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A Linear Amino Acid Sequence Involved in the Interaction of t-PA With Its Endothelial Cell Receptor

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Endothelial cell receptors for tissue plasminogen activator (t-PA) have been demonstrated recently, and we have sought to identify a region of the t-PA molecule involved in its interaction with these receptors on human umbilical vein endothelial cells. Of three monoclonal antibodies against various regions of t-PA, one directed against the finger region inhibited ^{125}I–t-PA binding to the cells. Synthetic peptides corresponding in amino acid sequences to segments from within the finger region were constructed, and one of these inhibited t-PA binding. This peptide corresponded to residues 7 through 17 of t-PA. The inhibition by this peptide was specific as other peptides from the finger region were inactive. The inhibitory peptide also did not affect the binding of another fibrinolytic ligand, urokinase, to the cells. Although a role for other regions of t-PA in binding to endothelial cells cannot be excluded, the results implicate a short span of linear amino acid sequence within the finger region in the interaction of t-PA with endothelial cells.

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DEMONSTRATION of specific receptors for fibrinolytic components has been a major recent development in the area of fibrinolysis. These receptors may not only contribute to the dynamics of clot lysis but may also regulate numerous cellular functions that are dependent upon cell-surface proteolytic activity. To date, interactions of the zymogen, plasminogen, and the plasminogen activators (urokinase [u-PA], and tissue plasminogen activator [t-PA]) with cells have been demonstrated. The receptors for these fibrinolytic components are widely distributed and are multifunctional.[1,2]

The interactions of these ligands with endothelial cells are particularly relevant in view of the proximity of these cells to sites of vascular injury and fibrin deposition. The exposure of these cells to blood-borne ligands, such as plasminogen, and the capacity of these cells to synthesize certain of these ligands, such as t-PA, ensure exposure of endothelial cells to fibrinolytic ligands. With plasma therapeutic levels sometimes reaching 0.1 μmol/L,[3] a new potential source of t-PA for receptor occupancy must also be considered. Endothelial cells express a high capacity (8.2×10^5 to 1.2×10^7 sites/cell) binding site for t-PA with a kd of 18 to 240 nmol/L.[4-6] In addition, these cells may also express a smaller number of higher affinity t-PA binding sites[5,6] that may involve an interaction of t-PA with its inhibitor, plasminogen activator inhibitor-1.[6] In the present study, we provide an essential step in the understanding of t-PA:endothelial cell interactions by pinpointing a specific, linear amino acid sequence involved in receptor recognition of this plasminogen activator.

MATERIALS AND METHODS

Peptides and monoclonal antibodies (MoAbs). Peptides, corresponding to amino acid sequences within t-PA or u-PA, were synthesized on an Applied Biosystems (Foster City, CA) model 430 peptide synthesizer using t-Boc amino acids and peptidylglycine a-amidating monooxygenase resins. The peptides were analyzed by high performance liquid chromatography (HPLC) for homogeneity using a Vydac C18 column with a linear gradient of 0% to 60% acetonitrile in 0.1% trifluoroacetic acid and, for some experiments, were purified by this protocol. For t-PA I, II, and III peptides, a single major peak was detected in the elution profiles monitored at 212 nm. Before use in binding assays, the peptides were dissolved in phosphate buffered saline (PBS), and the pH was adjusted to 7.6. To authenticate the peptides and determine concentrations of peptide stock solutions, amino acid compositions were performed after hydrolysis of the peptides for 24 hours in 6 N HCl.

The three MoAbs used in this study were purchased from American Diagnostica (New York). Their reactivities with individual domains of t-PA were established in previous publications,[7] or by the manufacturer's specifications. Their t-PA binding activity was assessed in a solid phase immunoassay, performed as previously described,[8] using microtiter plates coated with 100 μL t-PA at 2.5 μg/mL in PBS. Incubations with the first antibody (the t-PA MoAbs) and the second antibody (radioiodinated goat anti-mouse Ig) were carried out for 90 minutes at 37°C.

