Binding of tissue plasminogen activator to human aortic endothelial cells

Michael A. SANZO, Susan C. HOWARD, Arthur J. WITTEW and Helen M. COCHRANE

Department of Cell Culture and Biochemistry, Corporate Research Laboratories, Monsanto Company, 800 N. Lindbergh Boulevard, St. Louis, MO 63167, U.S.A.

The experiments described in this paper were designed to examine the specific binding of tissue plasminogen activator (tPA) to cultured human aortic endothelial (HAE) cells. When ^{125}I -labelled tPA was incubated with the cells at 4 °C, binding was found to plateau within 90 min after incubations were begun. Binding was saturable and the bound enzyme dissociated from the sites with a half-time of approx. 48 min. Scatchard analyses were performed using tPA molecules isolated from human melanoma and colon cells as well as from C127 and Chinese hamster ovary cells that had been transfected with the human tPA gene. These enzymes showed very similar binding characteristics in spite of the fact that they differ substantially in the types of sugars which comprise their side chains. Neither the chainedness of the molecules (one-chain or two-chain) nor the sites at which they are glycosylated (type I or type II) appear to affect their ability to interact with binding sites. The tPA molecules were found to have an average equilibrium dissociation constant of $(1.15 \pm 0.10) \times 10^{-9}$ M and HAE cells appeared to have a single, homogeneous population of independent binding sites present at a concentration of $(1.57 \pm 0.13) \times 10^6$ sites per cell. Lowering the pH of the binding buffer from 7.4 to 6.5 resulted in a reversible increase in specific binding of between 2-fold and 7-fold depending upon the particular preparation of cells. Preincubation of tPA with plasminogen activator inhibitor 1 (PAI-1) was found to have little effect on binding, suggesting that tPA interacts at sites distinct from surface-bound PAI-1. No evidence for either internalization or degradation of tPA was observed in assays run at 37 °C. This suggests that, like urokinase, tPA remains on cell surfaces for an extended period of time.

INTRODUCTION

There are two enzymes found in human plasma which are capable of converting plasminogen into plasmin; one is tissue plasminogen activator (tPA) and the other is urokinase (UK) (Wun *et al.*, 1981; Chmielewska *et al.*, 1983; Wiman *et al.*, 1983; Bachmann & Kruithof, 1984). These two plasminogen activators are thought to promote tumour cell invasiveness, tumour cell metastasis, tissue remodelling and other processes involving cell migration (Reich, 1978; Dano *et al.*, 1985; Blasi *et al.*, 1987). In addition, it has been well established that tPA plays a major role in controlling fibrinolysis (Verstraete & Collen, 1986; Collen, 1987). In the course of studying the way in which the plasminogen activators regulate cellular events, it has been found that many cultured cells have receptors for one or both enzymes (Beebe, 1987; Hajjar *et al.*, 1987; Blasi, 1988). Although the binding properties of the UK receptors found on various cell types have been well described, specific receptors for tPA have only recently been discovered and their characteristics remain largely unknown. Our particular interest has focused on receptors for tPA found on endothelial cells. These cells are known to exhibit dramatic differences in the proteins they express and in the receptors found on their membranes depending upon their specific location within the body (Simionescu *et al.*, 1982; Thilo-Korner *et al.*, 1982; Wojta *et al.*, 1988). In the case of tPA, van Zonneveld *et al.* (1986) have demonstrated that endothelial cells obtained from umbilical veins synthesize less than one-tenth of the mRNA and express less than one-tenth of the protein of endothelial cells obtained from aortas. This suggests that endothelial cells in different parts of the vasculature may be playing different roles in fibrinolysis. The present report examines, for

the first time, the binding of tPA to endothelial cells from human aortas. First, we define the basic binding parameters for interactions between tPA and our preparations of cells. Secondly, we examine a few characteristics which are likely to be of physiological importance.

MATERIALS AND METHODS

Reagents

Melanoma one- and two-chain tPA and plasminogen activator inhibitor 1 (PAI-1) purified from melanoma-cell-conditioned medium were purchased from American Diagnostica Inc. Recombinant human tPA from C127 cells was a gift from Invitron Inc. and Activase, a recombinant human tPA from Chinese hamster ovary (CHO) cells, was manufactured by Genentech and purchased from a local pharmacy. Growth medium MCDB107 (MCDB105 plus 0.015 g of glycine/litre and 0.015 g of KCl/litre) was purchased from Sigma Chemical Co. Endothelial cell growth supplement (ECGS) and human fibronectin (HFN) were from Collaborative Research Laboratories. Except where noted below, all other reagents were from Sigma or Fisher and were of the highest quality available.

Isolation and growth of human aortic endothelial (HAE) cells

HAE cells were generously provided by Dr. DiCorleto, Cleveland Clinic Foundation Research Institute. The cells had been cultured using a modification of the procedure of Chen *et al.*, 1986). Briefly, cells were isolated from human thoracic aorta pieces by collagenase digestion (2.0 mg/ml) and grown on fibronectin-coated plastic dishes in MCDB107 medium con-

Abbreviations used: tPA, tissue plasminogen activator; UK, urokinase; PAI-1, plasminogen activator inhibitor 1; ECGS, endothelial cell growth supplement; HFN, human fibronectin; HAE, human aortic endothelial; FEBS, fetal bovine serum; PBS, phosphate-buffered saline; NSB, non-specific binding; HAc, acetic acid; K_a , equilibrium association constant; K_d , equilibrium dissociation constant; CHO, Chinese hamster ovary.

taining heparin (90 µg/ml, ECGS (150 µg/ml) and fetal bovine serum (FBS, 10%). For routine maintenance, cells were fed every 2–3 days with MCDB107 containing heparin (180 µg/ml), ECGS (30 µg/ml), gentamycin (100 µg/ml) and FBS (10%). At confluence, the cells were harvested by treatment with 0.05% trypsin + 0.02% EDTA and expanded at 1–3 ratios.

