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R L Silverstein, … , R L Nicholas, R L Nachman

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Research Article

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Binding of Lys-Plasminogen to Monocytes/Macrophages

Roy L. Silverstein, Robert J. Friedlander, Jr., Robert L. Nicholas, and Ralph L. Nachman
Department of Medicine, Division of Hematology-Oncology, and the Specialized Center of Research in Thrombosis, Cornell University Medical College, New York, New York 10021

Abstract

The ability of mononuclear phagocytes to assemble and activate components of the fibrinolytic system on their surfaces may be crucial in effecting an efficient inflammatory response. Lys-plasminogen, the plasmin modified form of this zymogen, was found to bind specifically and with high affinity to murine peritoneal macrophages and to cells of the human monocytoid line U937. This modified plasminogen has been shown to be a more efficient substrate for plasminogen activators than native Glu-plasminogen. Binding was lysine binding site dependent, rapid and reversible. In contrast, although native Glu-plasminogen bound specifically to these cells, affinity was low. Lys-plasminogen inhibited the binding of Glu-plasminogen but the opposite was not true. Molecular analysis of the bound ligands indicated that Glu-plasminogen was converted to Lys-plasminogen and Lys-plasminogen to plasmin on the cell surface but not in the supernatant. Peritoneal macrophages from patients with indwelling catheters and tissue macrophages in chronic inflammatory lesions were shown to express immunologically identified Lys-plasminogen on their surfaces. Therefore binding and surface activation of kinetically favored Lys-plasminogen may provide an important physiological mechanism for localizing proteolytic activity on the surface of inflammatory cells.

Introduction

Lys-plasminogen, containing an NH_2-terminal lysine, is formed from native NH_2-terminal glutamic acid plasminogen by plasmin digestion (1). Although the physiological relevance of this modified zymogen is unknown, it has been shown kinetically to be a more favorable substrate for plasminogen activators (2) than Glu-plasminogen. In addition Lys-plasminogen binds to fibrin with 100-fold greater affinity than Glu-plasminogen (3). In purified systems, activation of Glu-plasminogen yields Lys-plasmin, suggesting that plasminogen activation occurs via formation of a Lys-plasminogen intermediate (4). Holvoet et al., however, using a sensitive monoclonal antibody for Lys-plasminogen, were consistently able to detect only small amounts of Lys-plasminogen in the plasma of patients receiving therapeutic doses of tissue plasminogen activator. They concluded that Lys-plasminogen was not a major physiological intermediate in plasmin formation (5).

In addition to its role in fibrinolysis, plasmin generation has been postulated to play an important role in biological events such as inflammation (6), tumor cell invasion and implantation (7), ovulation and implantation (8), and neurodevelopment (9). We have hypothesized that plasmin generation in these circumstances may occur on biological surfaces other than fibrin (10) and have demonstrated that plasminogen specifically binds to purified thrombospondin and can be activated with favorable kinetics (10). In like manner this has been demonstrated for endothelial cell monolayers (11) and vascular subendothelial extracellular matrix (12). As with fibrin, we also demonstrated that the Lys-form of plasminogen bound to thrombospondin to a greater extent than the native Glu-form (13). In addition our studies suggest that when Glu-plasminogen is activated on a matrix or on a thrombospondin coated surface, there is preferential formation of Lys-plasminogen (12, 13). Recently, it was reported that U937 cells, a human monocytoid cell line derived from a patient with histiocytic lymphoma (14), possess specific binding sites for both urokinase and plasminogen (15). In this study we demonstrate that native Glu-plasminogen binds with very low affinity to U937 cells and murine macrophages, while in sharp contrast, Lys-plasminogen binds specifically and with high affinity. Evidence will be presented that Glu- to Lys-plasminogen conversion and Lys-plasminogen to plasmin conversion occurs on the cell surface due to endogenous plasminogen activators. We also demonstrate the presence of Lys-plasminogen on the surface of activated macrophages from the peritoneal cavity of patients with indwelling catheters and from sites of chronic inflammation.

Methods

Materials. Lysine agarose was purchased from Pharmacia Fine Chemicals (Piscataway, NJ), ε-amino-*n*-caproic acid (EACA)[1] and D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) from Calbiochem-Behring (La Jolla, CA), BSA (Pentex) from Miles Laboratories (Naperville, IL). The fluorometric plasmin substrate, D-Val-Leu-Lys-7-amino-4-trifluoromethyl coumarin was from Enzyme Systems Products (Livermore, CA). Carrier-free ^{125}I-NaI was obtained from New England Nuclear (Boston, MA), silicone oil (Dow Corning 550 and 556) from Dow Corning Corp. (Midland, MI), *p*-nitrophenylphosphate and soybean trypsin inhibitor from Sigma Chemical Co. (St. Louis, MO), and 96 well microtiter plates (nunc-immunoplates) from Vangard International (Neptune, NJ). Sterile tissue cul-

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Address reprint requests to Dr. Silverstein, Department of Hematology, Room C606, Cornell Medical College, 1300 York Avenue, New York, NY 10021.

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1. *Abbreviations used in this paper:* EACA, ε-amino-n-caproic acid; OCT, ornithine carbamyl transferase; PPACK, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; TPA, tissue plasminogen activator.

ture plasticware was from Falcon Labware, Becton Dickinson & Co. (Cockeysville, MD). OCT embedding medium was from Miles Laboratories and the alkaline phosphatase substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate from Bio-Rad Laboratories (Richmond, CA).

