Functional Characteristics of Receptor-Bound Urokinase on Human Monocytes: Catalytic Efficiency and Susceptibility to Inactivation by Plasminogen Activator Inhibitors

By Johannes C. Kirchheimer and Heinz G. Remold

We compared urokinase-type plasminogen activator (u-PA) in fluid phase and u-PA bound with its receptor on human blood monocytes with respect to proteolytic activity and susceptibility to inactivation by the plasminogen activator inhibitors PAI-1 and PAI-2. Receptor-bound u-PA is catalytically twice as efficient as fluid-phase u-PA. Fluid-phase u-PA is susceptible to rapid inhibition by PAI-1 and PAI-2 at an estimated PAI-u-PA molar ratio of 2:1. In contrast, u-PA bound to endogenously occupied receptors is inhibited by PAI-2 only at PAI-u-PA molar ratios of 20:1, but is not inhibited by PAI-1. u-PA/PAI-1 and u-PA/PAI-2 complexes bind to the receptor with a tenfold lower affinity than u-PA itself. Thus, competition of u-PA/PAI complexes with fluid-phase u-PA for binding to the receptor is unlikely to affect the overall plasminogen activator activity of the monocyte. These findings demonstrate that the activity of receptor-bound u-PA can be modulated by PAI-2, but not by PAI-1, to adjust the cell’s proteolytic activity to different local situations.

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Materials. Tissue culture cluster plates (24-well flat-bottom plates; Costar, Cambridge, MA); medium RPMI 1640 (GIBCO Laboratories, Grand Island, NY); Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NY); bovine plasminogen-containing fibrinogen (Poviet, Amsterdam); thrombin (Hoffman-La Roche, Nutley, NJ); dialysis membranes, molecular weight (mol wt) cutoff 1,000 to 8,000 (Union Carbide, Chicago); cyanogen bromide, Triton X-100, benzamidine chloride, agmatine sulfate (Sigma Chemicals, St Louis); 1,3,4,6-tetracloro-3a-6a-diphenyl-glycouril (Pierce Chemicals, Rockford, IL); Sepharose 4B, Sephadex G-25 (Pharmacia AB, Uppsala, Sweden); sodium 125iodide (17.4 Ci/mg; New England Nuclear, Boston); acrylamide, bisacrylamide, sodium dodecyl sulfate (SDS), low and high mol wt weight protein standards for SDS gel electrophoresis (BioRad Laboratories, Richmond, CA).

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plasminogen-rich fibrin agarose plates. SDS-polyacrylamide gel electrophoresis (PAGE) was performed with 7.5% polyacrylamide gels. After electrophoresis, the gel was layered on top of a plasminogen-rich fibrin agarose plate for determination of plasminogen activator activity. Mol wt markers electrophoresed in parallel were stained with the silver stain method.

Plasminogen was purified from human plasma by affinity chromatography on lysine-Sepharose followed by gel filtration on Sephadex G-25 column and was obtained in its Glu form as judged by acid/urea electrophoresis. u-PA was isolated from human urine by affinity chromatography on antiurokinase monoclonal antibody (anti u-PA MoAb), MPW5UK-Sepharose followed by affinity chromatography on agmatine-Sepharose as described previously. Analysis by SDS-PAGE followed by silver staining showed under nonreducing conditions a single band corresponding to a mol wt of 54,000. u-PA activity was expressed in IU by comparison with the first International Reference Preparation for Urokinase (coded 68/48, Institute for Biological Standardization and Control, London).

The purified two-chain u-PA was 100,000 U/mg protein. PAI-1 purified from the human melanoma cell line MJZ3 was provided by Dr. B. R. Binder, University of Vienna. Its native inhibitory activity was 40,000 U/mg. The specific activity was ~62,000 U/mg protein after incubation with 4 mol/L guanidine hydrochloride for 12 hours, followed by dialysis against phosphate-buffered saline (PBS). (Unit of PAI-1 activity is defined by the amount of PAI-1 neutralizing 1 IU u-PA. PAI-2 was purified from supernatants of U-937 cells stimulated with PMA as described previously, except that the last step, affinity chromatography on immobilized Cibacron blue to remove albumin, was omitted.