HAE-cell-conditioned medium has been found to contain only very low levels of tPA antigen (10–20 ng/ml) after 48 h of conditioning, and the medium used for growing the cells has been found to greatly inhibit binding. Therefore we do not believe that tPA is binding to cells to a significant extent during the time they are in culture.

Quantification of tPA and PAI-1 antigen levels

ELISA kits for tPA and PAI-1 were purchased from American Diagnostica Inc. and assays were performed according to protocols supplied by the manufacturer. Differences were that a single lot of melanoma tPA from American Diagnostica Inc. (product no. 111, lot 47-01) was used as antigen standard throughout this work and BSA (5.0 mg/ml) was included in ELISA buffers.

Quantification of tPA enzymic activity

tPA enzymic activity was determined using a modified form of the parabolic assay first described by Rånby (Rånby & Wallén, 1981; Rånby, 1982). The assay was performed in 96-well microtitration plates (Immulon 1, Dynatech) in a final reaction volume of 100 µl. Final concentration and source of reagents in the reaction mix were as follows: 50 µg of human Glu-plasminogen/ml (product no. 400, American Diagnostica), 1.0 mM-S-2251 (H-D-Val-Leu-Lys-*p*-nitroanilide dihydrochloride; KabiVitrum) and 130 µg of human fibrinogen (HFN) fragments/ml (KabiVitrum). The buffer used was phosphate-buffered saline (PBS) (cat. no. 1000-3; Sigma) with 0.01% Na₂S₂O₃ and 0.01% Tween 80. The tPA used as a standard was melanoma 1-chain tPA purchased from American Diagnostica (product no. 111).

Purification of human colon fibroblast tPA

Both the large-scale culturing of human colon fibroblasts and the purification of tPA from the conditioned medium of these cells were carried out by previously published procedures (Harakas *et al.*, 1988; Parekh *et al.*, 1989a).

Isolation of different glycoforms of tPA

Type I tPA was separated from type II by chromatography on lysine-Sepharose essentially as described previously (Rijken *et al.*, 1985). A 1.6 cm × 96 cm column of lysine-Sepharose (Pharmacia) was equilibrated at 4 °C with buffer (0.15 M-KSCN, 120 mM-NaCl, 2.7 mM-KCl, 10 mM-phosphate buffer, pH 8.0, 0.01% Tween 20 and 0.01% Na₂S₂O₃). tPA samples dissolved in 1 M-NH₄HCO₃ were applied to the column which was then washed with at least 40 ml of equilibration buffer. The column was developed with a linear gradient (500 ml total volume) beginning with equilibration buffer and ending with an elution buffer consisting of 0.6 M-arginine chloride, 0.25 M-KSCN, 120 mM-NaCl, 2.7 mM-KCl, 10 mM-phosphate buffer, pH 8.0, 0.01% Tween 20 and 0.01% Na₂S₂O₃. Two well-resolved peaks of tPA enzymic activity were eluted from the column. In accordance with the observations of others, the first peak was designated as type I and the second peak as type II (Rånby *et al.*, 1982; Pohl *et al.*, 1984; Rijken *et al.*, 1985). Fractions comprising the peaks were pooled, concentrated and exchanged into storage buffer (0.02 M-phosphate buffer, pH 7.4, 0.15 M-arginine chloride and 0.01% Tween 80). Before use in binding assays, tPA samples were analysed by SDS/PAGE performed using Pharmacia's

Phast system and employing their protocols for 10–15% gradient gels and silver staining. Only samples which appeared to be free of contaminants and in which the proteins had the expected molecular masses (type I tPA is slightly larger than type II) were used in binding assays.

N-Terminal analyses have been performed on tPA molecules from many different sources, including all of those used in binding studies. Typically, between 10 and 20% of the total molecules within a preparation are cleaved at Arg-27. Certain preparations from C127 cells were found to have an exceptionally high degree of cleavage at this position. We examined the binding characteristics of both an intact preparation of C127 tPA and a cleaved preparation. The intact preparation had a percentage of cleaved molecules which was no greater than that normally found in tPA preparations (approx. 15%). The cleaved preparation had more than 90% of its tPA molecules cleaved.

tPA binding and dissociation

Cells were seeded in MCDB107 containing heparin (180 µg/ml), ECGS (30 µg/ml), gentamycin (100 µg/ml) and FBS (10%) at a density of between 2.0×10^4 and 4.0×10^4 cells per cm². Unless otherwise noted, all assays were carried out using 24-well plastic dishes in which the wells were coated with HFN. Cultures were fed every 2–3 days and were assayed within 7 days of plating. Immediately before assay, plates were cooled at 4 °C for 10–15 min. Growth medium was aspirated from the wells, and cells were washed twice with 500 µl aliquots of cold binding buffer (PBS, pH 7.2, containing 5.0 mg of BSA/ml, 10 mM-glucose and 0.01% Tween 80). Preincubation with buffers at lower pH values failed to increase binding, which suggests that receptors are not being masked by the presence of endogenously bound tPA. Binding buffer (500 µl) was then added to each well and plates were placed on a rotating platform for 15–20 min. Wells were aspirated and 600 µl of various samples was added. Typically these samples contained 5.0–50 ng of ¹²⁵I-tPA/ml of binding buffer. In experiments designed to go to equilibrium, incubations continued for at least 90 min with constant mixing. Cell counts taken before and after incubations and Trypan Blue exclusion experiments indicated that cells remained attached to plates and were viable for at least 2.5 h when incubated under these conditions. At the end of the incubation period, all wells were aspirated and were rapidly washed four times with 500 µl portions of binding buffer (total wash time was less than 20 s). Cells were solubilized by adding 300 µl of prewarmed solubilization buffer (0.01 M-Tris/Cl, pH 8.0, 10 mM-EDTA and 3% SDS). The buffer from each well was transferred to a polypropylene test tube and each well was then washed with a second 300 µl volume of solubilization buffer. Microscopic inspection of wells indicated that this procedure resulted in the complete removal of cells from plates. The samples from each well were combined and counted. Non-specific binding (NSB) was determined in two different ways. For Scatchard analyses, NSB was determined by simply carrying out titrations of ¹²⁵I-tPA with unlabelled tPA until saturation was reached (as indicated by graphs becoming parallel with the horizontal axis). Alternatively, NSB was determined by assaying samples in the presence of 2.0 µg of tPA/ml (a concentration equivalent to approx. $25 \times K_d$). Experiments run at 37 °C were performed in a similar manner except that all buffers were prewarmed before assay. In dissociation experiments, ¹²⁵I-tPA binding was allowed to come to equilibrium and cells were washed using the procedure described above. Fresh buffer containing 2.0 µg of unlabelled tPA/ml was then added and the amount of ¹²⁵I-tPA remaining bound to cells with respect to time was determined. Estimates of the time required for one-half of the bound ligand to dissociate (i.e. *t*_{1/2}) were obtained using the program 'Kinetic' (McPherson,