Purified proteins and antibodies. Plasminogen was prepared from platelet poor plasma by affinity chromotography on lysine-agarose by the method of Deutsch and Mertz (10, 16). All purification steps were performed in the presence of 0.01% soybean trypsin inhibitor. Lys-plasminogen was prepared by limited proteolysis of Glu-plasminogen by plasmin in a molar ratio of 1:50 for 90 min at 37°C (17). Plasmin was then inhibited by the addition of PPACK at 10^{-4} M and removed by batch adsorption with soybean trypsin inhibitor-agarose beads. The Glu- and Lys-plasminogen preparations were compared by SDS-PAGE with authentic Glu-plasminogen form 1 and Lys-plasminogen form 1 standards which were generously supplied by Dr. F. J. Castellino (Notre Dame University, South Bend, IN). Two additional procedures were carried out to distinguish Glu-plasminogen from Lys-plasminogen. First, the Lys-plasminogen preparation reacted with a monoclonal antibody specific for Lys-plasminogen provided by P. Holvoet, University of Leuven, Leuven, Belgium (5) in an ELISA system, while the Glu-plasminogen did not. Second, fluid phase activation by tissue plasminogen activator (TPA) using the fluorogenic plasmin substrate D-Val-Leu-Lys-7-amino-4-trifluoromethyl coumarin (10), showed favorable reaction kinetics for Lys-plasminogen but not for Glu-plasminogen. Furthermore, both plasminogen preparations contained < 0.01% plasmin. Recombinant TPA was supplied by Genentech (San Francisco, CA). High molecular weight urokinase (Winkinase; 60,200 U/mg) was from the Sterling-Winthrop Research Institute (Rensselaer, NY). Purified human Lys-plasmin was obtained by activation of purified Lys-plasminogen with insolubilized urokinase as described previously and stored at −70°C in 50% glycerol (18, 19). Purified protein S was kindly provided by Dr. Charles Esmon (Oklahoma Medical Research Foundation, Oklahoma City, OK). Murine monoclonal antiplasminogen (3642) reactive with both Glu- and Lys-plasminogen was obtained from American Diagnostica (New York, NY), and monoclonal IgG MPWIPG, reactive only with native Glu-plasminogen (20) was a gift of Dr. B. R. Binder (Vienna, Austria). Rabbit antiplasminogen Fab'_2 conjugated to alkaline phosphatase was prepared as previously described (10). Murine monoclonal antimonocyte/macrophage antibodies OKM6 and anti-Leu M3 were obtained from Ortho Diagnostics (Westwood, MA) and Becton Dickinson (Mountain View, CA), respectively, and anti-von Willebrand factor from Cappel Laboratories (Malvern, PA). FITC-conjugated goat antimouse IgG was purchased from Tago Inc. (Burlingame, CA), alkaline-phosphatase conjugated goat antimouse IgG was from Cappel and gold-conjugated goat antimouse IgG (aurogold-BL) with silver enhancement reagents were from Janssen Life Sciences (Piscataway, NJ). Biotinylated goat anti-mouse IgG and avidin-horseradish peroxidase development kit was from Vector Laboratories (Burlingame, CA).

Radioisotope labeling. Purified Glu- and Lys-plasminogen and control proteins were labeled with ^{125}I by the modified chloramine-T method (21). Specific activity for most studies was $0.5–2.4 \times 10^{-2}$ μCi/μg.

Cells and cell culture. U937 cells were obtained from Dr. J. Unkeles (Mt. Sinai Medical College, New York) and were maintained in culture in RPMI 1640 medium (M. A. Bioproducts, Walkersville, MD) supplemented with 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were subcultured 1:4 three times per week into 75-cm^2 flasks. Elicited macrophages were obtained from peritoneal cavities of 6-8 wk C57Black female mice (Jackson Laboratories, Mt. Desert Island, ME) as described previously (22). Cells were harvested in Dulbecco's modified Eagle's medium (DME) containing penicillin and streptomycin, washed, resuspended in DME containing 20% fetal calf serum and dispensed into 24-well sterile plates at 10^6 cells/well. Wells were then incubated for 2 h and the nonadherent cells were removed by washing three times in DME plus FCS. Adherent cells were counted as previously described (22) by electronic particle counts of nuclei released after hypotonic lysis. Cells were maintained in culture for 24–48 h before use.

^{125}I-Glu-plasminogen and Lys-plasminogen binding assays

U937 cells. U937 cells were washed twice and then incubated for 1 h in serum-free medium RPMI 1640 containing 2 mg/ml BSA (RPMI/BSA) before use. The cells were then rewashed and aliquoted into 1.5 ml polypropylene microcentrifuge tubes ($0.8–1.5 \times 10^6$ cells per aliquot; 0.1 ml per tube). ^{125}I- Glu- or Lys-plasminogen diluted in RPMI/BSA was added to make a final volume of 0.15–0.2 ml. Binding studies were done in the presence of PPACK at 10^{-5} M. After incubation, cell suspensions were layered onto 0.4 ml of silicone oil (67:33 vol/vol of Dow Corning 550 and 556 oils) and centrifuged at 14,000 *g* in a microfuge (Beckman Instruments, Inc., Fullerton, CA). The aqueous phases were removed, the tubes were inverted, and the tips containing the cell pellet were amputated and placed in a gamma-counter for measurement of cell-bound radioactivity. To demonstrate reversibility, cells were incubated with ^{125}I-plasminogen for 1 h at 4°C (50 μg/ml), at which point they were washed and resuspended in RPMI/BSA containing 20 mM EACA. Bound and free radioactivity was then measured at timed intervals as above. All points were measured in duplicate and in all cases nonspecific binding was evaluated in separate tubes containing 20 mM EACA. Specificity was also assessed by coincubation experiments in which labeled plasminogen and excess unlabeled plasminogen were added to U937 cells for 1 h at 4°C, at which point cell-bound radioactivity was measured as above.

