RAPID COMMUNICATION

Single-Chain Urokinase-Type Plasminogen Activator Bound To Its Receptor Is Relatively Resistant To Plasminogen Activator Inhibitor Type 1

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Urokinase-type plasminogen activator (uPA) has been implicated in the remodeling of extracellular matrix and in cell migration.1 uPA is synthesized as single-chain molecule (scuPA) with little intrinsic activity.2 scuPA is activated when it is converted to two-chain urokinase (tcuPA) by plasmin or when it binds as a single-chain molecule to its cellular receptor (uPAR). Previous data indicate that complexes between scuPA and its receptor have somewhat higher affinity for plasminogen than does tcuPA. The current study indicates that plasminogen activator activity of scuPA bound to recombinant, soluble uPAR (suPAR) is also fivefold less sensitive to inhibition by plasminogen activator type 1 (PAI-1) than is soluble or receptor-bound tcuPA. Binding of PAI-1 to suPAR/scuPA complexes is totally reversible and can be overcome by increasing the concentration of plasminogen, suggesting a competitive mechanism of inhibition (Kᵢ = 18 nM/L). Binding of scuPA to suPAR also retards its cleavage by plasmin. These results indicate that binding of single-chain urokinase to its receptor promotes its activity, retards its inhibition, and protects it from conversion to a two-chain form of the enzyme, a step that may precede its inactivation and clearance from cell surfaces. These results are consistent with a physiologic role for receptor-bound single-chain urokinase as a cellular plasminogen activator.

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EXPERIMENTAL PROCEDURES

Materials. Human scuPA was prepared and isolated as described.13 High-molecular-weight two-chain urokinase (tcuPA; specific activity 100,000 IU/mg), PAI-1 (specific activity 750,000 IU/mg), glu-plasminogen, and the chromogenic substrate of urokinase (Spectrozyme PL; H-D-norleucyl-hexahydrotyrosyl-lysine-p-nitroanilide diacetate salt) and the chromogenic substrate of plasmin (Spectrozyme UK; carboxbenzoyl-L-g-glutamyl-(a-t-butoxy)-glycyl-arginine-p-nitroanilide diacetate salt) were provided by American Diagnostica (Greenwich, CT).

Soluble recombinant urokinase receptor. Human uPAR cDNA (amino acids 1 through 281 with its signal peptide sequence) was prepared from U937 cells by reverse transcription-polymerase chain reaction (RT-PCR) and cloned into pDEMp, an expression vector for murine hybridoma cells, as described.14 The murine hybridoma line Sp2/0 was grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum and the cells were transfected by electroporation. Clones were selected by growing the cells in DMEM containing increasing concentrations of methotrexate (150 nM/L to 1 μM/L). The concentration of suPAR secreted into the culture medium was determined by enzyme-linked immunosorbent assay (ELISA; American Diagnostica). Large-scale expression was accomplished by growing the stably transfected cells in spinner flasks in serum-free hybridoma medium. Media from three large scale cultures (total of 100 L, each containing 20 to 40 mg/L) was obtained on day 8 after seeding. A protease inhibitor cocktail (5 mM/L disodium ethylenediamine tetracetate, 100 μM/L phenylmethylsulfonyl fluoride, 5 μM/L aprotinin, and 5 μM/L leupeptin, final concentrations) was added to the media. The media was concentrated 10-fold, aliquoted, and stored at −20°C until use.

To isolate uPAR, scuPA (500 mg) was immobilized on CNBr-Sepharose (Sigma, St Louis, MO) and equilibrated with phosphate-buffered saline (PBS). Concentrated media was applied to the column at a flow rate of 3 mL/min using a peristaltic pump. The column was washed sequentially with PBS, PBS containing 1 mol/L KCl, and finally with PBS alone until the flow through contained no detectable protein by UV adsorption. Soluble uPAR was eluted with

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The protein migrated as a single broad band between diol and progesterone, and stain positive for both the fifth passage, a uniform population of epithelioid cells was obtained that express an extensive number of microvilli on their apical surface characteristic of syncytiotrophoblasts, synthesize both estradiol and progesterone, and stain positive for both α- and β-human choriongonadotrophic hormone and cytokeratin-peptide 8, but not for vimentin and von Willebrand factor.

Measurement of plasminogen activator activity. Plasminogen activator activity was measured in PBS, pH 7.4. scuPA or tcuPA (50 nmol/L) were preincubated or coincubated in the presence or absence of an equimolar concentration of suPAR in PBS at 4°C for 15 minutes. The samples were diluted 1/10 in PBS containing the plasmin substrate (1,000 μmol/L) and plasminogen (5 to 30 nmol/L), and the optical density (OD) at 405 nm was measured at various times.