1985). The time course of binding was examined on four different occasions. Rates of dissociation were examined on three occasions. HAE cells do not appear to secrete tPA or PAI-1 during the course of binding assays in quantities large enough to affect binding.

Electrophoresis of ^{125}I -tPA before and after binding

Preparations of ^{125}I -tPA were examined by SDS/PAGE on 10–15 % gradient gels (Phastgel system, Pharmacia) followed by autoradiography. Samples taken before and after incubation with HAE cells at 4 °C appeared on autoradiographic films as single bands which had migrated to a position identical with that of a silver-stained tPA standard. In no instance did we see evidence of either tPA degradation or complex formation in response to incubation with cells.

Scatchard analyses

Scatchard analyses were performed by using fixed levels of ^{125}I -tPA and adding increasing amounts of unlabelled tPA. Results were analysed on an IBM personal computer using the program 'Ligand' (Munson, 1983; McPherson, 1985). Scatchard analyses on different forms of tPA were all performed a minimum of two times and all points within assays were run at least in duplicate.

Rate of internalization

In determining the rate of ^{125}I -tPA internalization, the procedure of Haigler *et al.* was followed (Haigler *et al.*, 1980; Wiley & Cunningham, 1982; Friesel & Maciag, 1988). After allowing ^{125}I -tPA to bind to cells for a specified time, buffer was removed and cells were washed rapidly four times. ^{125}I -tPA on the surface of cells was dissociated by incubation in 300 μl of acetic acid (HAc) buffer (0.5 M-sodium acetate, pH 2.5, 0.4 M-NaCl) for 5 min at 0–4 °C. The incubation with HAc buffer was repeated a second time and the replicates were pooled and counted. The amount of ^{125}I -tPA remaining associated with cells after the HAc wash was determined by carrying out the SDS solubilization procedure described above. Non-specific c.p.m. were determined from wells in which binding reactions were performed in the presence of 2.0 μg of unlabelled tPA/ml. In all of our calculations, non-specific radioactivity was subtracted from total radioactivity.

No internalization of receptors takes place at 4 °C, even in cases where the same receptors are rapidly internalized at 37 °C (McKanna *et al.*, 1979; Dickson *et al.*, 1980; Low *et al.*, 1981; Howard & Knauer, 1987; Owensby *et al.*, 1988). Therefore, in assays performed at 4 °C, the HAc/SDS solubilization procedures provide a measure of the surface-bound counts that are dissociable at pH 2.5 (HAc-solubilized counts) and the surface-bound counts that are resistant to dissociation at pH 2.5 (SDS-solubilized counts). Typically, 6–12 % of the total specific radioactivity bound to cell surfaces was resistant to HAc solubilization. By then running the assay at 37 °C and comparing the results obtained with those obtained at 4 °C, the extent to which internalization has occurred can be determined (internalization being reflected by an increase in the percentage of the total counts resistant to HAc solubilization). We examined cells both during ^{125}I -tPA binding and after the ^{125}I -tPA had bound and the binding mix was replaced with buffer containing unlabelled tPA (i.e. during ^{125}I -tPA displacement). In the latter samples, the binding buffer containing displaced ^{125}I -tPA was saved and the radioactivity present was included in the determination of percentages.

The trichloroacetic acid precipitability of ^{125}I -tPA samples was examined by incubating the samples at 0 °C for 10 min at a final trichloroacetic acid concentration of 10 % (w/v). Precipitate was pelleted by centrifugation, washed once with 10 % trichloroacetic

acid and counted for radioactivity. Assays at 4 °C and at 37 °C were each performed on two occasions.

