Binding of tissue plasminogen activator to human endothelial cells

Importance of the B-chain as a ligand

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The aim of the present study was to investigate the binding of tissue plasminogen activator (tPA) to cultured endothelial cells and to characterize binding structures present in the cultures. Studies on the binding of ^{125}I -tPA to cultured endothelial cells from human umbilical-cord veins (HUVEC) indicated that the number of sites for specific binding of tPA is 8×10^5 per cell. Treatment with an excess of antibodies against plasminogen-activator inhibitor type 1 (PAI-1) caused an 80% decrease in the binding, leaving about 1.6×10^5 unoccupied binding sites per cell, which appeared to be different from PAI-1. About 1.9×10^5 binding sites/cell for tPA were found on the surface of HUVEC that had been detached from the matrix. This indicates that only minor amounts of PAI-1 occur on the surface of the cells. In addition, immunocytochemical analysis showed that PAI-1 antigen is present almost exclusively in the cytoplasm but was not observed on the surface of the cells, whereas tPA antigen is abundant on the plasma membrane of tPA-treated cells as well as intracellularly. Competition studies using unlabelled compounds showed that native tPA and tPA B-chain (the proteinase domain), as well as the inactive derivatives, B-chain inactivated with D-Phe-Pro-Arg-chloromethane and tPA-PAI-1 complex, caused a considerable quenching of the binding of ^{125}I -tPA to HUVEC, whereas the isolated A-chain had no demonstrable effect. Two components (apparent molecular masses 38 kDa and 56 kDa) reacting with tPA but lacking PAI-1 antigen determinants were identified. Thus the data suggest that tPA binds to HUVEC by two principally different mechanisms. One is mediated by PAI-1, which binds and inactivates tPA with a functional active site. The other binding is achieved by components which react with sites on the activator molecule other than structures of the A-chain or the active site.

INTRODUCTION

Studies during the last few decades have shown that the endothelial cell lining of the blood vessels participates very actively in the dynamic balance between fibrin formation (thrombosis) and fibrinolysis (thrombolysis). Much interest has been devoted to the interaction between cells and the components of the fibrinolytic enzyme system. It has been found that many cultured cells have surface receptors for plasminogen (Tobler *et al.*, 1981; Miles & Plow, 1985; Hajjar *et al.*, 1986) and urokinase (Stoppelli *et al.*, 1985; Vassalli *et al.*, 1985). Some early studies on the interaction between tissue plasminogen activator (tPA) and endothelial cells, performed on cultures of endothelial cells from bovine aorta, indicated a specific binding of the activator. Furthermore, the assembly of an activation complex of tPA, plasminogen and fibrin could be generated on the cell surface (Liu *et al.*, 1985, 1986). Lately, experiments on the binding of proteins engaged in the fibrinolytic system to the endothelium have mostly been performed using endothelial cells from human umbilical cord veins (HUVEC). Thus plasminogen binds specifically to endothelial cells mainly by means of its lysine-binding sites (Hajjar *et al.*, 1986; Miles *et al.*, 1991) and is easily activated by plasminogen activators. Other components that have been reported to bind to the HUVEC cultures are plasminogen activator inhibitor type 1 (PAI-1) and tPA.

From studies on the binding of tPA to HUVEC cultures, at least two types of binding sites have been suggested. In one,

probably identical with PAI-1, the tPA is bound via its active site, yielding an inactive complex. Components responsible for the second type of binding do not require an active enzyme. They interact with structures other than the active site of tPA and may be regarded as receptors in the proper sense. It has been claimed that endothelial cells in culture contain tPA-binding membrane proteins which are different from PAI-1 and which form enzymically active complexes (Beebe, 1987; Hajjar *et al.*, 1987; Aerts *et al.*, 1989). Again according to other reports the predominant tPA-binding protein in endothelial cell cultures is PAI-1 whereas specific receptors, if they exist, are suggested to be of minor importance (Ramakrishnan *et al.*, 1990; Russell *et al.*, 1990). The descriptions of the molecular properties of specific receptors have been contradictory. According to one report (Hajjar & Hamel, 1990), one tPA receptor protein was found with a molecular mass of 40 kDa, whereas another publication reports two receptors with molecular masses of 56 and 95 kDa (Beebe *et al.*, 1990). Hajjar (1991) isolated a 42 kDa tPA-binding protein from placental tissue which seemed to be identical with a receptor protein found on endothelial cells.

In the present paper we have studied the binding of tPA to endothelial cell cultures, isolated endothelial cells and membrane components. Efforts have been made to distinguish between PAI-1 and proper receptors. Two membrane components which reversibly bind tPA and are distinct from PAI-1 have been identified. In addition, the distribution of tPA and PAI-1 in the endothelial cell cultures has been studied by using immunocytochemical techniques.