Binding of ligands to endothelial cells. t-PA (Alteplase, a generous gift of Genentech, Inc, South San Francisco, CA) was 70% single chain and radiolabeled using Iodogen (Pierce, Rockford, IL) as described[9] to a specific activity of 2.7 to 3.2×10^7 cpm/μg. The labeled protein was homogeneous on a TSK-3000 HPLC column, and retained more than 85% of its enzymatic activity, as assessed in a chromogenic assay.[10] u-PA (Winkinase, Winthrop Labs, New York, NY) was purified by passage over benzamidine-Sepharose (Pharmacia, Uppsala, Sweden), and was iodinated as for t-PA or by a chloramine T protocol[11] and was greater than 95% homogeneous, with a molecular weight of 55,000 as determined by polyacrylamide gel electrophoresis.

Primary cultures of human umbilical vein endothelial cells (HUVEC) were a generous gift of Dr Tom Lawley (National Institutes of Health, Bethesda, MD). Cells were cultured in M-199 media (Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum (FBS), 1 mmol/L glutamine, 10 μg/mL gentamicin, 50 U/mL heparin, (all from GIBCO, Grand Island, NY) and 5 μg/mL endothelial cell growth supplement (Collaborative Research, Bedford, MA). Culture surfaces were routinely pretreated with 10 μg/cm^2 gelatin (Sigma, St Louis, MO) before the addition of

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HUVEC. The procedures for t-PA and u-PA binding were as published previously.[4,11] Briefly, HUVEC were grown to confluence in 24-well tissue culture plates (Costar, Boston, MA) at an average density of $7.5 \times 10^4/cm^2$. The plates were washed three times with PBS pH 7.4 (Quality Biological, Gaithersburg, MD) containing 2.5% human albumin (Armour Pharmaceuticals, Kankakee, IL) (PBS-HA). PBS-HA and selected concentrations of synthetic peptides, MoAbs, or the unlabeled ligand were added to the wells. The pH of the reaction mixture was maintained at 7.6 to ensure peptide solubility. Then either 20 μL of ^{125}I–t-PA (to give 3 nmol/L) or 20 μL of ^{125}I–u-PA (to give 0.2 to 36 nmol/L) were added, maintaining a total volume of 200 μL. The plates were incubated at 4°C for 30 minutes. After three washes with PBS-HA, the cells in each well were solubilized with 1% sodium dodecyl sulfate (SDS), 1 mmol/L ethylenediamine tetraacetic acid (EDTA), and counted for radioactivity. Nonspecific binding was defined as the counts bound in the presence of a large excess (either 7.7 μmol/L t-PA or 860 μmol/L u-PA) of unlabeled ligand and subtracted from total counts bound to obtain specific binding. Under these conditions, nonspecific binding was 11% with ^{125}I-t-PA and 2% with ^{125}I-u-PA of total counts bound. Under the conditions of t-PA binding used in this study, the 3 nmol/L input concentration of ^{125}I–t-PA, the interaction was not affected by inactivation of the ligand with phenylmethylsulfonyl fluoride, ($\geq$95% inactivation as measured in a clot lysis assay[12] and in the chromogenic assay[10]) indicating that the measured binding was predominantly independent of the enzymatic activity of t-PA. In addition, when unlabeled t-PA was inactivated with phenylmethylsulfonyl fluoride (PMSF), its ability to compete with ^{125}I–t-PA for binding to cells was retained. Specific binding of ^{125}I–t-PA was 100% inhibited by either 7.7 μmol/L active or PMSF-treated unlabeled t-PA. In all analyses performed, only one low-affinity binding site was detected (kd = 18 to 240 nmol/L) and, therefore, all inhibitory activity (with peptides or MoAbs) relate to this site alone. A high-affinity, PAI-1-mediated interaction was not detected. In inhibition studies, the percentage bound was calculated relative to buffer controls. As the t-PA is supplied in diluent containing 200 mmol/L arginine, controls were performed to verify that arginine did not affect the ability of unlabeled t-PA to compete for ^{125}I–t-PA binding. t-PA was dialyzed to remove arginine and the solution was then titrated with 1 N HCl and diluted into PBS-HA. This treatment did not affect the ability of unlabeled t-PA to compete with ^{125}I–t-PA for binding to HUVEC. Furthermore, in the competition studies reported here, arginine was present at less than 2 mmol/L, a concentration which has minimal effects on ^{125}I–t-PA binding to cells.[4,5]