Labelling of tPA

tPA was labelled using immobilized lactoperoxidase (Enzymobeads, Bio-Rad) and following the protocol supplied by the manufacturer. The reaction was allowed to proceed for 40 min at room temperature. Free ^{125}I was then separated from tPA-bound ^{125}I by gel filtration on a Sephadex G-25 column. Electrophoretic analysis of the resulting product (approx. 15 ng per lane) followed by silver staining revealed a single band migrating with a molecular mass indistinguishable from that of unlabelled tPA. Autoradiography indicated that all of the radioactivity present was associated with the band and it was found that about 75 % of tPA enzymic activity was recovered after labelling. Based on the results of Scatchard analyses, it does not appear that the enzymically inactive form of the labelled tPA preparation is binding to cells with an affinity significantly different from that of active tPA. This tPA may be binding with the same affinity as the active form or, alternatively, it may not be binding at all. Scatchard analyses for colon, melanoma and CHO tPAs were performed by labelling 2-chain type I tPA from each cell type and then performing competition assays with other forms of tPA produced by the same cell type. The specific radioactivities for the tPA species used to obtain the results shown in Table 1 were as follows: colon tPA, 0.21 $\mu\text{Ci}/\text{pmol}$; melanoma tPA, 0.28 $\mu\text{Ci}/\text{pmol}$; CHO tPA, 0.56 $\mu\text{Ci}/\text{pmol}$. In assays of C127 tPA, it was the unfractionated form of the enzyme that was labelled. The intact enzyme preparation was labelled to a specific radioactivity of 1.05 $\mu\text{Ci}/\text{pmol}$ and the cleaved form of the enzyme was labelled to a specific radioactivity of 0.75 $\mu\text{Ci}/\text{pmol}$.

RESULTS

Binding parameters

Time course of binding and dissociation. The change in ^{125}I -tPA binding with respect to time was examined (Fig. 1). It was found that specific binding plateaued within 90 min after assays were begun and remained unchanged for at least another 60 min. NSB at each point was determined in samples run in the presence of 2.0 μg of unlabelled tPA/ml and was found to represent approx. 25 % of the total counts bound. The inset of Fig. 1 shows the results of an experiment designed to determine the rate at which tPA dissociates from cells. In this experiment ^{125}I -tPA was allowed to bind to HAE cells for 90 min at 4 °C. Wells were then aspirated, cells were washed rapidly with $2 \times 500 \mu\text{l}$ of binding buffer, and 600 μl of binding buffer containing 2.0 μg of unlabelled tPA/ml was added. The cells then underwent a second incubation at 4 °C to allow bound ^{125}I -tPA to dissociate. At various times wells were aspirated and cells were washed once in binding buffer and then solubilized and counted for radioactivity. NSB was determined for each time point in sets of wells that contained 2.0 μg of unlabelled tPA/ml during the initial 90 min binding reaction. Analysis of the data using the program 'Kinetic' (McPherson, 1985) indicated that the $t_{1/2}$ of tPA at 4 °C was 48 ± 7 min. When analyses were performed at 37 °C it was found that the binding reaction plateaued somewhat sooner (by 65 min after assays were begun) but that the rate of dissociation was unchanged ($t_{1/2} = 50 \pm 8$ min; results not shown).

Saturability of binding and Scatchard and Hill plots. Saturation curves were run in which a fixed amount of ^{125}I -tPA was combined with increasing amounts of unlabelled ligand and the total amount of radioactivity bound was determined. This results in a curve such as that shown in Fig. 2(a). Saturation of specific

Fig. 1. Time course of tPA binding and dissociation

^{125}I -tPA (25 ng/ml) was incubated with HAE cells at 4 °C and the change in the amount of ^{125}I -tPA bound with respect to time was examined. For each time point, NSB was determined by performing incubations in the presence of 2.0 μg of unlabelled tPA/ml. In order to study dissociation (inset), ^{125}I -tPA was allowed to bind to cells for 90 min. Cells were then washed with binding buffer and fresh binding buffer containing 2.0 μg of unlabelled tPA/ml was added. A second incubation at 4 °C was begun. At various times thereafter, incubations were stopped and the amount of ^{125}I -tPA remaining bound to the HAE cells was determined. Results are expressed as the percentage of specific counts remaining bound (ordinate) with respect to time (abscissa). All points for the binding were run in triplicate. All points for the dissociation portion of the curve were run in duplicate.

binding sites is shown by, and complete in, the portion of the curve which parallels the horizontal axis (last three points) and bound radioactivity here represents NSB. Using this estimate for NSB, the data can be transformed into the Scatchard and Hill plots, shown in Figs. 2(b) and 2(c) respectively. The Scatchard plot is indicative of a single class of binding site with a K_d of approx. 1.0 nM. In the experiment shown, a concentration of 25.0 ng of ^{125}I -tPA/ml was used. In other experiments, we have used concentrations as low as 5.0 ng/ml and have observed similar results. The Hill plot (Fig. 2c) indicates that the binding sites are acting independently (i.e. binding at one site is neither promoting nor inhibiting binding at other sites).

Scatchard analysis of structurally distinct forms of tPA. Structurally, tPA may be classified as either 1-chain or 2-chain, depending upon whether or not it has been cleaved by plasmin. tPA may also be classified as either type I or type II depending upon whether or not Asn-184 is glycosylated (Pohl *et al.*, 1984). In addition, it is known that melanoma, colon cell and recombinant C127 and CHO tPA differ significantly in both the proportions of type I and type II produced and in the type of carbohydrates present (Parekh *et al.*, 1989a,b). For example, colon and recombinant C127 cells synthesize somewhat more type I than type II, whereas in melanoma and recombinant CHO cells this is reversed. In addition, each cell type makes tPA with certain carbohydrate structures that are unique to it (discussed by Parekh *et al.*, 1989b). A variety of these structurally distinct tPA molecules was examined in our binding assay (Table 1). It is apparent that neither the nature of the glycosylation pattern associated with tPA nor whether tPA has been cleaved by plasmin is important in determining its ability to bind to the receptor. On average (mean $\pm$ S.E.M. for 13 experiments) the various tPA molecules were found to have a K_d of $(1.15 \pm 0.10) \times 10^{-9}$ M, and there were $(1.57 \pm 0.13) \times 10^6$ receptors for tPA per cell.