Abbreviations used: FITC, fluorescein 5-isothiocyanate; HUVEC, human umbilical-vein endothelial cells; PAI-1, plasminogen-activator inhibitor-1; PBS, phosphate-buffered saline (140 mM-NaCl/2.6 mM-KCl/1.4 mM- KH_2PO_4 /8.1 mM- Na_2HPO_4 , pH 7.4); PMSF, phenylmethanesulphonyl fluoride; PPACK, D-Phe-Pro-Arg-chloromethane ('D-Phe-Pro-Arg-chloromethyl ketone'); PVDF, poly(vinylidene difluoride); TBS, Tris-buffered saline (150 mM-NaCl/20 mM-Tris, pH 7.4); tPA, tissue plasminogen activator.

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MATERIALS AND METHODS

Dithioerythritol, benzamidine, phenylmethanesulphonyl fluoride (PMSF), 6-aminohexanoic acid, pepstatin A, CHAPS, thermolysin, endothelial cell growth supplement, isobutylmethylxanthine, thymidine and globulin-free bovine albumin were purchased from Sigma, St. Louis, MO, U.S.A. Tween 80, Tween 20 and Triton X-100 were obtained from Kebo Laboratories AB, Stockholm, Sweden. Trasylol (10000 Kallikrein-inhibitory units/ml) was obtained from Bayer AG (Leverkusen, Germany). Parker Medium 199 was purchased from SBL (Stockholm, Sweden). Pluronic 68 was from Basf Co., Parsippany, NJ, U.S.A. Iodogen was from Pierce Co. and Na¹²⁵I from du Pont Scandinavian AB, Stockholm, Sweden. Trypsin/EDTA, gentamicin, L-glutamine, fungizone and newborn calf serum were obtained from Gibco. Leupeptin was from Boehringer-Mannheim GmbH. Heparin was from KabiVitrum, Stockholm, Sweden. Pencillin was from Astra, Stockholm, Sweden. D-Phe-Pro-Arg-chloromethane (PPACK) was obtained from Bachem AG, Bubendorf, Switzerland. Culture flasks and plates were obtained from Nunc, Roskilde, Denmark. Antibodies to *Ulex europaeus* lectin and to von Willebrand factor were from Dakopatts, Copenhagen, Denmark. Fluorescent acetylated low-density lipoprotein was from Biomedical Technologies Inc., Stoughton, MA, U.S.A. Alkaline phosphatase-conjugated goat anti-rabbit IgG and substrates for immunoblots were from Bio-Rad. Horseradish-peroxidase-conjugated goat anti-tPA IgG was from Biopool AB, Umeå, Sweden. Poly(vinylidene difluoride) membranes (PVDF, Immobilon) were obtained from Millipore, Bedford, MA, U.S.A.

tPA and derivatives

Recombinant single-chain tPA (Actilyse) contaminated with about 10% two-chain tPA was obtained from Boehringer, Ingelheim, Germany. Two-chain tPA was prepared as described previously (Wallén *et al.*, 1983). Preparations of A- and B-chains were obtained by partial reduction of two-chain tPA as described previously (Ichinose *et al.*, 1986). The A- and B-chains were separated by gradient chromatography on DEAE-Sephadex. The amidolytic activity of the B-chain was practically identical with that of two-chain tPA (Wallén *et al.*, 1990). PPACK-inactivated tPA and B-chain was prepared by incubating 500 μ l of 0.8 μ M-tPA or B-chain with 10 μ l of 0.2 mM-PPACK for 15 min at room temperature. This treatment completely abolished the amidolytic activity of the B-chain. A complex between tPA and PAI-1 was prepared by incubating 750 μ l of 2 μ M-tPA in phosphate-buffered saline (PBS) with 1 ml of 4 μ M-PAI-1 for 30 min at room temperature. The mixture was adsorbed on 0.25 mg of Sepharose-coupled anti-tPA monoclonal antibody (Sepharose-1:2 B9), added to a column and washed with 4 ml of PBS. The tPA-PAI-1 complex was eluted with 2 ml of 0.05 M-sodium phosphate buffer, pH 7.3, containing 3 M-KSCN and 0.1 mg of Tween 80/ml and dialysed against Medium 199 overnight. The concentration of tPA-PAI-1 was about 0.7 μ M as judged from identical control experiments performed with ¹²⁵I-tPA instead of unlabelled tPA.

Various proteins

Recombinant human PAI-1 prepared from *Escherichia coli* (Lawrence *et al.*, 1989) as well as rabbit antiserum against PAI-1 were generously provided by Dr. Tor Ny (Department for Applied Cell and Molecular Biology, Umeå, Sweden). The IgG fraction was isolated by chromatography on Protein A-Sepharose (Pharmacia, Uppsala, Sweden) and was stored frozen as a stock solution (2.9 mg/ml). Goat antiserum against

human tPA as well as IgG from this serum was prepared as described (Wallén *et al.*, 1983). A monoclonal antibody, 1:2 B9 against tPA, suitable for purification of tPA was prepared as described (Stigbrand *et al.*, 1989) and coupled to CNBr-activated Sepharose 4B (Pharmacia, Sweden) according to the directions of the supplier.

