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Plasmin catalyzes binding of lipoprotein (a) to immobilized fibrinogen and fibrin

(atherosclerosis/vascular disease/inflammation/fibrinolysis/thrombosis)

PETER C. HARPEL, BRUCE R. GORDON, AND THOMAS S. PARKER

Division of Hematology-Oncology, Department of Medicine, Specialized Center of Research in Thrombosis and The Rogosin Institute, The New York Hospital–Cornell Medical Center, New York, NY 10021

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ABSTRACT **Lipoprotein (a) [Lp(a)] is a plasma component whose concentration is related to the development of atherosclerosis, although the underlying mechanisms are not known. Lp(a) contains a unique structure, apolipoprotein (a), that shares partial homology with plasminogen. We now report that plasmin catalyzes the binding of Lp(a) to both immobilized fibrinogen and fibrin in a manner analogous to our previously reported studies with plasminogen. Plasmin treatment of immobilized fibrinogen induces a 3.7-fold increase in Lp(a) binding. Low density lipoprotein, molecules similar to Lp(a) but lacking apolipoprotein (a), bind poorly to immobilized fibrinogen and binding is not increased by plasmin. Trypsin but not neutrophil elastase also increases the binding of Lp(a) to fibrinogen. Lp(a) also complexes to plasmin–fibrinogen digests, and binding increases in proportion to the time of plasmin-induced fibrinogen degradation. Lp(a) binding is lysine-binding site dependent as it is inhibited by ε-aminocaproic acid. Lp(a) inhibits the binding of plasminogen to plasmin-modified immobilized fibrinogen, indicating that both molecules compete for similar lysine-binding sites. These findings demonstrate an affinity between Lp(a) and protease-modified fibrinogen or fibrin and thereby provide a potential mechanism to explain the association between thrombosis, coronary atherosclerosis, and increased blood concentrations of Lp(a).**

Lipoprotein (a) [Lp(a)] was first identified by Berg in 1963 (1). The concentration of this lipoprotein varies widely in humans, and elevated plasma concentrations are correlated with an increased risk of cardiovascular disease (2). Structural studies of Lp(a) indicate that the molecule is closely related to low density lipoprotein (LDL). The unique characteristic of Lp(a) is that it contains another glycoprotein, apolipoprotein (a), that is disulfide-linked to the apolipoprotein B-100 molecule (3). Recent studies indicate a remarkable structural homology between Lp(a) and a portion of the plasminogen molecule (4, 5). Apolipoprotein (a) contains 37 copies of kringle 4, one copy of kringle 5, and the protease region of plasminogen, which cannot be activated because of substitution of arginine by serine in the active site (5). As yet, the mechanisms by which Lp(a) predisposes to vascular disease or of the function of the apolipoprotein (a) in the interaction of Lp(a) with other molecules is not known.

Many of the functions of plasminogen appear to be enhanced by its kringle structures. The lysine-dependent binding of plasminogen to fibrin is mediated by plasminogen kringle 1, whereas kringles 2–5 have less or no affinity (6). Although Lp(a) has been shown to bind to lysine-Sepharose (4), the binding to fibrin is not documented (5). Recent studies have demonstrated that plasminogen activators enhance the binding of Glu-plasminogen, the native form of the molecule, to a fibrin clot (7–9). Analysis of this interaction has demonstrated that the plasmin formed by plasminogen activation induces modifications in the forming fibrin substrate that results in the exposure of plasminogen-binding sites (9). Fibrinogen derivatives produced by incubating plasmin with native fibrinogen also demonstrated increased ability to incorporate Glu-plasminogen when clotted (9). The binding of Glu-plasminogen to plasmin-modified fibrinogen or fibrin was dependent on the lysine-binding sites of plasminogen since this molecule was eluted from the fibrin network by ε-aminocaproic acid (εACA), a lysine analogue that interrupts lysine-binding site interactions.

