H., Chang, L.C., Gong, C.C., Lou, K.L., and Shi, G.Y., 1990, Interaction of plasminogen and fibrin in plasminogen activation. J. Biol. Chem. 265, 19658–19664.