Endothelial cell cultures

Human umbilical cords were obtained within 1 h of delivery and the umbilical vein was cannulated and flushed with 100 ml of 0.15 M-NaCl at ambient temperature. The vein was then filled with 15 ml of 0.05% thermolysin in Dulbecco's PBS. After incubation at room temperature for 30 min, the vein was perfused with 50 ml of endothelial cell culture medium, consisting of Medium 199 supplemented with antibiotics [penicillin (100 i.u./ml) and gentamicin (5 μ g/ml)], 20% newborn calf serum, 40 units of heparin/ml, 2 mM-L-glutamine, 30 μ g of endothelial cell growth factor/ml, 15 μ M-thymidine, 2.5 μ g of fungizone/ml and 33 μ M-isobutylmethylxanthine. The endothelial cells were washed once with culture medium and seeded in 80 cm² cell culture flasks (T80 flasks), pretreated with 1% gelatine in PBS, and cultured at 37 °C in 5% CO₂ until confluence. The cultures were then split 1:4 after a short incubation at ambient temperature with trypsin/EDTA and seeded into 24-well plates or new T80 flasks. The cells showed cobblestone morphology and to confirm that they were endothelial cells, first-passage cells were cultured on glass slides and stained with antibodies to von Willebrand factor and *Ulex europaeus* lectin or incubated with fluorescent acetylated low-density lipoprotein.

Radiolabelling of tPA

Human recombinant one-chain tPA was labelled with ¹²⁵I using the iodogen method modified from the procedure of Salacinski *et al.* (1981) and earlier used for labelling of tPA by Liu *et al.* (1985, 1986). A mixture of 300 μ g of tPA in 200 μ l of 1 M-NH₄HCO₃, 20 μ l of 38 μ M Na¹²⁵I and 95 μ l of 0.25 M-sodium phosphate buffer, pH 6.5, containing 70 μ M-KI and 0.5 mg of Pluronic F 68/ml, was added to a glass tube coated with 50 μ g of iodogen. After incubation for 15 min at 25 °C, 2.815 ml of 0.05 M-sodium phosphate buffer, pH 7.3, containing 0.5 mg of Pluronic F 68/ml was added to dilute the reaction mixture. After a further 15 min, 2 g of Sepharose-1:2 B9 was added and the mixture stirred by end-over-end rotation overnight at 4 °C. The suspension was transferred to a small column and washed extensively with the phosphate/Pluronic buffer and 0.05 M-sodium phosphate buffer, pH 7.3, containing 0.1 mg of Tween 80/ml. The elution of labelled tPA was performed with 3 M-KSCN dissolved in the phosphate/Tween buffer. The labelled tPA was dialysed either against 1 M-NH₄HCO₃ containing 0.1 mg of Tween 80/ml or Medium 199. According to an e.l.i.s.a. the yield of tPA protein was 50–60% and it retained full enzyme activity (about 600 000 units/mg when compared with the International tPA Standard, coded 83/517, from the National Institute of Biological Standards and Control, Potters Bar, Herts., U.K.) as measured by fibrin-stimulated plasminogen activation (Rånby *et al.*, 1982). The incorporation of iodine was about 1.2 atoms/molecule of tPA (mean of six preparations).

Immunocytochemical localization of tPA and PAI-1

HUVEC were grown to confluency on gelatine-coated chamber glass slides. After being washed with Tris-buffered saline (TBS; 0.02 M-Tris/0.15 M-NaCl, pH 7.5) containing 1% BSA, the cells were incubated for 30 min at 37 °C with tPA (1 μ g of tPA/ml) in Medium 199. The cells were then washed with TBS/BSA and fixed with methanol for another 30 min. This was followed by incubation at ambient temperature with goat antiserum against

tPA or rabbit antiserum against PAI-1. The antisera were used at dilutions of 1:50–1:250. After 45 min the endothelial cells were rinsed in TBS/BSA and incubated for 45 min with fluorescein 5-isothiocyanate (FITC)-conjugated antibodies against goat IgG or rabbit IgG (Dakopatts). After a final wash with TBS/BSA, the cells were visualized in a fluorescence microscope and photographed.

Binding of ^{125}I -labelled tPA to HUVEC cultures

Confluent HUVEC in 24-well tissue culture plates were washed once with 400 μl of 1% BSA in PBS and incubated with 400 μl of 10 mM-6-aminohexanoic acid/1% BSA in PBS at 4 °C for 10 min. After the cells had been washed twice with 1% BSA in PBS, 400 μl of Medium 199 containing various concentrations of ^{125}I -tPA (0–50 nM) was added to duplicate wells and the plates were incubated at 4 °C for 30 min. The wells were then washed twice rapidly with PBS/BSA. The cells were removed from the wells by adding 100 μl of trypsin/EDTA (0.5 mg of trypsin/ml, 0.5 mg of EDTA/ml) and 100 μl of 1% BSA in PBS to each well. The molar amounts of tPA bound to the wells were calculated from measurements of the released radioactivity.