Fig. 2. Scatchard and Hill plots of ^{125}I -tPA binding

Samples were prepared by mixing a fixed concentration of ^{125}I -tPA (25 ng/ml) with increasing amounts of unlabelled tPA. These were then examined in the tPA binding assay at 4 °C for 90 min. The amount of tPA bound in each sample was determined and Scatchard analyses were performed using the program 'Ligand'. The leveling of the graph in (a) occurs at the point at which binding sites become saturated and provides an estimate of NSB. (b) The same data with NSB subtracted out. The last three points in (a), which are in the region where saturation is nearly complete, were deleted from (b). (c) Data presented in the form of a Hill plot. The symbol B/F used in (a) and (b) refers to the concentration of bound ligand divided by the concentration of free ligand.

Binding characteristics

Effect of pH on binding. Binding buffer (PBS containing 5.0 mg of BSA/ml, 10.0 mM-glucose and 0.1 % Tween 80) was adjusted to pH values ranging from 6.5 to 8.2 and the tPA binding

Table 1. Comparison of the binding constants for different forms of tPA

Scatchard analyses were performed on a variety of structurally distinct forms of tPA and results were analysed using the program 'Ligand'. Binding constants along with associated S.E.M.s are shown. CHO and C127 preparations contained a mixture of one- and two-chain forms of the enzyme (about 80% one-chain and 20% two-chain in each case). In the course of performing *N*-terminal sequence analyses on tPA preparations purified from C127 cells, it became clear that, in certain preparations the tPA had undergone an exceptional amount of cleavage at Arg-27. Two preparations were selected for analysis in the tPA binding assay: one cleaved preparation, in which more than 90% of the tPA molecules had been cleaved, and one intact preparation which showed a degree of cleavage typical of tPA preparations in general (approx. 15%).

tPA source	Type	K_a (M^{-1})	K_d (nM)	Number of binding sites per cell
Colon cells	1-Chain, unfractionated	$(6.70 \pm 0.90) \times 10^8$	1.49	$(1.30 \pm 0.09) \times 10^6$
	2-Chain, unfractionated	$(1.14 \pm 0.53) \times 10^9$	0.88	$(8.90 \pm 3.41) \times 10^5$
	2-Chain, type I	$(9.75 \pm 1.16) \times 10^8$	1.03	$(1.02 \pm 0.06) \times 10^6$
	2-Chain, type II	$(9.85 \pm 1.01) \times 10^8$	1.02	$(1.15 \pm 0.06) \times 10^6$
Melanoma cells	1-Chain, unfractionated	$(1.13 \pm 0.75) \times 10^9$	0.88	$(2.03 \pm 0.75) \times 10^6$
	2-Chain, unfractionated	$(8.08 \pm 2.21) \times 10^8$	1.24	$(1.97 \pm 0.17) \times 10^6$
	2-Chain, type I	$(9.33 \pm 0.63) \times 10^8$	1.07	$(9.35 \pm 0.34) \times 10^5$
	2-Chain, type II	$(1.17 \pm 0.10) \times 10^9$	0.85	$(9.68 \pm 0.41) \times 10^5$
CHO cells ('activase')	Unfractionated	$(5.73 \pm 0.74) \times 10^8$	1.74	$(1.92 \pm 0.20) \times 10^6$
	Type I	$(6.49 \pm 0.78) \times 10^8$	1.54	$(1.48 \pm 0.13) \times 10^6$
	Type II	$(1.06 \pm 0.13) \times 10^9$	0.94	$(1.54 \pm 0.16) \times 10^6$
C127 cells	Unfractionated (intact)	$(6.86 \pm 2.54) \times 10^8$	1.46	$(3.33 \pm 1.25) \times 10^6$
	Unfractionated (cleaved at Arg-27)	$(5.74 \pm 2.04) \times 10^8$	1.74	$(1.89 \pm 0.77) \times 10^6$

reaction was carried to equilibrium at 4 °C in each. The results shown in Fig. 3(a) indicate that lowering the pH from 7.4 to 6.5 results in about a 2.5-fold increase in tPA binding. In buffers with pH values above 7.4 there is a steady, progressive decline in specific binding. Although we have consistently seen the same pattern, the magnitude of the changes in binding with respect to pH has varied in different preparations of HAE cells. In going from pH 7.4 to 6.5, we have seen an increase in binding which has ranged from 2- to 7-fold.

Experiments were performed in order to determine if the observed pH effects were reversible (Figs. 3b and 3c). The effect of pH on tPA was examined by preincubating the labelled enzyme at pH 6.5 for 1 h and then adjusting the pH of the buffer to 7.4 just before assay. The amount of specific binding obtained with this sample was compared with that obtained from samples maintained at either pH 6.5 or pH 7.4 throughout the entire preincubation and assay. The results shown in Fig. 3(b) indicate that binding at pH 6.5 is about 5.5 times higher than at pH 7.4. The sample incubated at pH 6.5 and then adjusted to pH 7.4 showed a level of binding just slightly higher than the sample held at pH 7.4 throughout. These results suggest that any effects which the lowered pH had on tPA were reversible. Fig. 3(c) shows the results obtained when HAE cells are preincubated at pH 6.5, 6.8 or 7.4 for 1 h at 37 °C and then washed in buffer at pH 7.4 before the binding assay. It can be seen that the preincubation of the cells had no effect on subsequent binding, suggesting that any effects which the lowered pH had on the tPA receptor were also reversible.

Effect of PAI-1 on tPA binding. Purified PAI-1 from melanoma-cell-conditioned medium was mixed with ^{125}I -tPA at molar ratios ranging from 0 PAI-1 per tPA to 100 PAI-1 per tPA and incubated at 37 °C for 1 h. The amount of enzymic activity and binding activity obtained with no PAI-1 present were taken as 100% and the relative amount of activity remaining in each of the incubates was determined. It can be seen from Fig. 4 that decreases in enzymic activity (reflecting the formation of tPA-inhibitor complexes) are not accompanied by a corresponding decrease in tPA binding. At a concentration of PAI-1 that completely inhibits enzymic activity, more than 80% of the

tPA binding activity remains. Therefore it appears that the majority of the binding observed is occurring at sites distinct from any surface-bound inhibitor that may be present. This view is also supported by the observation that the inclusion of a high concentration (2.0 µg/ml) of polyclonal antibody to PAI-1 in assays had no effect on the amount of specific ^{125}I -tPA binding observed (results not shown).