These observations, that the serine protease plasmin participates in a positive feedback control mechanism that increases the binding of its zymogen, plasminogen, to fibrinogen and fibrin, suggested that similar mechanisms might be operative in reactions between Lp(a), fibrinogen, and fibrin. In the present study, we have examined the binding of Lp(a), and as a control, LDL, to surface-immobilized fibrinogen and fibrin. We have demonstrated that plasmin-induced modifications dramatically increase the binding of Lp(a), and not LDL, to the immobilized proteins. This binding is lysine-binding site dependent as it is inhibited by εACA. Furthermore, Lp(a) inhibits the binding of Glu-plasminogen to plasmin modified-immobilized fibrinogen. These studies provide a rationale for the relationship between increased blood concentrations of Lp(a) and coronary atherosclerosis since binding of Lp(a) to fibrin may potentiate incorporation of this lipoprotein into the vessel wall.

MATERIALS AND METHODS

Lp(a) and LDL Purification. Lp(a) was purified from plasma by minor modifications of described methods (10). Fresh plasma from a donor with an elevated Lp(a) concentration was treated with the following protease inhibitors and reagents: soybean trypsin inhibitor and pancreatic trypsin inhibitor (10 mg/dl), D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (Calbiochem; 0.1 mM), *p*-nitrophenyl-*p'*-guanidinobenzoate hydrochloride (NPGB) and phenylmethylsulfonyl fluoride (PMSF; 0.1 mM), εACA (0.005 M), and EDTA (0.005 M). After the two ultracentrifugation steps as detailed (10), the Lp(a) fraction was applied to a Sephacryl S-400 HR column (2.6 × 90 cm; Pharmacia) in Tris·HCl (0.05 M; pH 7.2) containing 0.15 M NaCl, 0.005 M EDTA, 0.02% sodium azide. The first peak contained Lp(a) separated from LDL as indicated by native acrylamide gel electrophoresis (see Fig. 2*A*). LDL was purified by density ultracentrifugation followed by Sephacryl S-400 column chromatography. Peak fractions did not contain Lp(a) as assessed by slab gel electrophoresis (see Fig. 2*A*) or by rocket immunoelectrophoresis, using an immunospecific sheep anti-human Lp(a)

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Abbreviations: Lp(a), lipoprotein (a); LDL, low density lipoprotein; εACA, ε-aminocaproic acid; PMSF, phenylmethylsulfonyl fluoride; NPGB, *p*-nitrophenyl-*p'*-guanidinobenzoate hydrochloride.

kindly provided by E. Molinari (Immuno AG, Vienna), methods that would detect Lp(a) contamination above ≈5%. Protein concentrations of the lipoprotein fractions were determined by the Lowry procedure in the presence of SDS. Protein concentration was converted from mg of protein into mg of lipoprotein by using the coefficients 2.8 and 4.8 for Lp(a) and LDL, respectively (11). Lp(a) and LDL were labeled with ^{125}I as described (10).

Protein Purification. Fibronectin and plasminogen were removed from human fibrinogen (Kabi type L) by affinity chromatography on insolubilized calf skin gelatin and lysine as detailed (9). Human Glu-plasminogen was prepared as described (9). Fibrinogen and plasminogen were labeled with ^{125}I as indicated (9). Plasmin was prepared by activation of plasminogen with agarose-immobilized urokinase as detailed (12), or it was obtained from American Diagnostica (New York, NY). Human neutrophil elastase and bovine β-trypsin were purified as detailed (13). Recombinant tissue plasminogen activator was a generous gift from Genentech. The immunoaffinity-purified IgG fraction of sheep anti-human LDL (a gift of Immuno AG) was labeled with alkaline phosphatase as described (12). Gradient gel electrophoresis on 2–16% acrylamide gels was performed according to the manufacturer's instructions (Pharmacia).