The effect of antibodies against PAI-1 on the binding of tPA to HUVEC was studied by preincubation of the cultures with an excess of anti-PAI-1 IgG (0.24 or 1.8 μM) or corresponding concentrations of normal rabbit IgG in Medium 199 at 4 °C for 45 min, before addition of ^{125}I -tPA to the wells. The procedure was in other respects as described for the binding assay above.

The ability of unlabelled forms and derivatives of tPA to compete with ^{125}I -tPA for binding to HUVEC was studied essentially as described above. Competing agents were: One- and two-chain tPA, A- and B-chain, PPACK-inactivated B-chain and tPA-PAI-1 complex. The washed wells were incubated for 30 min at 4 °C with 370 μl of Medium 199 containing the unlabelled compounds in various concentrations (0–440 nM) before addition of 30 μl of ^{125}I -tPA (final concentration 38 nM). The binding and competition experiments were repeated several times and the coefficient of variation for replicate values was less than 16% (range 8.4–16%) in all experiments.

Preparation and extraction of endothelial cell membranes

Endothelial cells were detached from cultures with 0.02% EDTA in PBS, washed with PBS and centrifuged (440 g). The pellet was suspended in PBS containing 2% Tween 20, 0.25 M-sucrose, 21 μM -leupeptin, 1 mM-PMSF, 15 μM -pepstatin A. The cells were disrupted in a Potter-Elvehjem homogenizer and centrifuged at 2500 g at 4 °C. The supernatant was centrifuged for 1 h at 100000 g to collect cell membranes. The membrane fraction was extracted sequentially with 15 mM-CHAPS and 1% Triton X-100 in PBS, containing the same inhibitor mixture as above. The extracts were dialysed against 5% acetic acid and freeze-dried. They were subsequently dissolved in 250 μl of SDS/PAGE sample buffer (62.5 mM-Tris/HCl, 12.5% glycerol, 1.25% SDS, 0.00125% Bromphenol Blue, pH 6.8).

Electrophoretic analysis

SDS/PAGE performed as described by Laemmli (1970) in 10% gels was used for the separation of proteins in cell membrane fractions. Separated proteins were transferred to PVDF membranes by the semi-dry blotting technique (Kyhse-Andersen, 1984) in a Sartoroblott II apparatus (Sartorius, Göttingen, Germany) using 48 mM-Tris/39 mM-glycine/1.3 mM-SDS/20% methanol, pH 9.2, as the blotting buffer (30 min, 4 mA/cm² gel). Reference protein strips were stained with Coomassie Brilliant Blue. Components with affinity to tPA were traced with ^{125}I -tPA.

The membranes were washed overnight with PBS at 4 °C, incubated with 5 ml of 5% (w/v) BSA in PBS for 3 h and with

5 ml of 1.35 nM- ^{125}I -tPA in PBS for 2 h. After the membranes had been washed three times with PBS, the radioactive ligands were detected by autoradiography using Hyperfilm (Kodak).

PAI-1 was detected in the membranes by immunoblotting. After transfer to a PVDF membrane as described above, the membranes were incubated in 1% solution of delipidized milk powder in Tris buffer (20 mM-Tris/500 mM- NaCl/5 mM- NaN_3 , pH 7.5) at 4 °C overnight. The membrane was washed twice for 5 min in the incubation buffer and was then incubated for 4 h in rabbit anti-PAI-1 IgG diluted to 30 $\mu\text{g}/\text{ml}$ with the milk powder solution. The membrane was washed four times in Tris buffer and incubated for 1 h with alkaline phosphatase-conjugated goat antibodies against rabbit IgG diluted in the milk powder solution. The membrane was again washed four times in TBS and finally incubated in a solution of 5-bromo-4-chloroindol-3-yl phosphate and Nitroblue Tetrazolium in 0.1 M- NaHCO_3 buffer/1 mM- MgCl_2 , pH 9.8. The reaction was stopped by washing twice with distilled water.

Various methods

Concentrations of total protein were determined either by a modification of the Lowry method (BCA, Pierce Europe, Oud-Beijerland, The Netherlands) or by amino acid analysis. The concentrations of tPA and PAI-1 antigen were determined by e.l.i.s.a. (Imulysse tPA and PAI-1 respectively; Biopool, Umeå, Sweden). Plasminogen activation was measured with a chromogenic assay (Rånby *et al.*, 1982) using fibrin as stimulator.

RESULTS

Binding of tPA to HUVEC monolayers

The binding of ^{125}I -tPA in concentrations from 4 to 50 nM was studied in 24-well culture plates. The non-specific binding, which was assessed by determination of binding in the presence of 1 μM -unlabelled tPA, amounted to about 45%. Saturation of the cell layer was obtained at concentrations of tPA exceeding 40 nM (Fig. 1). The maximal amount of tPA bound to the culture was about 0.07 pmol/well after correction for non-specific binding. The number of cells was about 44000/well indicating 8×10^5

Fig. 1. Specific binding of tPA to HUVEC

Monolayers of HUVEC of 24-well culture plates (44000 cells/well) were incubated with the indicated concentrations of ^{125}I -tPA in Medium 199 supplemented with 0.5% BSA at 4 °C for 30 min. After being washed, the wells were treated with EDTA/trypsin to release the bound tracer. The radioactivity was measured and the tPA bound/well was calculated. The data points (means of duplicates) in the curve represent specific binding determined as the difference between binding of ^{125}I -tPA in the absence and that in the presence of 1 μM -unlabelled tPA.