Radiolabeling of u-PA. u-PA was iodinated using iodogen. Thirty micrograms of u-PA in 50 µL was added to a vial coated with 1,3,4,6-tetrachloro-3a,6a-diphenylglycouril (Iodogen) together with 1 mCi Na125I. After a 20-minute incubation at room temperature, the reaction was terminated by addition of 350 µL 0.05 mol/L sodium phosphate buffer (pH 7.4). Free iodine was removed by gel filtration on a Sephadex G-25 column (15 × 0.8 cm) equilibrated with 0.05 mol/L Tris HCl buffer (pH 8.0) containing 0.1 mol/L NaCl. u-PA with a specific activity of ~9.6 × 104 cpm/µg was obtained. No alteration in mol wt as judged by SDS-PAGE, or changes in binding properties occurred after radiolabeling.

Isolation of monocytes. Mononuclear cells were isolated from heparinized venous blood (100 to 250 mL) of healthy volunteers using Ficoll-Hypaque density separation. Thereafter, 2.5 × 10⁸ cells/mL were resuspended in RPMI 1640 medium containing 10% autologous u-PA-depleted serum (obtained by chromatography of serum on anti-u-PA mAb Sepharose) and maintained at 37°C in a 5% CO2 atmosphere. Removal of nonadherent cells after 18 hrs resulted in a preparation that contained >95% monocytes as determined by morphology and esterase staining.

Kinetic experiments on plasminogen activation by fluid-phase and receptor-bound u-PA. Monocytes (1.0 × 10⁶/well) were washed three times with PBS, incubated with 5 IU u-PA for 30 minutes at 37°C, washed three times with PBS, and then incubated with human Glu-plasminogen (135 to 4,344 nmol/L) at 37°C. The plasmin formed was quantified using 0.05 mol/L synthetic substrate Spectrozyme P1 and cleavage of the substrate was monitored at 405 nm in a Gilford spectrophotometer. From the rate of increase in absorbance at 405 nm, the rate of formation of plasmin could be calculated. Kinetics of plasminogen activation were determined by plotting the data in a double-reciprocal way and calculating correlations by the least-squares fit; km and vmax values were obtained from plots of reciprocal of substrate concentration v reciprocal of rate of substrate cleavage. km Values were calculated from the vmax values obtained from the plots and the amount of u-PA present in the assay system. The quantity of cell-bound u-PA was determined by a 125I-u-PA binding assay and equivalent amounts of the enzyme were used in the fluid phase.

To evaluate inhibition of fluid-phase and cell-bound u-PA by PAIs, fluid-phase u-PA, the cultured u-PA-saturated (5.0 IU/mL) or the unsaturated cells were incubated for 30 minutes at 37°C with a two-, ten-, 20-, and 40-fold molar excess of PAI-1 or PAI-2, respectively, relative to u-PA. Then the cells were washed three times with PBS and assayed as described above. Inhibitory activity was calculated by the percentage of decrease of the absorbance at 405 nm.

Formation of u-PA/PAI-1 and u-PA/PAI-2 complexes. u-PA or 125I-labeled u-PA (100 IU/mL in PBS) was incubated for 60 minutes at ~22°C with 20 U guanidine-activated PAI-1 or PAI-2, respectively. This mixture was applied to a benzamidine-Sepharose 4B column. u-PA (mol wt 54,000) was eluted with 0.5 mol/L potassium chloride in 0.1 mol/L potassium phosphate buffer (pH 7.5). The u-PA/inhibitor complexes eluted in the nonadherent fraction. The quantity of u-PA/inhibitor complex was calculated from the amount of radiolabeled u-PA present, assuming a 1:1 complex formation. The purity of the u-PA, u-PA/PAI-1 and u-PA/PAI-2 complexes is shown in Fig 1, an autoradiography of an SDS-PAGE of u-PA, u-PA/PAI-1 and u-PA/PAI-2 complexes. Both the u-PA used in these experiments and the PAI-complexes consist of a single band.