Fibrin binding assay. Binding of ^{125}I–t-PA to fibrin clots was performed as described.[13] Briefly, 1.5 mg/mL fibrinogen were mixed with peptides or PBS and 50 ng/mL ^{125}I–t-PA. The mixtures (1 mL) were clotted with 20 U/mL thrombin. After 1 minute at 37°C, the incubation time used by Collen et al,[13] the samples were centrifuged for 10 minutes in a microfuge, the clots wrung out, and aliquots of the supernatants counted for radioactivity. Percent binding was calculated relative to a control without fibrinogen and thrombin.

RESULTS

The t-PA molecule is composed of several structural modules found in many proteins of the coagulation and fibrinolytic systems. In sequence from the N-terminus, t-PA contains a finger domain, homologous to the finger domain of fibronectin, an epidermal growth factor domain, two disulfide-looped kringle structures, and a B-chain region at the carboxy-terminus that contains a catalytic site, typical of serine proteases.[14,15] As an initial approach to identify regions involved in the interaction of t-PA with its receptor, the

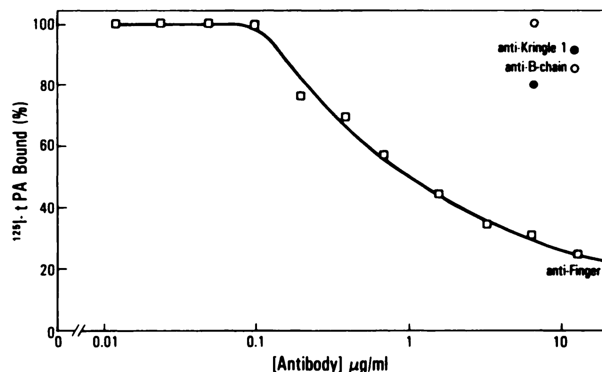

Fig 1. Effect of MoAbs to t-PA on ^{125}I–t-PA binding to HUVEC. Monoclonal antibodies were incubated with HUVEC, followed by addition of ^{125}I–t-PA, and binding was assessed as described in Materials and Methods. (○), PAM-1 to the B-chain region; (●), PAM-2 to the kringle 1 region; (□), 370 to the finger region.

effects of three MoAbs to specific domains on t-PA binding to HUVEC were examined. As shown in Fig 1, an MoAb to the finger region (no. 370) inhibited the binding of ^{125}I–t-PA to HUVEC in a concentration-dependent manner. At 12 μg/mL, this MoAb produced 74% inhibition of t-PA binding. In contrast, an MoAb to the kringle 1 region (PAM-2)[7] and one to the B-chain region of t-PA (PAM-1)[7] produced minimal inhibition of t-PA binding at this concentration. Nevertheless, when the capacity of these MoAbs to bind t-PA in a solid phase immunoassay was measured, the affinity of PAM 2 was approximately 11-fold higher than no. 370 and the affinity of no. 370 was 17-fold higher than PAM-1. When the antibodies were preincubated with ^{125}I–t-PA for 90 minutes at 37°C before addition to the endothelial cells, the concentration of the anti-finger MoAb (no. 370) required for 50% inhibition was decreased twofold to 0.78 μg/mL. Under this condition, the other two MoAbs, PAM-1 and PAM-2, still produced no inhibition of binding.