Fig. 2. Effect of preincubation of HUVEC with anti-PAI-1 IgG on the specific binding of tPA

The cell monolayers were preincubated with 36 μ g of anti-PAI-1 IgG/ml (hatched bars) or normal rabbit IgG (open bars) before 125 I-tPA at 38, 44 and 50 nM was added. Determination of specific binding was performed in duplicate as described in Fig. 1.

Fig. 4. Binding of tPA and PPACK-inactivated tPA to PAI-1

Samples of tPA (Δ) or PPACK-inactivated tPA ($\blacksquare$) (0.1 μ M final concentration) were mixed with the indicated concentrations of PAI-1 for 1 h at room temperature. The mixtures were added to microtiter wells that had been coated with anti-PAI-1 IgG. Bound complexes were detected by incubation with peroxidase-conjugated anti-tPA IgG.

Fig. 3. Competition of 125 I-tPA binding to HUVEC by active and inactive unlabelled tPA and tPA derivatives

The cell monolayers were preincubated with the indicated concentrations of the competing ligands for 30 min at 4 $^{\circ}$ C before 125 I-tPA (38 nM) was added. (a) Single-chain tPA (Δ); two-chain tPA ($\blacktriangle$); A-chain of tPA ($\blacksquare$); B-chain of tPA ($\bullet$). (b) tPA-PAI-1 complex ($\square$); B-chain of tPA ($\bullet$); PPACK-inactivated B-chain ($\circ$). Data points represent the means of duplicate samples.

binding sites/cell. The concentration at half-saturation suggests a dissociation constant of approx. 20 nM. It is well known that high concentrations of PAI-1 are secreted from endothelial cells in culture, and it is deposited in the matrix in an active form. This contributes markedly to the binding of tPA to the culture wells. In order to estimate the extent to which this occurs, the culture surface was preincubated with an excess of antibodies against human PAI-1 before the incubation with labelled tPA. Three concentrations of tPA were chosen, all at or above the limit for saturation. The results (Fig. 2) demonstrate that about 20% of the specific binding cannot be quenched by anti-PAI-1 IgG at a concentration of 0.24 μ M (36 μ g/ml). Similar results were obtained at a sevenfold higher concentration of the antibody preparation (not shown). Control experiments with the same amounts of normal rabbit IgG showed no significant effect on the unspecific binding. These results suggest the occurrence of about 1.6×10^5 binding sites for tPA that seem to be different from PAI-1 on the cell surface.

Specificity of the binding of tPA to HUVEC monolayers

The capacity of the following unlabelled derivatives of tPA to compete with 125 I-tPA for binding sites in endothelial cell cultures was studied (Figs. 3a and 3b): single-chain tPA, two-chain tPA, isolated A-chain and isolated B-chain. The total binding of 125 I-tPA at 38 nM was decreased to about 50% by preincubation with unlabelled single-chain or two-chain tPA at concentrations exceeding 50 nM, which implies almost complete quenching of specifically bound tracer (unspecific binding is defined as total binding in the presence of 1 μ M-unlabelled tPA, see above). The isolated B-chain, at concentrations above 150 nM, caused about a 60% decrease in total binding of 125 I-tPA. This equals 80% quenching of specifically bound tracer. The A-chain, at concentrations up to 150 nM, had no significant effect on the binding of labelled tPA (Fig. 3a). The ability of active-site-blocked B-chain to inhibit the binding of 125 I-tPA is compared with active B-chain in Fig. 3(b). The quenching by PPACK-inactivated B-chain amounted to 50–60% of the quenching caused by active B-chain, indicating that a considerable part of the tPA binding is distinct from binding to PAI-1. This is further supported by the ability of the tPA-PAI-1 complex to cause a similar magnitude of quenching of the tPA binding to that of the PPACK-inactivated B-chain (Fig. 3b).

Fig. 5. Components binding ^{125}I -tPA tracer in membrane fractions from HUVEC

The binding of ^{125}I -tPA to components separated by 10% SDS/PAGE of the CHAPS and Triton X-100 extracts from a cell membrane preparation was visualized by autoradiography after electroblotting. The membranes were first extracted with CHAPS buffer (lane 1) followed by Triton X-100 buffer (lane 2). The respective molecular masses (kDa) of the tPA-binding proteins, calculated from the mobilities of the reference proteins, are indicated.

A comparison between tPA and its PPACK-inactivated derivative with regard to their interaction with PAI-1 is shown in Fig. 4. Mixtures of tPA or PPACK-inactivated tPA ($0.1 \mu\text{M}$) were incubated with increasing amounts of PAI-1 (0 – $0.27 \mu\text{M}$) and the mixtures added to 96-well plates coated with anti-PAI-1 IgG. Adsorbed tPA-PAI-1 complex was assessed by peroxidase-conjugated anti-tPA IgG. The results show that PPACK-inactivated tPA, in contrast with active tPA, has virtually no reactivity with PAI-1. The decrease in absorbance observed with active tPA at the highest concentration of PAI-1 indicates a competition between excess PAI-1 and tPA-PAI-1 complex for the anti-PAI-1 IgG coat.