Demonstration of Binding of Plasminogen and Lp(a) to Immobilized Fibrinogen or Fibrin by ELISA. Assays were performed in triplicate by methods previously described (12). Microtitration plates (Nunc) were coated with fibrinogen or human albumin (Miles) (0.1 ml containing 5 μg of protein per ml of bicarbonate coating buffer) and incubated in a humid chamber overnight at 4°C. The wells were washed three times for 3 min each in 0.15 M phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20 (PBS/Tween). In some studies, human thrombin (0.1 ml containing 0.3 μg) was added for a 1-hr incubation to convert the absorbed fibrinogen to fibrin. To determine the effect of plasmin on the subsequent binding of the various ligands studied, plasmin (0.05 ml containing 100 ng/ml) in PBS/Tween or buffer alone was added to the wells. After incubation for 30 min at room temperature, the wells were incubated with a solution containing the serine protease inhibitors NPGB and PMSF (final concentration, 0.1 mM) for 20 min. After an additional wash, various concentrations of Glu-plasminogen, Lp(a), or LDL in 0.1 M Tris·HCl (pH 7.2) containing 0.005 M EDTA and 0.05% Tween 20 with or without 0.05 M εACA was added (0.05 ml) for a 90-min incubation at room temperature. After washing the detecting antibody, either rabbit anti-plasminogen IgG or sheep anti-LDL IgG labeled with alkaline phosphatase was added. After an 18-hr incubation in a humid chamber at 4°C, the wells were washed and 0.2 ml of the substrate *p*-nitrophenyl phosphate (Sigma) (1 mg/ml in 10% diethanolamine buffer, pH 9.8) was added. The color development at 405 nm was followed in a V_{max} microplate reader (Molecular Devices, Palo Alto, CA). The results are expressed as change in absorbance per min × 10^4.

Studies of the Binding of Lp(a) to a Progressive Fibrinogen Digest. The binding of Lp(a) and LDL to fibrinogen that had been degraded by plasmin in the fluid phase was studied. Plasmin (2.5 μg/ml) was incubated at room temperature with human fibrinogen (1.33 mg/ml) that contained a trace amount of ^{125}I-labeled fibrinogen. The reaction was stopped at various time intervals by the addition of NPGB and PMSF (final concentration, 0.1 mM). The digests were diluted to 10 μg/ml in coating buffer, and 0.1 ml was added to the wells of microtiter plates. After an 18-hr incubation, the wells were washed and the binding of Lp(a) and LDL was assessed, as detailed above, by ELISA. Clottability of the fibrinogen digest was determined by adding thrombin (1 unit/ml) and determining the incorporated radioactivity in the washed clot as described (9).

Effect of Trypsin or Neutrophil Elastase on the Binding of Lp(a) to Immobilized Fibrinogen. Microtitration plate wells were coated with fibrinogen, following which plasmin, bovine β-trypsin, or human neutrophil elastase (5 ng per well) was added for 30 min. After washing, an inhibitor solution containing NPGB and PMSF (0.1 mM) was added for an additional 20 min. Lp(a) with or without εACA was added for a 90-min incubation. After washing, the bound Lp(a) was determined by ELISA.

Inhibition of Glu-Plasminogen Binding to Plasmin-Treated Immobilized Fibrinogen by Lp(a). Microtiter plates were coated with fibrinogen as described above, and, after washing, the wells were incubated for 30 min with plasmin (5 ng). The wells were then incubated with the inhibitors NPGB and PMSF for an additional 10 min and then washed two times with PBS/Tween for 3 min each. Mixtures of plasminogen (8 μg/ml) with various concentrations of Lp(a) or LDL (0.05 ml per well) as indicated in Fig. 5 were added for 3 hr at 37°C. To produce a standard curve, various concentrations of plasminogen (1–8 μg/ml) were added to the plasmin-treated fibrinogen. After washing, 0.3 ml of a solution containing tissue plasminogen activator (100 ng) and the fluorometric plasmin substrate, D-Val-Leu-Lys-AFC (AFC, 7-amino-4-trifluoromethyl coumarin) (Enzyme System Products, Livermore, CA; final concentration, 10 μM) was added and incubated at 37°C for 3 hr. Fluorescence was measured by transferring well contents to cuvettes and recording relative fluorescence at an excitation wavelength of 400 nm and emission wavelength of 505 nm in a Perkin–Elmer 650-10S fluorescence spectrophotometer. Fluorescence was converted to percent activity by reference to the standard curve produced by plotting the amount (%) of plasminogen added (12.5–100%) against relative fluorescence units.