Murine peritoneal macrophages. Tissue culture wells containing adherent macrophages ($6–7 \times 10^5$/well) were incubated for 2 h in DME containing 2 mg/ml BSA, washed and then incubated for 1 h at 4°C with gentle agitation with radioiodinated ligands in 0.25 ml DME/BSA containing 10^{-5} M PPACK. Wells were then extensively washed and specific binding measured by elution of the bound radioactivity with DME/BSA containing 50 mM EACA. Total bound radioactivity was measured by solubilizing remaining well contents in 0.2 N NaOH. This procedure removed > 99% of the cells and radioactivity. Time course and reversibility experiments were carried out as described for the U937 cells.

Analysis of cell-bound plasminogen. ^{125}I Glu- or Lys-plasminogen was incubated with 2×10^6 U937 cells as described above at 37°C for 60 min. Included in the reaction mixture were equal volumes of either the serine protease inhibitor PPACK (10^{-4} M), TPA 10 U/ml, urokinase 10 U/ml, or buffer (PBS). After the incubation period cells were separated from unbound supernatant radioactivity by centrifugation through silicone oil and the cell bound ^{125}I-ligand eluted with 20 mM EACA in 100 μl Tris buffered saline for 30 min at 37°C. Both the cell bound and free ligands were then analyzed by SDS-PAGE using 7.5% polyacrylamide vertical slab gels. Gels were fixed in methanol/acetic acid, dried and autoradiographs obtained with Kodak XAR-5 film (Eastman Kodak, Rochester, NY). The autoradiographs were scanned in an LKB laser densitometer (model 2202; LKB Instruments, Gaithersburg, MD). Bound and free ligands were also adsorbed to polystyrene microtiter wells by incubation at 4°C for 16 h in carbonate buffer. Monoclonal antibody specific for the Lys-form of plasminogen was added for 2 h at 37°C. After extensive washing the bound antibody was quantified by sequential addition of alkaline-phosphatase goat anti-mouse IgG and a colorimetric phosphatase substrate (*p*-nitrophenyl-phosphate) as previously described (10).

Demonstration of Lys-plasminogen on human tissue macrophages

Cell preparation. Peritoneal exudates from patients with end-stage renal disease and indwelling catheters were obtained by centrifugation of 0.4–1.5 liters of peritoneal dialysate at 250 *g* for 10 min. To minimize ex vivo proteolysis, PPACK (10^{-5} M) was immediately added to the freshly removed dialysate and cells were washed three times in Hepes-buffered saline containing 10^{-4} M PPACK before analysis by flow cytometry, immunocytology, or immunoblot. In one study ^{125}I-Glu-plasminogen (5.5×10^6 cpm) was added to the dialysate at collec-

tion as a tracer, and after completion of all washes and incubations the cell-bound radioactivity was analyzed by SDS-PAGE/autoradiography.

Fresh tissue from patients with chronic inflammatory lesions (pilonidal cyst, an amputated diabetic leg, and inflamed tonsils) were obtained at surgery and snap frozen in OCT medium. 5-μm sections made on a cryostat, mounted on gelatinized glass slides and processed as described below for immunoperoxidase.

Immunofluorescence flow cytometry. Cells were resuspended in PBS containing 4% BSA and goat serum at 1–2 × 10^7/ml and incubated with monoclonal antibodies for 1 h at 22°C. The cells were then washed three times and resuspended in FITC-goat anti-mouse IgG for 1 h at 22°C. After repeated washing, the cells were resuspended in filtered PBS and analyzed using an Epics flow cytometer (Coulter Instruments, Hialeah, FL) equipped with a MDADS data handling computer and an argon laser. A sizing gate was set by examination of both right angle and forward angle light scatter signals from nonstained cells to eliminate granulocytes and small lymphocytes. For each sample 1–10 × 10^4 cells were counted within this gate.

Immunocytology. Peritoneal cells were incubated as above with monoclonal antibodies, washed and then resuspended in 20 nm gold-conjugated goat-anti-mouse IgG for 1 h at 22°C. After washing, the cells were resuspended in PBS containing 1% BSA and centrifuged onto poly-L-lysine and gelatin-coated glass slides using a Shandon Cytospin 2 centrifuge (Shandon Southern Instruments, Sewickley, PA). Slides were then fixed in iced methanol/acetone (50:50, vol:vol), silver enhanced using the Janssen EnhanCE kit, and then lightly counterstained with Giemsa.

Immunohistology. Frozen sections from surgical specimens containing chronic inflammatory lesions were incubated with monoclonal anti-Lys-plasminogen, anti-LeuM3, or anti-von Willebrand factor and then stained using an avidin-biotin-peroxidase complex (Vector ABC kit). Sections were counterstained with hematoxylin.

Immunoblot analysis. Washed peritoneal cells were resuspended in 100 μl Tris-buffered saline containing 50 mM EACA and incubated for 1 h at 22°C. The eluate was separated from the cells by high-speed centrifugation (15,000 g × 3 min), electrophoresed under nonreducing conditions on SDS-7.5% polyacrylamide gels, and then electrophoretically transferred to nitrocellulose membranes using an LKB Novablot horizontal system. The membranes were incubated in PBS containing 5% nonfat milk and 0.1% NP-40 for 1 h at 22°C and then in the same buffer containing alkaline phosphatase-conjugated rabbit antiplasminogen Fab'_2 for 4 h. After extensive washing the blots were developed with colorimetric substrates.

Results

Specific binding of Lys-plasminogen to U937 cells and murine peritoneal macrophages. ^{125}I-Lys-plasminogen bound specifically to murine peritoneal macrophages and to cells of the human monocytoid line U937. Two unrelated labeled control ligands, ^{125}I-albumin and ^{125}I-protein S, were shown in this and prior studies (22) not to bind to these cells. In addition, > 80% of ^{125}I-Lys-plasminogen binding was blocked by a 200-fold molar excess of unlabeled ligand, further demonstrating specificity. As shown in Fig. 1, ^{125}I-Lys-plasminogen bound in a time-dependent and reversible manner to both of these cells at 4°C. Equilibrium was achieved within 30 min for the U937 cells (*A*) and 40 min for the macrophages (*B*). For both cell types binding was > 90% reversible at both 4°C and 37°C after reincubation of washed ^{125}I-Lys-plasminogen coated cells in plasminogen-depleted media containing 20 mM EACA.