Binding of tPA to isolated cells in suspension and to cell fractions

Since it has been shown that the surface-bound PAI-1 of endothelial cell cultures is mainly adsorbed to the matrix, further

Fig. 6. Immunoreactive PAI-1 in membrane fractions from HUVEC

Samples of the same CHAPS (lane 1) and Triton X-100 (lane 2) extracts as in Fig. 5 were separated by SDS/PAGE and electroblotted on to a PVDF membrane. PAI-1 was detected after incubation with rabbit anti-PAI-1 IgG followed by alkaline phosphatase-conjugated goat anti-rabbit IgG. The molecular masses (kDa) of the immunoreactive proteins are indicated.

studies of the binding of tPA were performed on isolated cells in suspension. The detachment of cells from the matrix was made by treatment with 0.02% (w/v) EDTA, a procedure that yields viable cells. The capacity of the isolated cells to bind tPA was assessed by incubating a suspension of endothelial cells from 12 T80 flasks (about 2.2×10^7 cells) in 1.5 ml of a 70 nM solution of ^{125}I -tPA on ice for 30 min. The suspension was layered on top of 5 ml of a solution of high-density Lymphoprep (Nyegaard & Co., Oslo, Norway) diluted 1:2 with PBS and centrifuged at 400 *g* for 30 min at 4 °C. Cell-bound tPA was collected in the pellet and free tPA stayed at the top. Determination of the

Fig. 7. Immunocytochemical localization of PAI-1 in HUVEC monolayer

Confluent endothelial cells were incubated at 37 °C, first with tPA ($1 \mu\text{g}/\text{ml}$), followed by washing with TBS/BSA and fixation with methanol, then with rabbit anti-PAI-1 antiserum (dilution 1:50); they were finally visualized with FITC-conjugated swine anti-rabbit IgG. The nuclei are clearly identified as non-fluorescent areas, suggesting that the fluorescence is mainly cytoplasmic.

Fig. 8. Immunocytochemical localization of tPA in HUVEC monolayer

Confluent endothelial cells were treated with tPA, fixed with methanol and incubated with goat anti-tPA antiserum (1:250) and finally FITC-conjugated rabbit anti-goat IgG using the same conditions as for Fig. 7. The fluorescence is also homogeneous over the cell nuclei, indicating staining for immunoreactive tPA on the cell membrane.

radioactivity in the pellet indicated 1.9×10^6 binding sites per cell. This number, which should be compared with the number of non-PAI-1-related binding site (1.6×10^6) found for the cell culture (see above), indicates that the part of the binding of tPA to the cell culture, which is not caused by PAI-1, is mainly localized to the surface of the cells.

The CHAPS and Triton extracts of the crude membrane preparation obtained from lysed isolated cells were separated on SDS/PAGE and electroblotted on to PVDF membranes. Components binding tPA were detected by autoradiography using ^{125}I -tPA as tracer (Fig. 5). The CHAPS fraction contained three major bands with apparent molecular masses 67, 50 and 38 kDa whereas the Triton fraction only showed one component of about 56 kDa. Immunoblotting of an SDS/polyacrylamide gel run in parallel using rabbit antiserum against human PAI-1 (Fig. 6) indicated that the CHAPS fraction contained three main PAI-1-related components (molecular masses: 50, 71 and 97 kDa), whereas no PAI-1-reactive material was found in the Triton fraction. The results indicate the presence of at least two components in the plasma membrane (38 and 56 kDa) interacting with tPA but distinct from PAI-1. With regard to the other tPA-binding compounds in the CHAPS extract, it seems likely that the 50 kDa component is identical with PAI-1, whereas the nature of the 67 kDa component is more uncertain.

The association of PAI-1 and tPA with endothelial cells was demonstrated using FITC-labelled antibodies. The incubation with antibody against PAI-1 (Fig. 7) shows bright fluorescence of the cytoplasm but areas corresponding to the nuclei are dark. The staining pattern was independent of the temperature during the experiment (4 °C or 37 °C). Antibodies against tPA give a strong fluorescence over the cell surfaces but also over the cell nuclei (Fig. 8). These results suggest that PAI-1 is primarily localized inside the cells or in the extracellular matrix under the cells, whereas binding sites for tPA are abundant on the cell surface.

DISCUSSION

This study shows that the distribution of PAI-1 and tPA differ in endothelial cell cultures. PAI-1 is confined to the intracellular compartment of the cells and to the extracellular matrix (Fig. 7), whereas tPA can be visualized both intracellularly (like PAI-1) and on the cell surface (Fig. 8). The latter finding supports earlier reports on specific receptors for tPA on the endothelial cell surface (Liu *et al.*, 1985, 1986; Beebe, 1987; Hajjar *et al.*, 1987).