RESULTS

Binding of Glu-Plasminogen, Lp(a), and LDL to Immobilized Fibrinogen and the Effect of Plasmin. To determine whether plasminogen would bind to immobilized fibrinogen as described for a fluid-phase fibrin-clotting system (9), increasing concentrations of Glu-plasminogen were added to the wells of microtitration plates coated with fibrinogen preincubated either with buffer or with plasmin (Fig. 1). The plasminogen bound to the fibrinogen was quantified by ELISA. Plasmin treatment of the surface-bound fibrinogen increased the subsequent binding of plasminogen by ≈3.2-fold at the highest concentration of ligand added, as compared to the binding to untreated fibrinogen. εACA inhibited plasminogen binding to both untreated and plasmin-treated fibrinogen.

An ELISA was also used to determine the binding of purified Lp(a) or LDL to immobilized fibrinogen. With increasing concentrations of Lp(a) added, there was an increase in binding that approached saturation, with an apparent K_d of 2 nM (Fig. 2*A*). Plasmin treatment of immobilized fibrinogen markedly increased the binding of Lp(a) (Fig. 2*B*). The binding of Lp(a) to plasmin-treated fibrinogen was greater than that observed with the untreated fibrinogen surface at all concentrations tested. At the highest concentration of Lp(a) studied, 22.4 nM, ≈3.7-fold more lipoprotein bound to the plasmin-treated fibrinogen than to the unmodified fibrinogen. εACA inhibited the increase in binding of Lp(a) to plasmin-treated fibrinogen. In studies not shown, the binding of Lp(a) to immobilized fibrin was found to be identical to that described for fibrinogen, with a similar increase in binding after modification of the fibrin surface by plasmin, and a similar inhibition of binding by εACA.

The binding of LDL to immobilized fibrinogen was also studied by ELISA as detailed for Lp(a). In contrast to the binding of Lp(a), only trace amounts of LDL bound to the fibrinogen surface at a concentration of ligand below 4 nM.

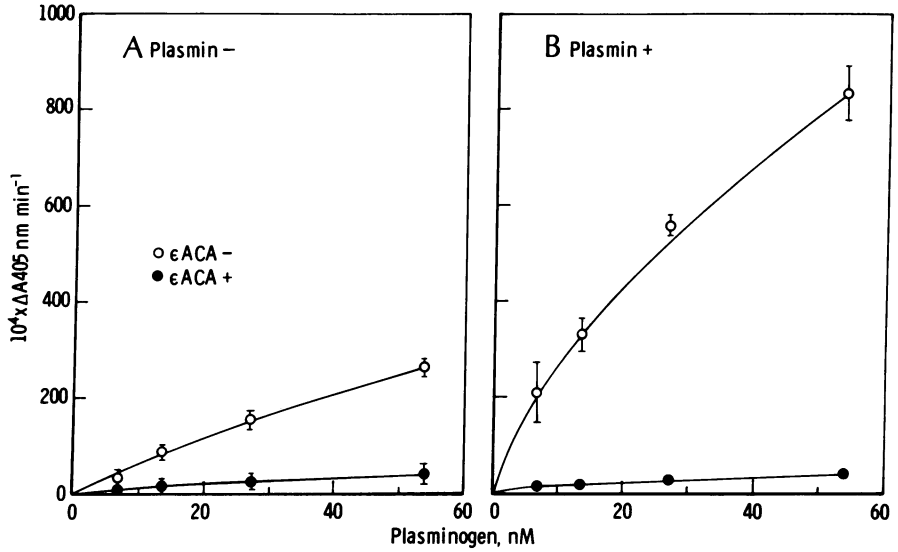

FIG. 1. Binding of Glu-plasminogen to immobilized fibrinogen. Microtiter plate wells coated with human fibrinogen were incubated with buffer (*A*) or with plasmin (*B*). After incubation for 30 min and neutralization of plasmin with protease inhibitors, increasing concentrations of plasminogen in Tris·HCl/EDTA/Tween (○) or in buffer containing 0.05 M εACA (●) were added. After incubation for 90 min, the wells were washed, and the binding of plasminogen was determined by ELISA. The SEM is indicated.