Rate of internalization. In order to study the rate at which ligand is internalized, we used a method first described by Haigler *et al.* (1980) and recently applied to human endothelial cells (Friesel & Maciag, 1988). The method depends on the observation that surface-bound ligand can be displaced from receptors on the surface of cells by briefly exposing the cells to low-pH buffers. Since internalized ligand remains associated with cells under these conditions, the acid treatment may be used to distinguish surface-bound from internalized ligand.

Fig. 5(a) shows the results of an experiment run at 4 °C. At each time point, the ^{125}I -tPA incubation buffer was removed and cells were washed with binding buffer before the acid solubilization procedure. Results for acid-solubilized and SDS-solubilized counts are expressed as percentages of the total bound (i.e. acid-solubilized specific counts + SDS-solubilized specific counts). It can be seen that during the first 90 min of the incubation both percentages remained essentially constant, with acid-solubilized counts comprising 90–95% of the total. At 90 min, the ^{125}I -tPA binding mix was replaced with displacement buffer which contained 1.0 µg of unlabelled tPA/ml but no labelled enzyme. At subsequent time intervals, the displacement buffer was removed to test tubes and counted for radioactivity and the percentages of acid-solubilized and SDS-solubilized counts were again determined. It was found that the percentage of acid-solubilized counts decreased rapidly as unlabelled tPA displaced cell-surface-bound ^{125}I -tPA and that the percentage of SDS-solubilized counts remained constant. The SDS-solubilized counts are taken to represent surface-bound counts which are not dissociated by the acid treatment (see the Materials and methods section). The samples of displacement buffer obtained at the various time intervals were precipitated with 10% trichloroacetic acid and these results are also shown in Fig. 5(a).

Fig. 3. Effect of pH on tPA binding

The tPA binding assay was performed in binding buffer which had been adjusted to pH values between 6.5 and 8.2 (a). After 90 min at 4 °C, the incubation was stopped and the amount of ^{125}I -tPA specifically bound to HAE cells was determined. Results for each sample are expressed as a percentage of the maximum c.p.m. bound (i.e. the amount bound at pH 6.5). (b) Results obtained when tPA was preincubated at pH 6.5 or 7.4 for 1 h and then examined in the binding assay at the same pH as the preincubation. Also shown are the results obtained when tPA was preincubated for 1 h at pH 6.5 and then adjusted to pH 7.4 before assay. (c) HAE cells were preincubated at pH 6.5, 6.8 or 7.4 for 1 h and the buffer pH was adjusted to 7.4 before assay. In both (b) and (c) results are expressed as a percentage of the ^{125}I -tPA bound to HAE cells when both preincubations and binding assays are performed at pH 7.4. Error bars show the amount of variation present in triplicate measurements.

Fig. 4. Effect of PAI-1 on tPA binding

Samples of ^{125}I -tPA and PAI-1 were incubated together at 37 °C for 1 h. The ratio of PAI-1 to ^{125}I -tPA (mol/mol) varied from 0:1 up to 100:1. At the end of the incubation, samples were collected and examined in both the tPA enzyme assay and the binding assay. Results are expressed as percentages of the total enzymic or binding activity present in each sample relative to that seen when ^{125}I -tPA was preincubated in buffer alone (i.e. in the absence of PAI-1). The effect of PAI-1 on tPA binding was examined on two occasions. All points in the assay depicted were run in duplicate.

Fig. 5. Rate of internalization of ^{125}I -tPA by HAE cells at 4 °C and 37 °C

HAE cells were incubated with ^{125}I -tPA at either 4 °C (a) or 37 °C (b). At various times incubations were stopped, cells were washed with binding buffer and plates were put on ice. Surface-bound ^{125}I -tPA was dissociated by incubation in cold HAc buffer (0.5 M-sodium acetate, pH 2.5, and 0.5 M-NaCl). The radioactivity remaining associated with the cells was then determined using samples solubilized in warm SDS buffer (0.01 M-Tris/Cl, pH 8.0, 10 mM-EDTA, and 3% SDS). Results are expressed as the percentage of the total c.p.m. in the SDS-solubilized material (curves C) and in the HAc-solubilized material (curves B). NSB was determined from wells run in parallel but which contained 1.0 μg of unlabelled tPA/ml. After 90 min. the ^{125}I -tPA buffer was replaced with buffer containing 1.0 μg unlabelled tPA/ml and incubations were resumed in order to determine the distribution of counts as tPA dissociated from cells. During the dissociation portion of the experiment, the buffer from the wells was collected and counted. The c.p.m. in this buffer were included in determining the total radioactivity present. Trichloroacetic acid was subsequently added to the buffer samples to a final concentration of 10% (w/v) and the percentage of the total counts in the buffer which was precipitated was determined. These percentages are also shown in the Figure (curves A). All points in the assay were run in triplicate.

The results indicate that all of the radioactivity is being precipitated, which suggests that the tPA has not been degraded. This result, along with those described above, is what would be expected of a ligand which binds to cell surface receptors but which is not internalized.

The experiment was repeated at 37 °C (Fig. 5b). The most remarkable feature of this graph is that it is essentially the same as that obtained at 4 °C. At no point do we see a rise in the percentage of SDS-solubilized counts which would be indicative of internalization. Overall, the results suggest that tPA is either not internalized at all or internalized only very slowly.