As shown in Fig. 2, binding of ^{125}I-Lys-plasminogen to U937 cells was concentration-dependent and saturated at ~ 100 μg/ml. In this and all further studies, specific binding was considered as that degree of binding inhibited by 20 mM

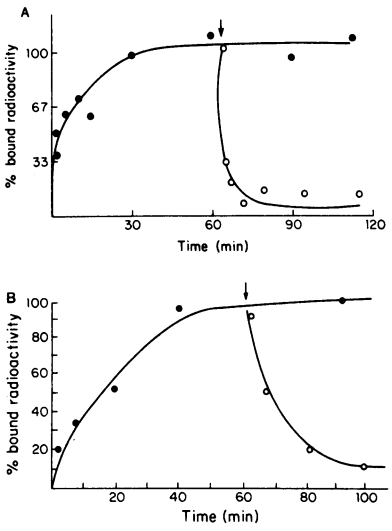

Figure 1. Time dependence and reversibility of Lys-plasminogen binding to U937 cells and murine peritoneal macrophages. U937 cells (*A*) or elicited murine peritoneal macrophages (*B*) were incubated at 4°C with ^{125}I-Lys-plasminogen (50 μg/ml). At timed intervals specifically bound radioactivity was counted (●). To assess reversibility ^{125}I-Lys-plasminogen coated cells were washed after 60 min incubation (*arrow*) and reincubated in plasminogen-free media containing 20 mM EACA. Bound radioactivity was then measured at timed intervals (○). For both experiments, points represent the mean of duplicate tubes.

EACA. Scatchard analysis of this data (*inset*) revealed a K_d of 540 nM with 1.8 × 10^6 binding sites per cell. Computer-assisted data analysis using the ligand program of Munson and Robard (23) for nonlinear curve fitting gave the best fit for a single class of binding sites. No significant difference was noted in these binding parameters at 37°C.

As with U937 cells, we demonstrated specific binding of Lys-plasminogen on the surface of murine peritoneal macrophages. Fig. 3 depicts a typical binding isotherm and shows that specific binding approached saturation at a concentration of ~ 150 μg/ml. Computer-assisted scatchard analysis of the data (*inset*) gave best fit for two classes of binding site. One, as

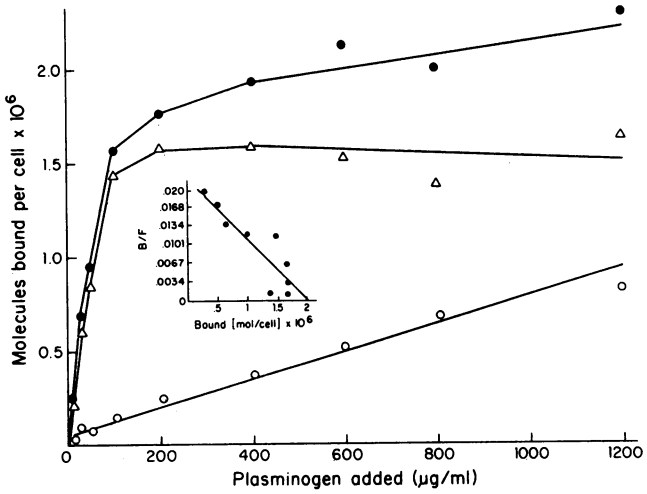

Figure 2. Binding of ^{125}I-Lys-plasminogen to U937 cells. U937 cells (1.2 × 10^6) were incubated with ^{125}I-Lys-plasminogen in RPMI/BSA for 1 h rotating at 4°C. Bound and free ligands were then separated by centrifugation through silicone oil and radioactivity was counted. Each point represents the mean of duplicate tubes. Binding is expressed as total cpm bound (●), cpm bound in the presence of 20 mM EACA (○), and EACA-inhibitable or specific binding (△). Inset is a Scatchard plot calculated using the Ligand program of Munson and Robard (23).

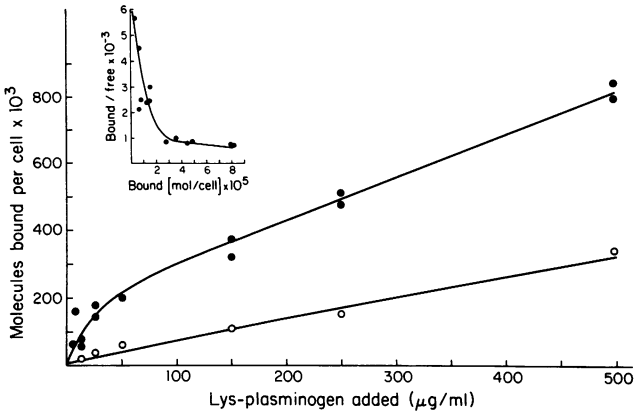

Figure 3. Binding of ^{125}I-Lys-plasminogen to murine peritoneal macrophages. Adherent elicited murine peritoneal macrophages (6.5 $\times$ 10^5/well) were incubated with ^{125}I-Lys-plasminogen in DME/BSA for 1 h at 4°C with gentle agitation. Wells were then washed three times to remove unbound radioactivity and then incubated in DME/BSA containing 50 mM EACA for 1 h to elute specifically bound material (●). Total bound material was then determined by solubilizing remaining well contents in 2 N NaOH. Nonspecifically bound radioactivity (○) was defined as total minus specific. Each point represents the mean of duplicate wells.

with U937 cells, of high affinity (K_d = 640 nM) and moderate capacity ($\sim$ 3 $\times$ 10^5 binding sites/cell), and one of markedly lower affinity and higher capacity. At plasminogen concentrations available we were unable to saturate these low affinity binding sites. Since the macrophage studies were carried out on adherent cells the number of binding sites may be underestimated because the basal surface of the cells is not available to the ligand.