Although at higher concentrations there was a progressive increase in the amount of LDL that bound to the immobilized fibrinogen, plasmin-treatment did not increase binding, nor did εACA affect the binding of LDL to the immobilized fibrinogen.

Lysine-dependent binding of plasminogen, Lp(a), and LDL to immobilized fibrinogen or to albumin was quantified (Table 1). The ratio of lysine-site-dependent binding of plasminogen and Lp(a) to plasmin-modified immobilized fibrinogen as compared to untreated fibrinogen was 3.6 and 4.3, respectively. Only small amounts of plasminogen or Lp(a) bound to immobilized human albumin. In contrast to our observations with fibrinogen, plasmin treatment of the albumin surface did not increase the subsequent binding of Lp(a). Negligible binding of LDL to immobilized fibrinogen or albumin was observed. These studies indicate the relative specificity and the lysine-binding site dependence of the interaction between both plasminogen and Lp(a) and fibrinogen or fibrin.

Binding of Lp(a) to Plasmin–Fibrinogen Digests. Fig. 3*A* documents that Lp(a) binds to fibrinogen degraded by plasmin prior to immobilization. In this study, catalytic amounts of plasmin were incubated with fibrinogen for various time periods, and the reaction was stopped with serine protease inhibitors. The thrombin clottability of the fibrinogen decreased linearly with increasing incubation time (96–7.4%). SDS/polyacrylamide slab gel electrophoresis analysis documented progressive degradation of fibrinogen, with the final 10-min incubation mixture containing mainly fibrinogen fragments Y, D, and E. The timed digests were absorbed to microtiter plate wells, and either Lp(a) or LDL was added. Increasing amounts of Lp(a) bound to the fibrinogen digests as compared to the intact fibrinogen. As the time of interaction between plasmin and fibrinogen increased, the amount of Lp(a) bound to the immobilized digest also increased. εACA inhibited this increase in binding. The specific lysine-site-dependent binding of Lp(a) increased ≈12-fold during the course of plasmin digestion. No increase in the binding of LDL to the progressive fibrinogen digest was observed (Fig. 3*B*). In a study not shown, a terminal fibrinogen digest containing primarily fragments D and E also bound Lp(a) but not LDL.

Effect of Trypsin and Neutrophil Elastase on the Binding of Lp(a) to Fibrinogen. Immobilized fibrinogen was treated with trypsin or neutrophil elastase and the subsequent binding of Lp(a) was compared to the binding to plasmin-treated fibrinogen. Plasmin-treated fibrinogen demonstrated the greatest capacity to bind Lp(a). Fibrinogen incubated with trypsin bound ≈71% of the Lp(a) bound to the plasmin-modified fibrinogen. In contrast, neutrophil elastase did not increase the binding of Lp(a) to fibrinogen. Quantification of radiolabeled fibrinogen remaining surface bound after incubation with the proteinases documented a similar 15–20% decrease, indicating that the observed differences in binding of Lp(a) were not due to a differential loss of immobilized fibrinogen.