DISCUSSION

The binding reaction described in this paper exhibits the characteristics that would be expected from the interaction between a ligand and its receptor. Binding is saturable, dissociable

and occurs with high enough affinity ($K_d = 1.2$ nM) to be of physiological significance. Binding sites interacted equally well with each type of tPA tested regardless of the nature of its glycosylation or whether it had undergone cleavage by plasmin, and the Scatchard curves indicated the presence of a single class of independently acting site. The binding sites were present in very large numbers (1.57×10^6 sites per cell) suggesting that they may be of considerable biological importance. The results may be compared with results reported by Hajjar *et al.* (1987), Barnathan *et al.* (1988), and Beebe (1987), each of whom examined the binding of tPA to endothelial cells from umbilical veins. Hajjar *et al.* (1987) reported that the umbilical vein cells have two classes of tPA binding sites, a high-affinity class with a K_d of 2.87×10^{-11} M and a low-affinity class with a K_d of 1.81×10^{-8} M. Barnathan *et al.* (1988) also found that there were two classes of sites; a K_d for the high-affinity site was not determined, but the low-affinity site was reported to have a K_d of 1.70×10^{-7} M. In contrast, Beebe (1987) found only a single class of binding site of very low affinity ($K_d = 2.38 \times 10^{-7}$ M) which was present in enormous numbers (1.2×10^7 sites per cell). The results reported here regarding the dissociation constants and receptor number in the aortic cells do not appear to correspond well with any of the results reported for the umbilical vein cells.

In addition to determining the basic characteristics of the binding of tPA to endothelial cells from the human aorta, we examined a few of the features of binding which are likely to be of biological significance. It was found that the specific binding of tPA to HAE cells progressively increased as the pH of the binding buffer was lowered from 8.2 to 6.5. It is worth noting that significant changes occur in a part of the pH range that is at least physiologically possible. For example, although the pH of plasma is normally about pH 7.4, it may drop below 6.9 in cases of extreme acidosis or rise above 7.6 in cases of extreme alkalosis (Levinsky, 1977). Special conditions may also exist which cause localized changes in pH. The results shown in Fig. 3 indicate that physiological events which lead to a drop in pH would tend to promote the binding of tPA to endothelial cells, whereas events which lead to an increase in pH would tend to decrease binding.

Both Sakata *et al.* (1988) and Barnathan *et al.* (1988) have presented very convincing evidence that umbilical vein endothelial cells have PAI-1 bound to their surface and that this PAI-1 is capable of interacting with tPA in the surrounding buffer. This prompted us to examine the effect of preincubating 125 I-tPA with PAI-1 before use in the HAE cell binding assay. The results shown in Fig. 4 indicate that the majority of the specific binding observed in the assay was unaffected by this treatment. This suggests that, for the most part, the binding observed in the assay is occurring at sites on the HAE cells distinct from surface-bound PAI-1. It is of course possible that cells cultured under different conditions might produce different results (i.e. PAI-1 interactions might play a greater role in binding) and this is probably a point that warrants further investigation.

Similar to what has been reported for UK (Stoppelli *et al.*, 1985; Vassalli *et al.*, 1985), it appears that tPA remains bound to the surface of HAE cells for long periods of time without being either internalized or degraded. In contrast, Kuiper *et al.* (1988) have presented data from experiments *in vivo* suggesting that endothelial cells associated with the liver internalize tPA by means of a carbohydrate-specific receptor system. It may be that, in the case of the aortic cells, tPA is important in events occurring at or mediated by the cell surface, whereas endothelial cells associated with the liver behave differently because of a role they play in clearance (Nilsson *et al.*, 1985; Beebe & Aronson, 1986; Rijken & Emeis, 1986).

The endothelial cells which line the aorta undoubtedly have a number of different physiological roles, and a great deal of future

study will be required before these are fully understood. In terms of tPA binding, an examination of the extent to which the various structural forms of the enzyme maintain proteolytic activity after binding is clearly warranted. There are reports that indicate that, at least in some systems, the binding of tPA and UK leads to increased enzymic activity and a decreased rate of inactivation by inhibitors (Hajjar *et al.*, 1987; Kirchheimer & Remold, 1989). An examination of the effect of binding on the extracellular matrix of the aortic cells would also be of interest. Finally, studies aimed at determining how binding changes in response to the conditions under which cells are grown and assayed may provide new insights to how the cells behave *in vivo*.