Measurement of PAI-1 inhibitor activity. PAI-1 was added to the reaction mixture containing plasminogen and the plasmin chromogenic substrate immediately before adding a source of urokinase (scuPA, tcuPA, or preformed complexes between suPAR and scuPA or tcuPA). In one set of experiments PAI-1 was incubated with these enzymes for various periods of time before adding plasminogen and chromogenic substrate. In a second set of experiments, trophoblastic cells were incubated with scuPA or tcuPA (10 nmol/L), the chromogenic substrate of urokinase Spectrozyme UK (60 μmol/L) was added alone or in the presence of PAI-1 (20 nmol/L) at 37°C as described and the OD at 405 nm was measured.

RESULTS AND DISCUSSION

We first compared the plasminogen activator activity of complexes between suPAR and the two principal forms of intact urokinase, scuPA and tcuPA, after 15 minutes of incubation. suPAR/scuPA complexes expressed approximately 46% more plasminogen activator activity than did soluble tcuPA alone at a plasminogen concentration of 5 nmol/L (Fig 1). suPAR caused a minimal or no increase in the activity of tcuPA under these conditions, whereas the activity of soluble scuPA was essentially unmeasurable. This stimulatory effect of suPAR on scuPA was maintained at plasma concentrations of plasminogen, as shown previously, while suPAR had no effect on the activity of tcuPA under these conditions (not shown).

We then compared the susceptibility of suPAR/scuPA, suPAR/tcuPA, and soluble tcuPA to inhibition by equimolar concentrations of PAI-1. PAI-1 almost totally inhibited the plasminogen activator activity of tcuPA and preincubation with suPAR afforded little or no protection (Fig 2). In contrast, the activity of suPAR/scuPA was essentially unaffected (Fig 2). The protection of receptor-bound scuPA from PAI-1 was relative. The activity of suPAR/scuPA was almost totally inhibited at fivefold molar excess PAI-1 (Fig 3). The same pattern was seen when the susceptibility of cell-associated scuPA or tcuPA to inhibition by PAI-1 was studied. Binding of scuPA to cells expressing uPAR increased the amidolytic activity fivefold, consistent with previously reported results. Addition of equimolar concentrations PAI-1 almost totally inhibited the enzymatic activity of cell-associated tcuPA, but had no effect on the activity of cell-associated scuPA (not shown).

We then compared the mechanism of inhibition of tcuPA
and scuPA/suPAR complex by PAI-1. Equal amounts of scuPA/suPAR or tcuPA (50 nmol/L) were incubated in the presence and absence of 250 nmol/L PAI-1 (a concentration that almost totally inhibits the activity of both entities) for various lengths of time up to 3 hours. The reaction mixtures were then diluted fivefold and the residual plasminogen activator activity was measured. The enzymatic activity of tcuPA remained unmeasurable at each time point tested in the presence of PAI-1, whereas the activity of the complex was restored (Fig 4A). The results suggest that the interaction of PAI-1 with receptor bound scuPA is reversible, as has been reported for soluble scuPA and for the initial step in the interaction between PAI-1 and tcuPA. Further, this result also indicates that PAI-1 eluted from suPAR/scuPA lost activity, consistent with conversion to the latent state. The reversibility enables the k of the reaction to be calculated.

A Dixon plot of the inhibition data indicated that the k of PAI-i for suPAR/scuPA is 18 nmol/L under these conditions (Fig 4B). Further, the addition of plasminogen (5 to 30 nmol/L) attenuates the inhibitory effect of PAI-1 (20 nmol/L) on the suPAR/scuPA complex (5 nmol/L) in a dose-dependent manner (Fig 5), consistent with a competitive pattern of inhibition.

Lastly, we examined the susceptibility of receptor bound scuPA to proteolytic cleavage by the end-product, plasmin, to determine whether any of the observed activity was due to its conversion to receptor-bound tcuPA. scuPA and suPAR/scuPA complexes were incubated with plasminogen and plasmin substrate in the absence or in the presence of equimolar concentrations of PAI-1 for 18 hours. As before, the activity of suPAR/scuPA complex became apparent well before the activity of scuPA alone (Fig 6). However, by 4 hours plasmin activity was clearly measurable in the mixture of scuPA and plasminogen in the absence of added suPAR.