Binding studies with u-PA and u-PA/inhibitor complexes. Binding of u-PA, u-PA/PAI-1, and u-PA/PAI-2 complex to monocytes was quantified by adding 125I-labeled u-PA or 125I-u-PA/PAI complex (1,000 pmol/L) in 1 mL PBS to acid-treated (acid treatment with 0.05 mol/L glycine HCl, pH 3.0, for five minutes was performed to remove endogenously bound u-PA) cells (1.0 × 10⁷/well) for five to 60 minutes at 37°C with and without 20-fold excess of unlabeled ligand, to account for nonspecific binding. No appreciable internalization of the occupied receptor or dissociation of u-PA from the receptor occurred. Cell-bound radioactivity was determined after cells were washed three times with PBS and lysed by addition of 1 mL 0.1 mol/L NaOH, 0.5 mol/L EDTA, and 1% Triton X-100. Radioactivity was measured in a gamma counter. Binding of u-PA and the u-PA/inhibitor complexes reached plateau levels after 20 minutes and remained unchanged for up to two hours. Therefore, binding experiments were performed after incubation with the ligand for 30 minutes. Nonspecific binding with excess unlabeled ligand added was 5% to 10% of total binding in the case of u-PA and 5% to 20% of total binding in the case of the u-PA/inhibitor complex. To determine binding parameters, the cells were incubated with increasing concentrations of labeled ligand (400 to 6,000 pmol/L). Binding parameters were determined by the method of Scatchard. From www.bloodjournal.org by guest on November 10, 2017. For personal use only.
was reflected in a 1.8-fold increase in catalytic efficiency ($k_{cat}/k_m$) ($P = .01$; Student's $t$ test for paired observations.

The observed effect was not due to secretion of plasminogen activator by monocytes into the cell supernatant because human monocytes produced negligible amounts of fluid-phase plasminogen activator as determined by fibrin overlay assay (data not shown) for a period of three hours, whether activated or not. Receptor-bound u-PA therefore retains full functional activity and is catalytically more efficient than fluid-phase u-PA.

Inhibition of u-PA by PAIs. Next, we tested whether the two forms of u-PA interact differently with PAI-1 and PAI-2. u-PA in the fluid phase was susceptible to rapid inhibition by PAI-1 and by PAI-2 at an estimated molar ratio of 2:1 (Fig 2, panel B). Receptor-bound u-PA on saturated cells did not react with PAI-1 at molar ratios of up to 40:1 (PAI-1:u-PA) (Fig 2, panel A). However, PAI-2 caused a maximal inhibition of 25% of receptor-bound u-PA on saturated cells at an estimated molar ratio of PAI:u-PA of 20:1. Higher amounts of PAI-2 did not increase the inhibitory effect (Fig 2, panel A). In addition, preincubation of PAI-2 with human monocytes for 60 minutes at 37°C did not affect its activity toward u-PA, suggesting that PAI-2 was not bound to the cell surface and that cells did not produce a product that competes with PAI-2.

Since 30% of the total u-PA receptors on human monocytes is endogenously occupied with u-PA and ~25% of total receptor-bound u-PA were inhibited by PAI-2, we questioned whether inhibitable u-PA molecules are identical to endogenously bound u-PA. Inhibition by PAI-2 of endogenously bound u-PA resulted in a 75% decrease of plasminogen activation (PAI:u-PA ratio of 20:1). The remaining 25% of bound u-PA could not be inhibited even at a ratio of inhibitor to enzyme of 40:1 (Fig 3). Neither u-PA nor the u-PA/PAI complex could be detected in the supernatants by the fibrin overlay assay, indicating that neither u-PA nor the complex dissociates from the receptor.