Kinetics of the binding of ^{125}I native Glu-plasminogen to U937 cells was markedly different from that of ^{125}I-Lys-plasminogen. Binding was specific as shown by 80% inhibition in the presence of a 200-fold excess of unlabeled ligand, but as shown in Fig. 4, we were unable to demonstrate saturability up to concentrations of 7 μM. Similar results were obtained for murine peritoneal macrophages (data not shown).

Binding of both Glu- and Lys-plasminogen to macrophages and U937 cells was inhibited by EACA, suggesting that the phenomenon was mediated by plasminogen lysine-binding sites. Lys-plasminogen has been shown to contain multiple lysine-binding sites, one of K_d 35 μM, one of K_d 260 μM, and four additional sites of average K_d 10 mM (24). Glu-plasminogen contains two noninteracting classes of lysine-binding sites, one of K_d 9 μM and a set of five weaker sites of average K_d 5 mM (25). EACA titration studies revealed an I_{50} of 50 μM for Lys-plasminogen binding to U937 cells and 200 μM to macrophages suggesting that this interaction is mediated by the high-affinity lysine-binding site. The significantly higher I_{50} observed for Glu-plasminogen (> 2 mM) strongly suggests that its binding is mediated by lower affinity lysine-binding sites. Importantly, coincubation experiments with both Lys- and Glu-plasminogen revealed that incubation of ^{125}I-Lys-plasminogen with a 200-fold molar excess of Glu-plasminogen had little inhibitory effect on ^{125}I-Lys-plasminogen binding (Fig. 5). In contrast coincubation of ^{125}I-Glu-plasminogen with a 20-fold molar excess of unlabeled Lys-plasminogen resulted in 90% inhibition of ^{125}I-Glu-plasminogen binding. Therefore, while Lys-plasminogen did compete with Glu-plasminogen for U937 binding sites, Glu-plasminogen failed to compete with Lys-plasminogen.

Surface activation of Glu- and Lys-plasminogen on U937 cells. U937 cells and macrophages secrete both plasminogen activator and plasminogen activator inhibitor (26), and also express a cellular receptor for urokinase (27, 28). We therefore studied the molecular form of the bound plasminogen species by SDS-PAGE and autoradiography. Experiments were carried out in the presence and absence of protease inhibitors as well as exogenous plasminogen activators to determine whether cell-bound plasminogen could act as a substrate for plasmin and plasminogen activator. As shown in Fig. 6, in the presence of protease inhibitors the bound Glu-plasminogen (lane *B*) was identical to that added (lane *A*). However, as can be seen in lane *C*, in the absence of protease inhibitors, when Glu-plasminogen was added to the cells the Lys form was detected on their surface (43% conversion after 1 h incubation). The conversion of Glu- to Lys-plasminogen was accelerated by addition of catalytic concentrations of either urokinase (lane *D*) or TPA (lane *E*). 59% of the Glu-plasminogen was

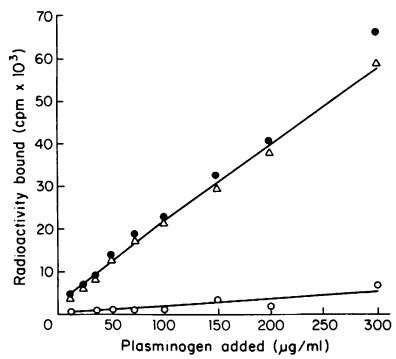

Figure 4. Binding of ^{125}I-Glu-plasminogen to U937 cells. U937 cells (1.2 $\times$ 10^6) were incubated with ^{125}I-Glu-plasminogen in RPMI/BSA for 1 h at 4°C. Bound and free ligands were then separated by centrifugation through silicone oil and radioactivity was counted. Each point represents the mean of duplicate tubes. Binding is expressed as total cpm bound (●), cpm bound in the presence of 20 mM EACA (○), and EACA-inhibitable or specific binding (△).

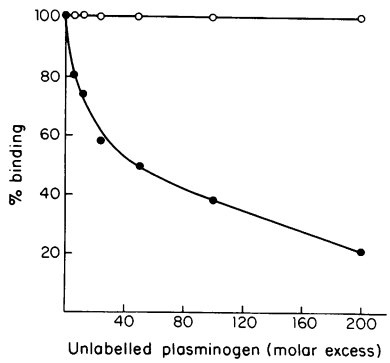

Figure 5. Inhibition of ^{125}I-Lys-plasminogen binding to U937 cells by nonlabeled Lys-plasminogen and Glu-plasminogen. U937 cells were incubated at 4°C with ^{125}I-Lys-plasminogen (2 μg/ml) for 1 h in the presence of excess nonlabeled Glu-plasminogen (○) or Lys-plasminogen (●). Bound radioactivity was then determined after centrifugation through silicone oil and is expressed as % of maximal specific binding.

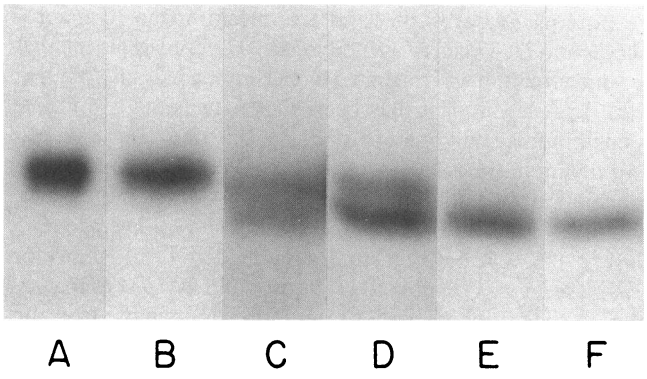

Figure 6. Molecular identity of bound plasminogen species. ^{125}I-Glu-plasminogen (100 μg/ml) was incubated with 2×10^6 U937 cells for 60 min at 37°C. After the incubation period cells were separated from unbound radioactivity as described in Fig. 1 and the cell-bound ^{125}I-ligand was eluted with 20 mM EACA. This material was then analyzed by SDS-PAGE and autoradiography. Lane *A* represents the starting material, lane *B* material eluted after incubation of Glu-plasminogen with the cells in the presence of 10^{-4} M PPACK, lane *C* after incubation without PPACK, lane *D* after incubation in the presence of 10 U/ml tPA, and lane *E* after incubation in the presence of 10 U/ml urokinase. Lane *F* contained purified Lys-plasminogen.