To further explore the role of the finger domain (residues 6 through 43) of t-PA in receptor recognition, a synthetic peptide approach was taken. By predictive analyses,[16] the N-terminal aspect of the finger region is the most likely to be surface oriented. Therefore, three overlapping peptides corresponding to this region were synthesized and tested for function. The structures of these peptides and other peptides used in this study are indicated in Table 1, and the effects of these peptides on ^{125}I–t-PA binding to HUVEC are shown in

Table 1. Characteristics of Synthetic Peptides Studied

	Sequence	Amino Acid Residue Number in Mature Protein
t-PA Peptides		
t-PA I	RDEKTQMIYQQ	7-17
t-PA II	YQQHQSWLRPVL	15-26
t-PA III	VLRSNRVEY	25-33
t-PA IV	RDEKTQMIYQQHQS	7-20
u-PA Peptides		
u-PA I	DCLNGGTCVSNKYFSNIHWCN	12-32
u-PA II	RNPDNRRRPW	103-112

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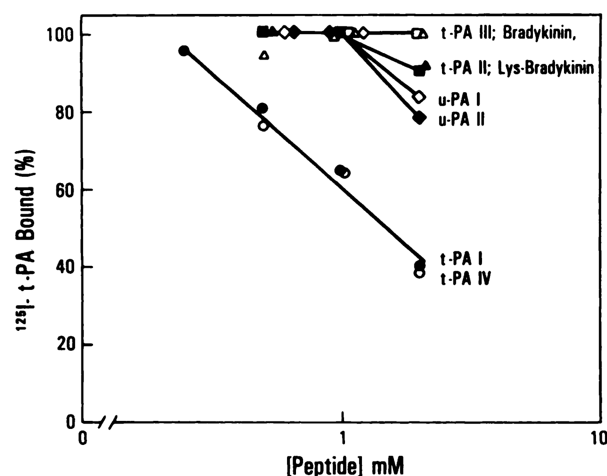

Fig 2. Effects of peptides on ^{125}I-t-PA binding to HUVEC. Peptides, at the indicated concentrations, and ^{125}I-t-PA were added to HUVEC and incubated on ice for 30 minutes. (●), t-PA I; (■), t-PA II; (□), t-PA III; (○), t-PA IV; (△), bradykinin; (▲), Lys-bradykinin; (◇), u-PA I; (◆), u-PA II.

Fig 2. t-PA I, corresponding to residues 7 through 17 of t-PA, inhibited binding of the ^{125}I–t-PA ligand in a dose-dependent manner. At the highest concentration testable, 2 mmol/L, t-PA I inhibited ligand binding by 60%. In contrast, t-PA II and III, containing sequences more C-terminal, were minimally inhibitory, even at 2 mmol/L. t-PA I could also dissociate the bound t-PA from the cells. ^{125}I–t-PA was bound to HUVEC for 30 minutes at 4°C, and 0.2 mmol/L t-PA I in a 15-fold volume excess was added. After 5 minutes, 35% of the bound ligand was dissociated. This extent of dissociation was intermediate to that produced by buffer (17%) and 154 nmol/L nonlabeled t-PA (70%). The difference in dissociation of t-PA in the presence of t-PA I compared with buffer was significant at the $P \leq .05$ level when analyzed by a paired t-test. These data are consistent with a role of the t-PA I sequence in t-PA binding to HUVEC. When the sequence of t-PA I was extended at its C-terminal (t-PA IV), inhibitory potency was retained, but not enhanced (Fig 2).