Our data suggest that the part of the tPA binding to HUVEC cultures that can be quenched by antibodies against PAI-1 is mainly caused by extracellular matrix-bound PAI-1, whereas the binding sites for tPA that are not affected by the anti-PAI-1 antibodies are present on the cell surface. These findings are in agreement with the results of other studies which have shown that PAI-1 is mainly bound to the extracellular matrix of endothelial cell cultures in a complex with vitronectin (Mimuro & Loskutoff, 1989*a,b*). Furthermore Schleef *et al.* (1990), using immunogold electron microscopy, reported that most of the PAI-1 in HUVEC cultures is located to the extracellular matrix under the cells whereas less than 1 % was found on the dorsal cell surface. Similar distribution patterns (PAI-1 in the matrix under the cells and tPA on the dorsal cell surface) have also been established for cultured bovine capillary endothelial cells (Murata *et al.*, 1991).

The number of binding sites that could not be blocked by PAI-1 antibodies (about 20 % of the total specific binding) correlated well with the number of binding sites calculated from binding experiments performed with cells in suspension, without the interference of matrix proteins. Furthermore, preliminary studies with PPACK- and di-isopropyl phosphorofluoridate-inactivated ^{125}I -tPA which does not interact with PAI-1, displayed a binding that was 20–30 % of the specific binding of corresponding concentrations of active tPA tracer (G. Pohl & X.-F. Cheng, unpublished work). The presence of two types of binding sites for

tPA in HUVEC cultures which is suggested from our data is also in agreement with reports from others (Hajjar *et al.*, 1987; Barnathan *et al.*, 1988). Barnathan *et al.* (1988) found the active-site-dependent binding (PAI-1) to saturate at 10 nM and calculated a K_D of about 170 nM for the non-active-site-dependent binding site. Such low-affinity binding sites have also been observed by other groups (Aerts *et al.*, 1989; Beebe, 1987). Ramakrishnan *et al.* (1990) and Russell *et al.* (1990) studied the binding of tPA to PAI-1 in HUVEC using an active-site mutant (Ser-478 changed to Ala). This mutant forms a non-covalent complex with PAI-1, which is not released to the medium. They found a very large number of binding sites (10^6 – 10^7 sites/cell) which according to Russell *et al.* (1990) were characterized by a K_D of 1.5 nM. However, they did not explore binding sites of lower affinity.

In the present study, we observe saturation of specific tPA binding to HUVEC at 40 nM, similar to the data obtained by Hajjar & Hamel (1990) who identified a tPA-binding site in HUVEC membrane preparations that saturated between 15 and 40 nM and which was concluded not to be related to PAI-1. Thus it is possible that HUVEC cultures express at least three different binding sites for tPA: one high-affinity active-site-dependent site, probably identical with PAI-1, and two non-active-site-dependent sites with dissociation constants of about 10–20 nM and 150–250 nM respectively.

We found that the binding of tPA to the endothelial cell culture could be quenched with single-chain tPA, two-chain tPA and the tPA B-chain, but not with the tPA A-chain at the concentrations used (Fig. 3). These findings disagree to some extent with a publication by Beebe *et al.* (1989), in which synthetic peptides from the 'Finger domain' in the A-chain were found to inhibit binding to endothelial cells. However, the concentrations of the peptides used were very high (more than 1 mM) compared with the concentrations of competing ligands used in our experiments (less than 0.2 μ M). The inability of the A-chain to inhibit the binding of tPA cannot, on the other hand, completely rule out the possibility of binding structures in the A-chain part of tPA. The preparation procedure may have caused misfolding of the protein or modifications, such as carboxymethylation of the 'free' thiol group in the EGF domain. In addition, we cannot rule out that low-affinity binding sites ($K_D > 50$ nM) may be directed against structures in the A-chain. Such low-affinity sites were not explored in this study.

The immunofluorescence studies failed to detect surface-bound PAI-1 (Fig. 7) whereas surface-bound tPA was readily observed (Fig. 8). Further observations in the present study that argue in favour of the existence of non-PAI-1 tPA-binding components were: (1) that there was a substantial residual binding to cell cultures even after treatment with excess antibody against PAI-1 (Fig. 2), (2) that the binding could also be demonstrated in cell suspensions where matrix-bound PAI-1 would not be expected to interfere and (3) that PPACK-inactivated B-chain as well as preformed tPA-PAI-1 complex had a considerable quenching effect on the binding of 125 I-tPA to cell cultures.

The separation of cell membrane extracts by SDS/PAGE, followed by electroblotting and tracing of 125 I-tPA-binding compounds identified two bands with approximate molecular masses 38 and 56 kDa respectively, which could not be detected by immunoblotting using anti-PAI-1 antibodies. Hajjar & Hamel (1990) reported the presence in endothelial cells of a 40 kDa tPA-binding membrane protein, which reacted with native as well as di-isopropyl phosphorofluoridate-inactivated tPA. This agrees with the present findings except that the authors did not report on any 57 kDa component. On the other hand Beebe *et al.* (1990)

reported a tPA-binding protein of 54 kDa but no 40 kDa component.