We confirmed the data obtained by colorimetric assay by analysis of receptor-bound u-PA and of the receptor-bound u-PA/PAI-2 complex on fibrin overlay gels. u-PA bound to endogenously occupied and total receptors exhibited a mol wt of 54,000 on SDS-PAGE, representing either intact active u-PA or single-chain u-PA (Fig 4, lanes 1 and 4). Incubation

Table 1. Plasminogen Activation by Fluid-Phase and Cell-Bound Urokinase

<table>
<thead>
<tr>
<th>u-PA</th>
<th>$k_m$ (μmol/L)</th>
<th>$k_{cat}$ (min⁻¹)</th>
<th>$k_{cat}/k_m$ (μmol/L⁻¹/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluid-phase (n = 5)</td>
<td>8.1 ± 1.3</td>
<td>54.8 ± 6.9</td>
<td>6.9 ± 1.2</td>
</tr>
<tr>
<td>Cell-bound (n = 5)</td>
<td>7.7 ± 1.4</td>
<td>90.1 ± 18.4</td>
<td>11.8 ± 1.9*</td>
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</tbody>
</table>

The $k_{cat}$ values were calculated from $v_{max}$ values, and the amount of receptor-bound u-PA was determined by a radiolabeled anti-u-PA antibody binding assay and the equivalent amount of u-PA in the fluid phase. Values are means ± SD of five experiments; $k_{cat}/k_m$ values for fluid-phase and cell-bound u-PA were calculated from the single values obtained in each of the five experiments separately. Significant differences between the $k_{cat}/k_m$ values obtained for fluid-phase and cell-bound u-PA were calculated using Student's $t$ test for paired samples.

*Significant differences ($P < .01$).
of 20-fold molar excess of PAI-2 with u-PA bound to saturated receptors resulted in a partial shift of the mol wt of the enzyme to 96,000, representing an enzyme–inhibitor complex. Most of the enzyme did not undergo complex formation with the inhibitor (Fig 4, lane 3). When u-PA bound to endogenously occupied receptors was treated with PAI-2, the lysis band revealed shift of nearly all the enzyme to a mol wt of 96,000 (Fig 4, lane 5) showing almost complete complex formation. Treatment of receptor-bound u-PA with PAI-1 did not result in a change in mol wt of the bound enzyme (Fig 4, lane 2).

Binding of u-PA/PAI-1 and u-PA/PAI-2 complexes to human monocytes. Complexes of u-PA with PAI-1 or PAI-2, occurring under physiologic conditions,30,44 might compete with free u-PA for binding to the receptor. The results of saturation analysis for binding of radiolabeled u-PA and u-PA/PAI-2 complex to human acid-treated monocytes are shown in Fig 5 (inset); saturation occurred at 4 nmol/L in the case of u-PA and at >6 nmol/L in the case of the u-PA/PAI-2 complex. Scatchard analysis of these data (Fig 5) showed linearity, suggesting a single class of binding sites in both cases. However, the u-PA/PAI-1 complex bound with an affinity ten times lower than that of the free enzyme. The number of binding sites and affinities (kd) of the enzyme and of the u-PA/PAI-1 and u-PA/PAI-2 complexes was calculated from three experiments (Table 2). These data indicate that both u-PA/PAI-1 and u-PA/PAI-2 complexes bind to the receptors with much lower affinity and are therefore probably not of importance in controlling extracellular proteolysis.

**DISCUSSION**

In this study, we investigated whether the activity of u-PA is altered when bound to receptors on human monocytes and whether the interaction of receptor-bound u-PA with PAIs is different from that of fluid-phase u-PA.