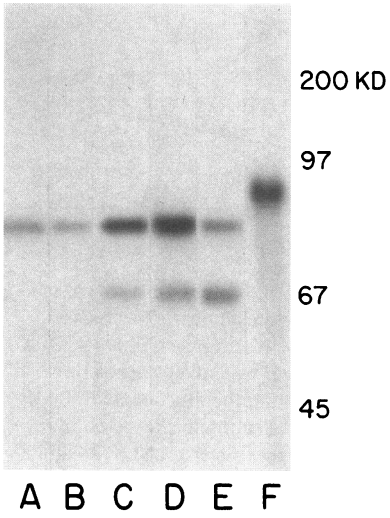

Figure 7. Molecular identity of bound plasminogen species. ^{125}I-Lys-plasminogen (100 μg/ml) was incubated with U937 cells and then eluted with EACA as described in Fig. 8. Lane *A* represents the starting material, lane *B* material eluted after incubation of Lys-plasminogen with the cells in the presence of 10^{-5} M PPACK, lane *C* after incubation without PPACK, lane *D* after incubation in the presence of 10 U/ml urokinase, and lane *E* after incubation in the presence of 10 U/ml TPA. Lane *F* contained purified Glu-plasminogen standard.

converted to Lys- in the presence of 10 U/ml urokinase and almost 100% by 10 U/ml TPA. We identified the molecular plasminogen species by comparison with ^{125}I-conjugated standards (lanes *A* and *F*) under nonreducing conditions. In addition the Glu to Lys conversion was confirmed by reactivity with a monoclonal anti-Lys-plasminogen antibody in an ELISA assay. Thus a portion of the EACA eluate from the U937 cells was coated on ELISA plates and probed with the monoclonal anti-Lys-plasminogen antibody. Only those eluates shown in lanes *C–E* of Fig. 6 reacted with the monoclonal antibody, further confirming Glu- to Lys-plasminogen conversion. Although this assay is not quantitative, the extent of reactivity of the EACA eluates with the monoclonal anti-Lys-plasminogen antibody paralleled the gel findings in that reactivity was greatest in the sample shown in lane *E*, followed by lane *D* and was least for the sample shown in lane *C*.

Identical results were obtained for Lys-plasminogen binding to the cells (Fig. 7). When Lys-plasminogen was incubated with the cells in the presence of protease inhibitors the bound form (lane *B*) was identical to that added (lane *A*). However, in the absence of protease inhibitors (lane *C*) plasmin was detected (16% conversion). Lanes *D* and *E* demonstrate that 24% of the Lys-plasminogen was converted to plasmin by urokinase and 51% by TPA. Plasmin generation was confirmed by the presence of its constituent plasmin light chain on reducing gels (data not shown). No comparable conversion of Glu- to Lys-plasminogen or Lys-plasminogen to plasmin was detected in the supernatants from these binding experiments (i.e., the unbound material).

Surface expression of Lys-plasminogen on human tissue macrophages. To discern whether human tissue macrophages can express Lys-plasminogen on their surfaces, immunofluorescence flow cytometry, immunogold light microscopy, and immunoblot analysis were carried out with macrophages obtained from patients undergoing chronic peritoneal dialysis. Cells were isolated from freshly removed peritoneal dialysates from patients with indwelling catheters. Serine protease activity in the fluid was immediately neutralized by the addition of PPACK to minimize ex vivo proteolytic activity. Flow cytometry, as shown in Table I, demonstrated that up to 76% of a cell population gated to exclude polymorphonuclear cells and small lymphocytes expressed OKM6 antigen, a specific monocyte/macrophage marker (29). All of the samples within this gated population contained cells reactive with monoclonal anti-Lys-plasminogen antibody. Reactivity ranged from 4 to 29% and was blocked by preincubation of the cells in 50 mM EACA, further demonstrating specificity. Less than 2% of the cells reacted with control antibodies or with a monoclonal

Table I. Lys-Plasminogen Surface Expression by Human Peritoneal Macrophages

Experiment	Peritoneal fluid cell count	Antibody reactivity (%)	
		Anti-mono	Anti-Lys-PLG
	($\times 10^4$/ml)		
1	1.6	56	14
2	60	74	20
3	3.1	47	11
4	0.25	9	4
5	6.3	76	20
6	12.9	71	29

Freshly obtained peritoneal dialysate fluid from six patients was washed in buffer containing PPACK 10^{-4} M and then incubated with nonimmune mouse IgG, murine monoclonal antimonocyte IgG (OKM6), or murine monoclonal anti-Lys-plasminogen IgG for 1 h at 22°C. After washing the cells were incubated in FITC-conjugated goat anti-mouse IgG and then analyzed by fluorescence activated flow cytometry. Data is expressed as percent reactive cells minus percent reactive with nonimmune mouse IgG.