We conclude from the present study that endothelial cells in culture contain tPA-binding receptor activity different from PAI-1. It is present in the cell membrane, is characterized by a dissociation constant of about 20 nM and binds tPA via domains at least partly localized within the B-chain part of tPA.

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REFERENCES

- Aerts, R. J., Gillis, K. & Pannekoek, H. (1989) *Thromb. Haemost.* **62**, 699–703
- Barnathan, E. S., Kuo, A., Van der Keyl, H., McCrae, K. R., Larsen, G. R. & Cines, D. B. (1988) *J. Biol. Chem.* **263**, 7792–7799
- Beebe, D. P. (1987) *Thromb. Res.* **46**, 241–254
- Beebe, D. P., Miles, L. A. & Plow, E. F. (1989) *Blood* **74**, 2034–2037
- Beebe, D. P., Wood, L. L. & Moos, M. (1990) *Thromb. Res.* **59**, 339–350
- Hajjar, K. A. (1991) *J. Biol. Chem.* **266**, 21962–21970
- Hajjar, K. A. & Hamel, N. M. (1990) *J. Biol. Chem.* **265**, 2908–2916
- Hajjar, K. A., Harpel, P. C., Jaffe, E. A. & Nachman, R. L. (1986) *J. Biol. Chem.* **261**, 11656–11662
- Hajjar, K. A., Hanel, N. M., Harpel, P. E. & Nachman, R. L. (1987) *J. Clin. Invest.* **80**, 1712–1719
- Ichinose, A., Takio, K. & Fujikawa, K. (1986) *J. Clin. Invest.* **78**, 163–169
- Kyhse-Andersen, J. (1984) *J. Biochem. Biophys. Methods* **10**, 203–209
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lawrence, D., Strandberg, L., Grundström, T. & Ny, T. (1989) *Eur. J. Biochem.* **186**, 523–533
- Liu, C. Y., Wallén, P. & Handley, D. A. (1985) *Thromb. Haemost.* **54**, 60, abstract no. 356
- Liu, C. Y., Wallén, P., Handley, D. A. & Smith, J. (1986) in *Fibrinogen and its Derivatives: Biochemistry, Physiology and Pathophysiology*; Excerpta Medica, International Congress Series 722 (Muller-Berghaus, G., Scheffers-Borchel, U., Selmayr, E. & Henschen, A., eds.), pp. 319–322, Excerpta Medica, Amsterdam
- Miles, L. A. & Plow, E. F. (1985) *J. Biol. Chem.* **260**, 4303–4311
- Miles, L. A., Dahlberg, C. M., Plescia, J., Felez, J., Kato, K. & Plow, E. F. (1991) *Biochemistry* **30**, 1682–1691
- Mimuro, J. & Loskutoff, D. J. (1989a) *J. Biol. Chem.* **264**, 936–939
- Mimuro, J. & Loskutoff, D. J. (1989b) *J. Biol. Chem.* **264**, 5058–5063
- Murata, T., Nakashima, Y., Yasunaga, C., Maeda, K. & Sueishi, K. (1991) *Exp. Mol. Pathol.* **55**, 105–118
- Ramakrishnan, V., Sinicropi, D. V., Dere, R., Darbonne, W. C., Bechtol, K. B. & Baker, J. B. (1990) *J. Biol. Chem.* **265**, 2755–2762
- Rånby, M., Norrman, B. & Wallén, P. (1982) *Thromb. Res.* **27**, 743–749
- Russell, M. E., Quertermous, T., Declercq, P. J., Collen, D., Haber, E. & Homcy, C. J. (1990) *J. Biol. Chem.* **265**, 2569–2575
- Salacinski, P. R. P., McLean, C., Sykes, J. E. C., Clement-Jones, V. V. & Lowry, P. J. (1981) *Anal. Biochem.* **117**, 136–146
- Schleef, R. R., Podor, T. J., Dunne, E., Mimuro, J. & Loskutoff, D. J. (1990) *J. Cell Biol.* **110**, 155–163
- Stigbrand, T., Frängsmyr, L., Bergsdorf, N. & Wallén, P. (1989) *Thromb. Haemost.* **62**, 742–747
- Stoppelli, M. P., Corti, A., Soffientini, A., Cassani, G., Blasi, F. & Assoian, R. K. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4939–4943
- Tobler, J., Krieger, M. & Stroud, R. M. (1981) *J. Cell. Physiol.* **108**, 277–290
- Vassalli, J. D., Baccino, D. & Belin, D. (1985) *J. Cell Biol.* **100**, 86–92
- Wallén, P., Pohl, G., Bergsdorf, N., Rånby, M., Ny, T. & Jörnvall, H. (1983) *Eur. J. Biochem.* **132**, 681–686
- Wallén, P., Cheng, X. F. & Ohlsson, P. I. (1990) *Adv. Exp. Med. Biol.* **281**, 185–194