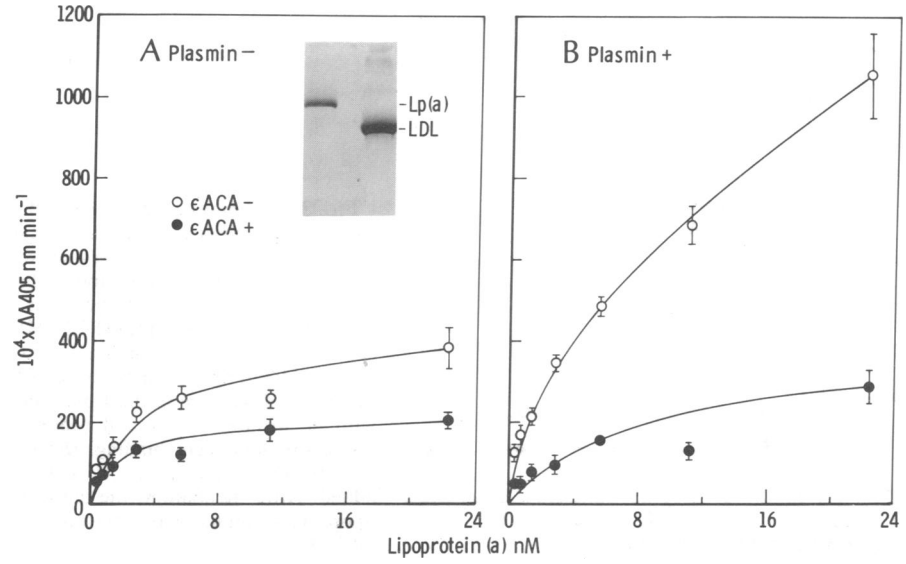

FIG. 2. Binding of Lp(a) to immobilized fibrinogen. Microtiter plate wells coated with human fibrinogen were incubated with buffer (*A*) or with plasmin (*B*). Lp(a) with (●) or without (○) εACA was added, and the binding was detected by ELISA. (*Inset*) Native polyacrylamide gradient gel (2–16%) electrophoresis of Lp(a) and LDL.

Table 1. Lysine-dependent binding of plasminogen, Lp(a), and LDL to immobilized fibrinogen or albumin

Immobilized protein	Ligand added	Lysine-dependent binding	
		+ plasmin	− plasmin
Fibrinogen	Plasminogen	1.75 ± 0.17	0.49 ± 0.02
Albumin		0.2 ± 0.03	0.08 ± 0.07
Fibrinogen	Lp(a)	2.3 ± 0.6	0.53 ± 0.18
Albumin		0.1 ± 0.2	0
Fibrinogen	LDL	0.06 ± 0.08	0.04 ± 0.04
Albumin		0.08 ± 0.1	0.04 ± 0.04

Microtiter plate wells were coated with human fibrinogen or albumin followed by incubation with plasmin (+) or buffer (−). Plasminogen (60 nM), Lp(a), or LDL (22.4 nM) was added in a vol of 0.05 ml of buffer, or in buffer containing εACA, and the ligand bound after a 90-min incubation was detected by ELISA. Incubations were done in triplicate. The SD is indicated. Lysine-dependent binding was calculated by subtracting the ng of ligand bound in the presence of εACA from that without εACA. The ELISA absorbance values were converted to ng of ligand bound with curves constructed by comparing the amount of radiolabeled ligand bound to the wells of the microtiter plate with the color developed by the detecting sheep anti-LDL immunoglobulin labeled with alkaline phosphatase.

Inhibition of Binding of Glu-Plasminogen to Plasmin-Treated Immobilized Fibrinogen by Lp(a). Since the binding of both plasminogen and Lp(a) to plasmin-treated fibrinogen or fibrin is lysine-binding site dependent, the possibility that both molecules compete for the same binding sites was examined. Glu-plasminogen (90 nM) with increasing concentrations of either Lp(a) or LDL (1.6–50 nM) was added to wells containing immobilized plasmin-treated fibrinogen (Fig. 4). The amount of plasminogen bound to the fibrinogen surface was then quantified by activation with tissue plasminogen activator. Increasing concentrations of Lp(a), but not LDL, progressively inhibited the amount of plasmin activity subsequently generated, indicating an inhibition in the binding of Glu-plasminogen to the plasmin-modified fibrinogen surface. At the highest concentration of Lp(a) added (50 nM), in the presence of 90 nM plasminogen, the plasminogen bound was 36.9% of that bound in the absence of the lipoprotein. The apparent K_i for the reaction was 30 nM. In studies not shown, Lp(a) inhibited the binding of ^{125}I-labeled plasminogen to plasmin-treated immobilized fibrinogen in a quantitatively similar manner.