We then asked whether this activity was attributable solely to scuPA or scuPA converted to tcuPA by the small amount of plasmin that had been generated. To distinguish between these possibilities, the experiment was repeated in the presence of equimolar concentration of PAI-1 which inhibits tcuPA but not scuPA or suPAR/scuPA, as shown above. PAI-1 totally suppressed the plasminogen activator activity of suPAR at 4 hours and by 57% at 18 hours. This result suggests that the activity attributed to scuPA was actually due primarily to trace amounts of tcuPA generated by plasmin, although the possibility that scuPA

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**Fig 3.** Effect of increasing PAI-1 concentration on the activity of suPAR/scuPA. Preformed suPAR/scuPA complexes (5 nmol/L final concentration) were incubated with the indicated concentrations of PAI-1, plasminogen (5 nmol/L) and plasmin chromogenic substrate (1,000 pmol/L) for 1 hour. The mean ± SD of three separate experiments is shown.

**Fig 4.** Inhibition of suPAR/scuPA by PAI-1: (A) The effect of time. Preformed complexes between suPAR and scuPA (*scuPA; □) or suPAR and tcuPA (*tcuPA; □) were preincubated alone or with fivefold molar excess PAI-1 (*suPAR/PAI-1, □; *tcuPA/PAI-1, □) for the indicated times. Each sample was diluted 1:5 and incubated with plasminogen (10 nmol/L final concentration) and plasmin chromogenic substrate (1,000 μmol/L). The mean ± SD of three separate experiments is shown. (B) The effect of concentration. Preformed suPAR/scuPA complexes (5 nmol/L final concentration) were incubated with the indicated concentrations of PAI-1, plasminogen (10 nmol/L) and the plasmin chromogenic substrate (1,000 μmol/L) for 1 hour. The results were analyzed using a Dixon plot.
assumes a novel conformation during prolonged incubation that renders it susceptible to PAI-1 cannot be formally excluded. What is clear is that the plasminogen activator activity of suPAR/scuPA in the presence of plasminogen was inhibited minimally by PAI-1 under the same conditions (Fig 6). This result suggests that the enzymatic activity of the complex did not result from the conversion of receptor-bound scuPA to receptor-bound tcuPA. These results also provide support for the concept that scuPA binds to its receptor is less susceptible to cleavage by plasmin than is soluble scuPA.20

These data, taken together with those reported previously by others,21 suggest that the suPAR/scuPA complex expresses several of the important biochemical prerequisites for it to be biologically active at the sites of cell-cell and cell-matrix contact where plasminogen concentrations are low: (1) Receptor-bound scuPA expresses comparable or greater plasminogen activator activity than does receptor-bound or soluble tcuPA; (2) this activity is less susceptible to inhibition by PAI-1 than is receptor-bound tcuPA; (3) inhibition of the activity of receptor bound scuPA by PAI-1 is reversed more rapidly than is receptor-bound tcuPA; and (4) receptor-bound scuPA is less susceptible to conversion to tcuPA than is soluble scuPA. The uPA receptor may thereby retard the rate with which complexes between tcuPA and PAI-1 form and are cleared from the cell surface. Additional experiments will be needed to determine whether scuPA bound to its native, glycosyl phosphatidyl inositol-anchored receptor subserves this proposed function. The data also suggest that a major biologic activity of PAI-1 on cell surfaces may be to facilitate the clearance of tcuPA rather than solely to inhibit urokinase activity directly. Finally, the fact that suPAR/scuPA complexes are less susceptible to PAI-1 may be relevant to the future design of inhibitors of cell-associated urokinase activity.

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![Fig 5. PAI-1 inhibition of suPAR/scuPA: The effect of increasing plasminogen concentration. Plasminogen (5 to 30 nmol/L) was added to a fixed concentration of suPAR/scuPA (5 nmol/L) in the absence or in the presence of PAI-1 (20 nmol/L) and the plasmin chromogenic substrate. The data are expressed relative to plasmin activity generated in the absence of PAI-1. The mean ± SD of two separate experiments performed in triplicate is shown.](image-url)

![Fig 6. Inhibition of suPAR/scuPA by PAI-1: The effect of prolonged incubation. suPAR/scuPA (*suPA), scuPA, suPAR/tcuPA (*tcuPA), and tcuPA were incubated in absence or in the presence of equimolar concentrations of PAI-1, plasminogen (5 nmol/L) and plasmin chromogenic substrate (1,000 μmol/L) for the indicated times. Column 1, *suPA (W); column 2, *suPA/PAI-1 (W); column 3, scuPA (W); column 4, scuPA/PAI-1 (W); column 5, *tcuPA (W); column 6, *tcuPA/PAI-1 (W); column 7, tcuPA (W); column 8, tcuPA/PAI-1 (W). The mean ± SD of three separate experiments is shown.](image-url)
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Single-chain urokinase-type plasminogen activator bound to its receptor is relatively resistant to plasminogen activator inhibitor type 1

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