REFERENCES

- Bachmann, F. & Kruithof, E. K. O. (1984) *Semin. Thromb. Hemostasis* **10**, 6-17
- Barnathan, E. S., Kuo, A., Van der Keyl, H., McCrae, R., Larsen, G. R. & Clines, D. B. (1988) *J. Biol. Chem.* **263**, 7792-7799
- Beebe, D. P. (1987) *Thromb. Res.* **46**, 241-254
- Beebe, D. P. & Aronson, D. L. (1986) *Thromb. Res.* **43**, 663-674
- Blasi, F. (1988) *Fibrinolysis* **2**, 73-84
- Blasi, F., Vassalli, J.-D. & Dano, K. (1987) *J. Cell Biol.* **104**, 801-804
- Chen, J.-K., Hoshi, H., McClure, D. B. & McKeehan, W. L. (1986) *J. Cell. Physiol.* **129**, 207-214
- Chmielewska, J., Rånby, M. & Wiman, B. (1983) *Thromb. Res.* **31**, 427-436
- Collen, D. (1987) in *Tissue Plasminogen Activator in Thrombolytic Therapy* (Sobel, B. E., Collen, D. & Grossbard, E. B., eds.), pp. 1-24, Marcel Dekker, New York
- Dano, K., Andreasen, P. A., Grondahl-Hansen, J., Kristensen, P., Nielsen, L. S. & Skivers, L. (1985) *Adv. Cancer Res.* **44**, 139-266
- Dickson, R. B., Willingham, M. C. & Pastan, I. (1980) *J. Biol. Chem.* **256**, 3454-3459
- Feder, J., Tolbert, W. R., Rademacher, T. W., Parekh, R. B. & Dwek, R. A. (1988) U.S. Patent 4571084
- Friesel, R. & Maciag, T. (1988) *Biochem. Biophys. Res. Commun.* **151**, 957-964
- Haigler, H. T., Maxfield, F. R., Willingham, M. C. & Pastan, I. (1980) *J. Biol. Chem.* **255**, 1239-1241
- Hajjar, K. A., Hamel, N. M., Harpel, P. C. & Nachman, R. L. (1987) *J. Clin. Invest.* **80**, 1712-1719
- Harakas, N. K., Schaumann, J. P., Connolly, D. T., Wittwer, A. J., Olander, J. V. & Feder, J. (1988) *Biotechnol. Prog.* **4**, 149-159
- Howard, E. H. & Knauer, D. J. (1987) *J. Cell. Physiol.* **131**, 276-283
- Kirchheimer, J. C. & Remold, H. G. (1989) *Blood* **74**, 1396-1402
- Kuiper, J., Otter, M., Rijken, D. C. & van Berkel, T. (1988) *J. Biol. Chem.* **263**, 220-224
- Levinsky, N. G. (1977) in *Harrison's Principles of Internal Medicine* (Thorn, G. W., Adams, R. D., Braunwald, E., Isselbacher, K. J. & Petersdorf, R. G., eds.), pp. 375-382, McGraw-Hill, New York
- Low, D. A., Baker, J. B., Koonce, W. C. & Cunningham, D. D. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2340-2344
- McKanna, J. A., Haigler, H. T. & Cohen, S. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5689-5693
- McPherson, G. A. (1985) *Kinetic, EBDA, Ligand, Lowry-A Collection of Radioligand Binding Analysis Programs*, Elsevier-Biosoft, U.K.
- Munson, P. (1983) *Methods Enzymol.* **92**, 543-577
- Nilsson, S., Einarsson, M., Ekvarn, S., Haggroth, L. & Mattsson, C. (1985) *Thromb. Res.* **39**, 511-521
- Owensby, D. A., Sobel, B. E. & Schwartz, A. L. (1988) *J. Biol. Chem.* **263**, 10587-10594
- Parekh, R., Dwek, R., Thomas, J., Opdenakker, G., Rademacher, T., Wittwer, A., Howard, S., Nelson, R., Siegel, N., Jennings, M., Harakas, N. & Feder, J. (1989a) *Biochemistry* **28**, 7644-7662
- Parekh, R., Dwek, R., Rudd, P., Thomas, P., Rademacher, T., Warren, T., Wun, T., Hebert, B., Reitz, B., Palmier, M., Ramabhadran, T. and Tiemeier, D. (1989b) *Biochemistry* **28**, 7670-7679
- Pohl, G., Kallstrom, M., Bergsdorf, N., Wallén, P. & Jornvall, H. (1984) *Biochemistry* **23**, 3701-3707

- Rånby, M. (1982) *Biochim. Biophys. Acta* **740**, 461–469
- Rånby, M. & Wallén, P. (1981) *Prog. Fibrinolitics* **5**, 233–235
- Rånby, M., Bergsdorf, N., Pohl, G. & Wallén, P. (1982) *FEBS Lett.* **146**, 289–292
- Reich, E. (1978) in *Molecular Basis of Biological Degradative Processes* (Berlin, R. D., Herrmann, H., Lepow, I. H. & Tanzer, J. M., eds.), pp. 155–169, Academic Press, New York
- Rijken, D. C. & Emeis, J. J. (1986) *Biochem. J.* **238**, 643–646
- Rijken, D. C., Emeis, J. J. & Gerwig, G. J. (1985) *Thromb. Haemostasis* **54**, 788–791
- Sakata, Y., Okada, M. & Matsuda, M. (1988) *J. Biol. Chem.* **263**, 1960–1969
- Simionescu, N., Heltianu, C., Antoché, F., & Simionescu, M. (1982) *Ann. N. Y. Acad. Sci.* **401**, 132–149
- Stoppelli, M. P., Corti, A., Soffrentini, A., Cassani, G., Blasi, F. & Assoran, R. K. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4939–4943
- Thilo-Korner, D. G. S., Heinrich, D. & Lash, H. G. (1982) in *The Endothelial Cell - A Pluripotent Control Cell of the Vessel Wall* (Thilo-Korner, D. G. S., Giessen, F. R. G. & Freshney, R. I., eds.), pp. 29–54, Karger, Basel
- van Zonneveld, A.-J., Chang, G. T. G., van den Berg, J., Kooistra, T., Verheijen, J. H., Pannekoek, H. & Kluft, C. (1986) *Biochem. J.* **235**, 385–390
- Vassalli, J. D., Baccino, D. & Belin, D. (1985) *J. Cell Biol.* **100**, 86–92
- Verstraete, M. & Collen, D. (1986) *Blood* **67**, 1529–1541
- Wiley, H. S. & Cunningham, D. D. (1982) *J. Biol. Chem.* **257**, 4222–4229
- Wiman, B., Melbring, G. & Rånby, M. (1983) *Clin. Chim. Acta* **127**, 279–288
- Wojta, J., Hoover, R. L. & Daniel, T. O. (1988) *J. Biol. Chem.* **264**, 2846–2852
- Wun, T.-C., Schleuning, W.-D. & Reich, E. (1981) *J. Biol. Chem.* **257**, 3276–3283

Received 2 January 1990/26 March 1990; accepted 2 April 1990