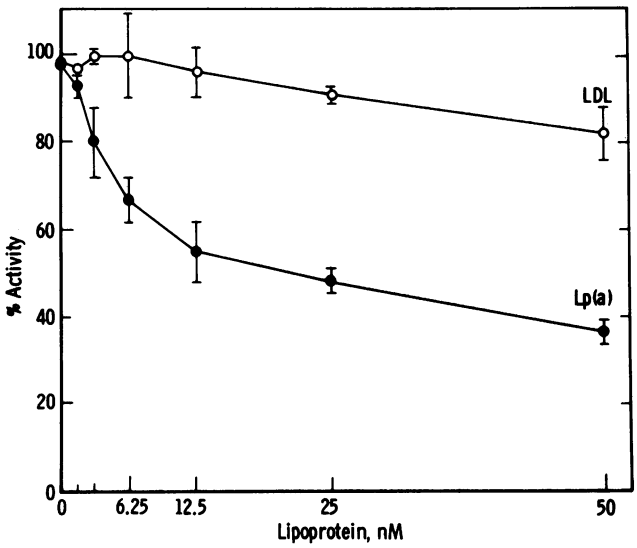

FIG. 4. Inhibition of binding of plasminogen to immobilized fibrinogen by Lp(a). Microtiter plate wells were coated with human fibrinogen and then incubated with plasmin. After inhibition of the plasmin and washing, mixtures of plasminogen (final concentration, 90 nM) with various concentrations of LDL (○) or Lp(a) (●) were added for a 3-hr incubation. After washing, a solution containing tissue plasminogen activator and substrate, Val-Leu-Lys-AFC, was added for 3 hr at 37°C. Release of 7-amino-4-trifluoromethyl coumarin was determined, and the binding of plasminogen was expressed as percent activity. The SD is indicated.

DISCUSSION

The present study is based on our prior observations that plasmin-induced modifications in fibrinogen dramatically increase Glu-plasminogen binding sites (9). We have found that the reaction of plasminogen with surface-bound fibrinogen is similar to that previously detailed for the binding of plasminogen to fibrinogen or fibrin in a fluid-phase clotting system. Plasmin pretreatment of the fibrinogen surface markedly increases the binding the Glu-plasminogen, and binding is almost completely abolished by εACA, indicating the lysine-binding site dependence of the plasminogen immobilized fibrinogen interaction. Since apolipoprotein (a) contains repetitive kringle 4 structures (5), and since kringle 4 on the plasminogen molecule has lysine-binding site properties, we have explored whether Lp(a) would also bind to plasmin-modified immobilized fibrinogen and fibrin.

We have found that Lp(a), similar to plasminogen, displays increased binding to immobilized fibrinogen and fibrin that have been plasmin-treated as compared to the untreated immobilized proteins. This increase in binding is inhibited by