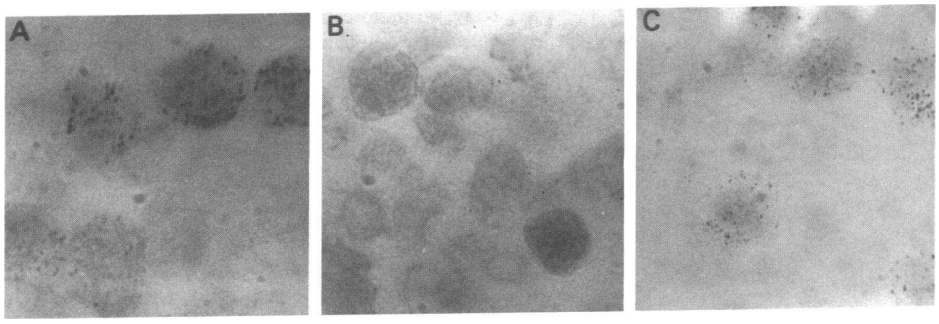

Figure 8. Immunogold microscopy of peritoneal exudates. Cells from peritoneal dialysates of patients undergoing chronic peritoneal dialysis were washed three times, incubated with monoclonal antibodies for 2 h at 22°C, rewashed and incubated for 1 h with 20 nm gold-conjugated goat anti-mouse IgG. After washing cytospin slides were prepared, fixed, silver enhanced and counterstained lightly with Giemsa. (*A*) A representative field from cells incubated with murine anti-plasminogen IgG; (*B*) from cells incubated with nonimmune mouse IgG and *C* from cells incubated with anti-LeuM3, a monocyte/macrophage specific murine monoclonal IgG. (× 1,000).

antibody reactive with Glu- but not Lys-plasminogen. Examination of these cells by phase-contrast immunofluorescence microscopy confirmed that the anti-Lys-plasminogen reactive cells were macrophages. In addition, to verify that these immunoreactive cells were morphologically macrophages indirect immuno-gold microscopy was done as shown in Fig. 8. Panel *A* demonstrates that a population of cells in the exudate reacted with the anti-plasminogen antibody (shown as dark grains). Cells in this population were morphologically identified as macrophages. In *B* there were no reactive cells with control antibody and in *C* a morphologically identical population reacted with anti-LeuM3, a marker specific for mononuclear phagocytes.

To characterize further the molecular form of this bound plasminogen, washed peritoneal exudates were incubated with EACA and the eluate analyzed by immunoblot using a specific rabbit anti-plasminogen Fab'_2 conjugated to alkaline phosphatase. Fig. 9 shows one such blot comparing the EACA eluate (lane *3*) to authentic Glu- and Lys-plasminogen (lanes *1* and *2*). In this gel system the major glycosylated forms of plasminogen (forms 1 and 2 as described by Castellino [30]) separated sufficiently so that both Glu- and Lys- appear as doublets. This blot demonstrates that the EACA eluate (lane *3*) reacted with the antiplasminogen Fab'_2 and that the bound plasminogen comigrated with authentic Lys-plasminogen (lane *2*), not Glu-plasminogen (lane *1*).

Additional studies were performed to address the question of whether the cell-bound Lys-plasminogen was the result of ex vivo proteolytic activity. ^{125}I-Glu-plasminogen was added to freshly obtained peritoneal fluid at the time of collection as a tracer and then after carrying the cells through all the washes and incubations described above the cell-associated radioactivity was analyzed by SDS-PAGE/autoradiography. Less than 0.014% of the added radioactivity was cell associated. Prolonged exposure of these gels revealed that this trace bound material comigrated with native Glu-plasminogen (not shown), demonstrating that the immunologically detected Lys-plasminogen seen on these cell surfaces was generated before the collection of the fluid.

In addition fresh frozen sections of human inflammatory tissues were evaluated by immunohistochemical techniques using the Lys-plasminogen specific monoclonal antibody. As shown in Fig. 10 (*A*), macrophage-associated Lys-plasminogen was detected in granulation tissue obtained from a pilonidal cyst. Adjacent sections showed that the same cell population reacted with the antimacrophage antibody anti-LeuM3 (*B*), but not with control anti-von Willebrand factor antibody (*C*). Similar Lys-plasminogen positive macrophages were also detected in inflamed tonsils and in granulation tissue from an amputated lower extremety of a diabetic patient. The monoclonal anti-Lys-plasminogen did not react with lymphocytes in frozen sections of lymph node, tonsil, and spleen.

Discussion

Surface deposition of Lys-plasminogen on mononuclear phagocytes may be an important mechanism for generating localized proteolytic activity. Using purified cell populations we have demonstrated that the kinetically favored Lys-plasminogen species binds specifically to U937 cells (a human monocytoid line) and to murine peritoneal macrophages. Binding was time dependent, saturable, reversible, and depended on the plasminogen high affinity lysine-binding sites. Although we found, as did Plow et al. (15) that native Glu-

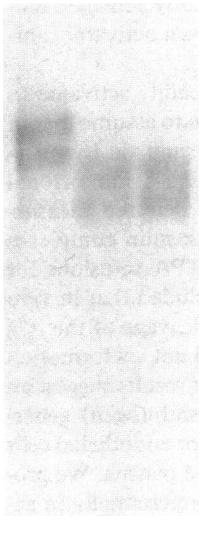

Figure 9. Immunoblot analysis of human peritoneal macrophage-associated plasminogen. Washed peritoneal exudates were incubated for 1 h in 50 mM EACA and the eluates then electrophoresed under nonreducing conditions in SDS-7.5% polyacrylamide gels. Proteins were then electroblotted onto nitrocellulose and probed with an alkaline phosphatase-conjugated antiplasminogen Fab'_2. Immunoreactive proteins were detected with a colorimetric substrate. Lane *1* contained Glu-plasminogen standard (30 ng), lane *2* Lys-plasminogen standard (30 ng) and lane *3* the EACA eluate.