A series of experiments were undertaken to validate the specificity of the effect of t-PA I on t-PA binding to the cells. (1) To verify that inhibition was an intrinsic property of the peptide, t-PA I was purified by HPLC. The amino acid composition of the isolated peptide corresponded to that predicted, and the purified peptide inhibited ^{125}I–t-PA binding by 68% and 32% at 2 and 1 mmol/L, respectively, similar to the potency of the starting material (Fig 2). (2) Because the binding of t-PA to HUVEC is inhibited by Arg and Lys,[2] residues present in t-PA I, other peptides containing these residues were tested. u-PA II, with both N-terminal and internal Arg residues, bradykinin (RPPGFSPFR), with both N- and C-terminal Arg, and Lys-bradykinin (KRPPGFSPFR), with N-terminal Lys and an internal and C-terminal Arg, failed to inhibit ^{125}I–t-PA binding to HUVEC at 2 mmol/L concentrations (Fig 2). (3) To demonstrate selectivity, the effect of t-PA I on u-PA binding to HUVEC was assessed. At a saturating input concentration (36 nmol/L) of ^{125}I–u-PA, 1 mmol/L t-PA I produced no inhibition of ligand binding to the cells. When ^{125}I–u-PA was incubated with the cells at 0.2 nmol/L, at approximately one tenth of its kd,[11] inhibition by t-PA I was also minimal. Specifically, at this concentration of ^{125}I–u-PA, unlabeled u-PA inhibited binding by greater than 98% and u-PA I, corresponding to a region involved in u-PA binding to cells,[17] inhibited binding by 95%. In contrast, only 10% inhibition was observed at 1 mmol/L t-PA I and a similar low level of inhibition was observed with u-PA II and lys-bradykinin. In a related experiment, the effect of u-PA I on ^{125}I–t-PA binding was tested. This peptide produced minimal inhibition of t-PA binding to HUVEC at a 2 mmol/L concentration (Fig 2). Nevertheless, u-PA I produced 50% inhibition of ^{125}I–u-PA binding to the cells at 0.25 mmol/L. Thus, u-PA I and t-PA I exert selective effects on the binding of their parent ligands to HUVEC. (4) t-PA I was not toxic or lytic to the HUVEC as assessed by trypan blue exclusion and microscopic verification of the integrity of the cell monolayers. The failure of the peptide to affect u-PA binding also indicates that t-PA I did not disrupt the integrity of the endothelial cells.

The relationship between the finger peptides and the epitope recognized by MoAb no. 370 was explored. When peptides I, II, or IV were coupled to wells of microtiter plates and under conditions where the binding of the MoAb to insolubilized t-PA was 10-fold above background, no binding of MoAb no. 370 to the peptides was detected. Furthermore, when the MoAb was mixed with the peptides, at 1 to 1.5 mmol/L, no inhibition of binding to t-PA on the microtiter plates was observed. Thus, either the MoAb reacts with sites distinct from those defined by these peptides or the epitope requires secondary structural determinants within the finger region.

As the finger region and the kringle 2 region appear to mediate the fibrin affinity of t-PA,[18] we tested whether t-PA I could inhibit binding of ^{125}I–t-PA to fibrin clots. In the assay system used, 34% of ^{125}I–t-PA was bound to the clot within 1 minute in the absence of peptides. As a positive control, MoAb no. 370, at 8.5 μg/mL, reduced the binding of ^{125}I–t-PA to 2%. In the presence of 2 mmol/L t-PA I 37% of the ^{125}I–t-PA was bound to the clot. This result contrasts with the effects of this peptide on inhibition of cell binding, which can be observed at concentrations as low as 0.25 mmol/L, thus distinguishing these two functions.

DISCUSSION

In this study, an MoAb directed against the finger region of t-PA and peptides corresponding to a sequence within the finger domain of t-PA inhibited the binding of t-PA to HUVEC. The peptide data suggest that a specific linear amino acid sequence contained within residues 7 through 17 of t-PA is involved in receptor recognition. Although the finger region is involved in binding of t-PA to fibrin,[18] the t-PA I peptide did not inhibit this interaction. This suggests a possible independence of the regions within the finger domain involved in these two functions of the native molecule. Alternatively, the fibrin binding of t-PA may be a complex function and the assay used in our study may not be

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sufficiently sensitive to detect an effect of the peptide. For example, the same amino acid sequence may be involved in both functions, but the free peptide may have a higher affinity for cells than for fibrin, or the interaction of t-PA with fibrin may require the peptide sequence and additional structural and/or conformational aspects of t-PA.

In a recent preliminary report, a deletion mutant lacking both the finger and growth factor regions has been shown to have diminished ability to bind to the catalytic site-independent t-PA binding site on endothelial cells.[19] Our data are consistent with this observation and do not exclude the participation of the growth factor region and/or other regions of the molecule in its interaction with HUVEC. One explanation for the high concentrations of t-PA I required to inhibit t-PA binding is that other regions of the molecule may participate in the interaction. Nonetheless, in the presence of the peptides corresponding to a portion of the finger region, t-PA binding to HUVEC was markedly reduced, implicating this linear amino acid sequence in the interaction. Thus, this region of t-PA, residues 7 through 17, becomes an interesting target for further structure–function studies using peptide and mutational approaches.

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