Our results indicate that u-PA remains active when bound to the receptor. This is consistent with previous studies showing that the catalytic site of u-PA is not necessary for receptor binding.30,25 Examination of supernatant demonstrated that u-PA is not released into the supernatant. We noted a 1.8-fold higher catalytic efficiency of receptor-bound u-PA as compared with fluid-phase u-PA. This effect was caused by an increase in the catalytic rate constant (kcat) accompanied by an almost unchanged apparent km.

u-PA in the fluid phase is highly sensitive to PAIs (Fig 1B).30,31 In contrast, receptor-bound u-PA is sensitive only to high concentrations of PAI-2, which appears to act exclusively on endogenously bound u-PA. The reason for this behavior is not known. We assume that single-chain u-PA bound to the endogenously occupied u-PA receptors is activated by cell-bound plasmin in a manner described recently.45 u-PA activated on the receptor may be sufficiently different from u-PA activated in fluid phase and then bound to the receptor with regard to its structure to interact with PAIs in a different way.

PAI secreted by mouse peritoneal macrophages after endotoxin stimulation reportedly is able to block 90% of the soluble plasminogen activator activity but only 15% of membrane associated plasminogen activator activity.46 The small amount of inhibited membrane-bound enzyme may reflect the u-PA on human monocytes which can be inhibited by PAI-2.
u-PA and u-PA/PAI-2 complexes (abscissa) are plotted with or without excess of unlabeled ligand. Bound radiolabeled radiolabeled u-PA and u-PA/PAI-2 complexes (400 to 6,000 pmol/cell) were incubated for 30 minutes with varying amounts of PAI-2 complexes to human PB monocytes. Cells (1.0 x 10^6) are calculated.

Saturation occurs at 4 nmol/L in the case of u-PA and at >6 nmol/L in the case of u-PA/PAI-2 complex. Saturation curves are calculated.

<table>
<thead>
<tr>
<th>Substance Tested</th>
<th>kd (x 10^{-10} mol/L)</th>
<th>Binding Sites per Cell</th>
</tr>
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<tbody>
<tr>
<td>u-PA (n = 5)</td>
<td>1.2 ± 0.3</td>
<td>14,300 ± 3,000</td>
</tr>
<tr>
<td>u-PA/PAI-1 complex (n = 3)</td>
<td>17.8 ± 3.4</td>
<td>16,700 ± 3,600</td>
</tr>
<tr>
<td>u-PA/PAI-2 complex (n = 3)</td>
<td>10.9 ± 2.1</td>
<td>18,100 ± 5,200</td>
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*Values are means ± SD.

Fig 4. SDS-PAGE of receptor-bound enzyme (recovered by acid treatment) under the following conditions. Lane 1, u-PA bound to enzyme-saturated cells; lane 2, u-PA bound to enzyme-saturated cells incubated with 20-fold molar excess of PAI-1 for 30 minutes; lane 3, u-PA bound to enzyme-saturated cells incubated with 20-fold molar excess of PAI-2 for 30 minutes; lane 4, u-PA bound to endogenously occupied receptors; lane 5, u-PA bound to endogenously occupied receptors incubated with 20-fold molar excess of PAI-2. Numbers on right indicate the mol wt (x 10^3) of the lytic zones as calculated from protein standards run in parallel.

This finding parallels those of experiments showing that the invasiveness of B16-BL6 cells into amniotic membranes can be blocked by protease inhibitors, such as inhibitors of u-PA and collagenase.51 Thus the results we report have physiologic relevance on the cellular level. Moreover, monocytes release increased amounts of PAI-2 when exposed to lipopolysaccharides (LPS) and tumor necrosis factor (TNF-α), a situation commonly occurring in inflammatory loci.52,53 Inhibition of cell-bound proteolytic activity may therefore inhibit the cells' mobility and allow the cells to exert their specific function.

The monocyte may encounter different conditions depending on the balance of the concentration of u-PA, PAI-2, and PAI-3.
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This situation and the modulation of the endogenously saturated receptors by cytokines might allow the monocytes to adjust their invasiveness to pathologic conditions and to exert their multiple functions in an optimal way.

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