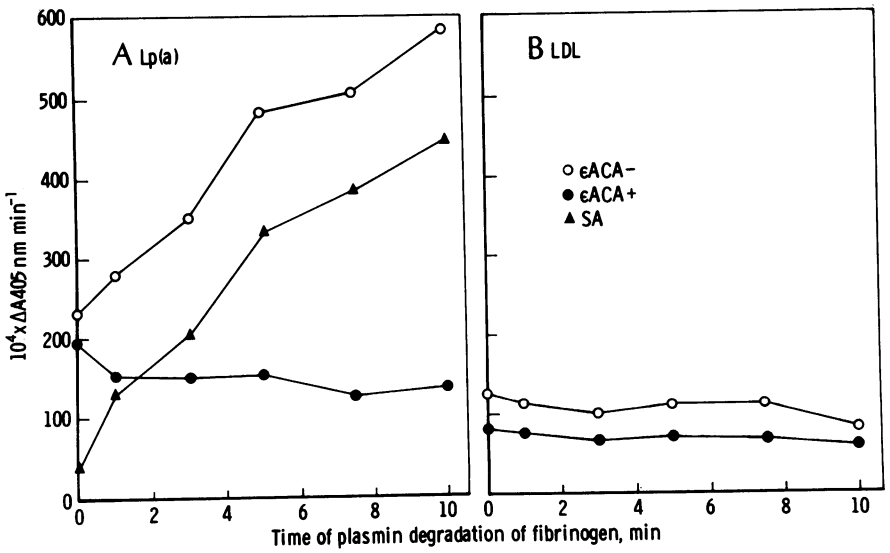

FIG. 3. Binding of Lp(a) and LDL to immobilized fibrinogen plasmin digests. Human fibrinogen was digested with plasmin for various time periods. These digests were absorbed to the wells of microtiter plates. (*A*) Lp(a) (11.2 nM) in buffer (○), or in buffer containing εACA (●) was added. Specific activity (▲) is the calculated difference of the Lp(a) bound with or without εACA and represents lysine-site-dependent binding. (*B*) LDL (11.2 nM) in buffer (○) or εACA (●) was added. After incubation, the amount of lipoprotein bound was determined by ELISA.

εACA, indicating the lysine-binding site dependence of the reaction. The specificity of the Lp(a) fibrinogen interaction is underlined by parallel studies that have found that only small amounts of LDL bind to immobilized fibrinogen, and that this binding is not increased by plasmin pretreatment. Furthermore, Lp(a) fails to bind significantly to plasmin-treated immobilized human albumin.

The ability of Lp(a) to bind to plasmin-treated immobilized fibrinogen also extended to the binding of Lp(a) to fibrinogen degraded by plasmin in solution and subsequently bound to the wells of microtitration plates. With increasing degradation of fibrinogen, there is a progressive increase in the amount of Lp(a) bound to the immobilized digests. This interaction between Lp(a) and the surface-bound fibrinogen digest is inhibited by εACA. These studies suggest that there is an increase in newly exposed lysine residues on the fibrinogen molecule as plasmin digestion occurs, and that these lysyl groups have the capacity to interact with the Lp(a) molecule. Whether Lp(a) binds to all products of plasmin digestion of fibrinogen or fibrin remains to be determined. Our results indicate, however, that Lp(a) will bind to an immobilized terminal fibrinogen digest, indicating that fibrinogen fragments D or E, or both, possess Lp(a) binding sites. Our studies also demonstrate that Lp(a) and plasminogen share similar binding sites on the fibrinogen/fibrin molecule. Increasing concentrations of Lp(a), but not LDL, were found to progressively inhibit the binding of Glu-plasminogen to the fibrinogen surface. These studies parallel a recent report that Lp(a) inhibits the fibrin-dependent enhancement of tissue plasminogen activator activity (14).

The interaction between immobilized fibrinogen and plasmin is relatively specific since human neutrophil elastase does not cause an augmentation of Lp(a) binding when incubated with immobilized fibrinogen. Trypsin, a proteinase that cleaves both arginyl and lysyl bonds, displayed an activity 71% of that of plasmin in promoting Lp(a) binding, indicating that serine proteinases with lysine specificity other than plasmin may also affect Lp(a) binding to fibrinogen/fibrin.

Our studies have thus demonstrated that Lp(a) possesses lysine-binding site structures that interact with fibrinogen or fibrin, and that this interaction is catalyzed by plasmin. These observations provide a potential mechanism for the association between elevated Lp(a) blood levels and vascular disease. The forming fibrin thrombus at the damaged vessel wall has the capacity to bind Lp(a). Activation of plasminogen at the fibrin surface would substantially increase Lp(a) binding. Paradoxically, the binding of Lp(a) to fibrin would impair plasminogen binding and thereby might inhibit further thrombus degradation. Repair mechanisms would then incorporate the persisting thrombus with its bound Lp(a) into the blood vessel wall, resulting in the accumulation of this lipoprotein in the forming atherosclerotic plaque.

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