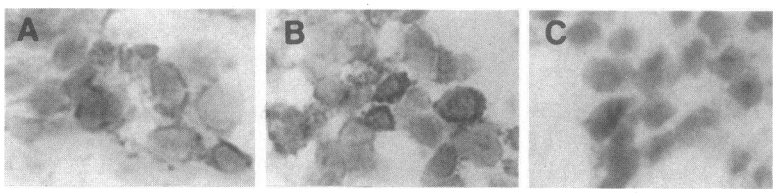

Figure 10. Immunohistochemical identification of Lys-plasminogen in mononuclear infiltrate from frozen section of a pilonidal cyst. (*A*) A section probed with monoclonal anti-Lys-plasminogen, (*B*) with the antimacrophage antibody anti-LeuM3, and (*C*) with control anti-von Willebrand factor monoclonal antibody. Immunoreactive material was detected with a peroxidase-conjugated avidin-biotin complex and is seen as a dark precipitate in *A* and *B*. (× 1,000).

plasminogen also bound specifically to these cells, the affinity was at least 10-fold lower than that of Lys-plasminogen. In fact, we were unable to demonstrate saturability of Glu-plasminogen binding. Furthermore, both EACA titration studies (I_{50} of 2 mM for Glu-plasminogen vs. 50 μM for Lys-plasminogen) and coincubation experiments demonstrated consistent differences between the binding of Lys- and Glu-plasminogen to the cell surfaces suggesting that Lys-plasminogen binding occurred via high affinity lysine-binding sites that may not be accessible on native Glu-plasminogen. Thus small concentrations of Lys-plasminogen easily attainable in vivo could bind to the cell surface in the presence of high concentrations of native Glu-plasminogen.

Since U937 cells and macrophages have been shown to secrete both plasminogen activators and plasminogen activator inhibitors the biochemical fate of the bound plasminogen was also studied. When ^{125}I-Glu-plasminogen was added to U937 cells in the absence of protease inhibitor, Lys-plasminogen was detected on the cell surface. Lys-plasminogen was not detected in the supernatants indicating that this Glu- to Lys-plasminogen conversion, catalyzed by endogenous plasminogen activator, probably occurred on the cell surface. Similarly, when ^{125}I-Lys-plasminogen was added to the cells, plasmin was detected on the cell surface, but not in the supernatant. Addition of TPA or urokinase resulted in accelerated Glu- to Lys-plasminogen and Lys-plasminogen to plasmin conversion, indicating that the bound species could be readily activated and may be "protected" from cellular plasminogen activator inhibitors produced by these U937 cells (25, 26).

Since Lys-plasminogen is much more readily activated to plasmin than is Glu-plasminogen, it is logical to assume that in vivo activation of the fibrinolytic system occurs via Lys-plasminogen intermediates. Holvoet et al., using a monoclonal antibody specific for Lys-plasminogen (5), detected measurable levels of circulating Lys-plasmin-antiplasmin complexes in only three of seven patients receiving TPA infusions for thromboembolic disease. The authors concluded that in vivo activation of Glu-plasminogen occurs by cleavage of the Arg 560-Val 561 bond yielding Glu-plasmin and not via formation of Lys-plasminogen as an intermediate. Our results suggest an alternative interpretation that the Lys-plasmin(ogen) generated remained surface-bound to monocytes or endothelial cells and therefore was not measurable in cell-free plasma. We propose that Lys-plasminogen may function preferentially in activation of native plasminogen in close association with specific cell surfaces.

To define the relationships of these in vitro experiments to in vivo reality, we have studied human macrophages from inflammatory lesions. Immunocytologic and immunohistologic techniques were used to show that macrophages from peritoneal lavage of patients with indwelling peritoneal catheters (presumably a low-level inflammatory stimulus) and within mononuclear cell infiltrates from fresh frozen surgical specimens obtained from patients with chronic inflammatory lesions expressed Lys-plasminogen on their surfaces. In addition, using monoclonal antibodies specific for Glu- or Lys-plasminogen we showed that peritoneal macrophages did not express native Glu-plasminogen. Immunoblot analysis of EACA eluates from these cells also confirmed the presence of Lys-plasminogen, but not Glu-plasminogen.

Flow cytometric analysis of the peritoneal exudates (Table I) revealed that ~ 10–30% of the macrophages expressed Lys-plasminogen on their surfaces. Whether this represents a specific subpopulation remains to be determined. It is probable that the Lys-plasminogen is deposited or forms on the monocyte/macrophage surface as the cells leave the circulation and enter the tissues, and then is gradually lost as the cells are exposed to the relatively plasminogen-depleted dialysis fluid. The possibility that ex vivo proteolytic activity contributed to Lys-plasminogen expression on these cells cannot be ruled out with 100% certainty. Protease activity in the peritoneal fluid was immediately neutralized by the addition of PPACK. In addition the fate of exogenously added ^{125}I-Glu-plasminogen was studied by SDS-PAGE/autoradiography. This was found to remain as Glu-plasminogen, suggesting that the Lys-plasminogen detected on the cells was generated before the collection of the fluid. Definitive proof, however, awaits perfusion fixation studies in animals.

The data presented in these studies show that Lys-plasminogen binds specifically to mononuclear phagocytes in vitro and is expressed on the surface of inflammatory macrophages in vivo. We suggest that this modified zymogen is the surface-associated form of plasminogen and may thus play a critical role in plasmin generation on biological surfaces. Plasmin, in addition to its role as initiator of fibrinolysis, has been postulated to participate in biological events requiring cellular migration through tissues, such as the inflammatory response (6), tumor cell invasion and implantation (7), ovulation and implantation (8), and neurodevelopment (9). Recent studies from this laboratory have demonstrated that the endothelial cell membrane is also a site of preferential conversion of Glu- to Lys-plasminogen conversion (31). The affinity of Lys-plas-

minogen for cell surfaces may thus extend beyond mononuclear phagocytes to tumor cells, endothelial cells, platelets, and extracellular matrix.

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