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Interaction of plasmin with endothelial cells

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Interaction of human plasmin with a monolayer culture of mini-pig aortic endothelial cells was studied by using the ^{125}I-labelled enzyme. The binding of plasmin was time- and concentration-dependent. Equilibrium between bound and free enzyme was obtained within 90s, and Scatchard analysis indicated a high- and a low-affinity population of binding sites of approx. 1.24×10^4 sites/cell having a K_d of 1.4×10^{-9}M and 7.2×10^4 sites/cell with a K_d of 2×10^{-8}M respectively. Plasmin, bound to cell, was spontaneously released within 2min, suggesting a rapid equilibrium. Chemical modification of the enzyme with phenylmethanesulphonyl fluoride or pyridoxal 5'-phosphate revealed that neither the active centre nor the heparin-binding site of plasmin was involved in the interaction with the endothelial cell. In terms of endothelial-cell receptors, the binding sites of cells for plasmin and thrombin were different: the two enzymes did not compete with each other, and the pretreatment of cells with neuraminidase or chondroitin ABC lyase resulted in a 50% decrease of thrombin or plasmin binding respectively. Arachidonic acid incorporated into phospholipids of the cell was released by plasmin, but a change in the rate of prostacyclin formation was not measurable. The interaction of plasmin with endothelial cells seems to be specific in the fibrinolytic system, since plasminogen did not bind to these cells under similar conditions.

Maintenance of the blood fluidity is one of the primary tasks in endothelial-cell functions (Barnhart & Baechirer, 1978). Endothelial cells are capable of producing molecular constituents which tend either to decrease or to increase coagulant activity. The synthesis and release of prostacyclin (Weksler *et al.*, 1978; Mareus *et al.*, 1979), plasminogen activator (Loskutoff & Edington, 1977), as well as the increased inactivation of endothelial-cell-bound thrombin by antithrombin III (Bauer *et al.*, 1983) are important functions of the endothelium. Furthermore, thrombin-catalysed activation of Protein C is accelerated by endothelial-cell surface cofactor, resulting also in inhibition of blood coagulation (Owen & Esmon, 1981). On the other hand, endothelial cells may produce tissue factors (Maynard *et al.*, 1977) and proteinase(s) to activate Factor XII (Wiggins *et al.*, 1980), thus triggering blood coagulation. In addition, β-thromboglobulin secreted by platelets inhibits the prostacyclin release of endothelial cells (Hope *et al.*, 1979). The plasminogen activators, also secreted by endothelial cells, may be involved in the initiation of arteriosclerosis (Smokovits, 1980).

Thus the regulation of the various functions of endothelial cells is essential for controlling haemostasis. It is well documented that thrombin binds tightly to endothelial cells (Lollar *et al.*, 1980), resulting in prostacyclin release (Weksler *et al.*, 1978) and a decrease in intracellular plasminogen-activator activity (Loskutoff, 1979). The binding and the biological function of the key enzyme of fibrinolysis, plasmin, however, is not known. Since endothelial cells produce plasminogen activator(s), the local plasmin level will rise. It is an interesting question how the newly formed plasmin binds to endothelial cells, and how it may influence its own formation, or some other cell

Abbreviations used: Δ_4Ach, arachidonic acid; PMSF, phenylmethanesulphonyl fluoride; pyridoxal-*P*, pyridoxal 5'-phosphate; PGI_2, prostacyclin; phosphate-buffered saline, 140mM-NaCl/10mM-sodium phosphate, pH7.2; Bz-Arg-OEt, *N*-benzoyl-L-arginine ethyl ester; S-2251, *H*-D-valyl-leucyl-lysine *p*-nitroanilide dihydrochloride; DMEM, Dulbecco's Modified Eagle Medium; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; PGF_1, prostaglandin F_1.

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functions. Therefore, in the present work, we examined the possible interaction between plasmin and endothelial cells.

Our results show that plasmin binds tightly to endothelial cells, with a K_d of approx. 10^{-9}M.

A preliminary report of this work was presented at the 22nd Meeting of the Hungarian Biochemical Society held at Debrecen in August 1982 (Csonka & Bauer, 1982).

Experimental

Materials

Lysine–Sepharose, and Bz-Arg-OEt were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. S-2251 and streptokinase were the products of Kabi AB Vitrum, Stockholm, Sweden. Sephadex G-25 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Foetal-calf serum was the product of Seromed, München, Germany. The silica-gel t.l.c. plates were obtained from Merck, Darmstadt, Germany. The $Na^{125}I$ (tracer free) and [5,6,8,9,11,12,14,15-^{3}H]Δ_4Ach (6.6TBq/mmol) were the products of the Isotope Institute of the Hungarian Academy of Sciences. Other chemicals were purchased from Reanal, Budapest, Hungary.

Methods

Plasminogen was purified from citrated human plasma by using lysine–Sepharose 4B affinity chromatography as described by Deutsch & Mertz (1970). Plasminogen was activated before starting the experiment with streptokinase (10μg/mg of plasminogen) at 22°C in 0.1mM-6-aminohexanoic acid containing 0.1M-sodium phosphate buffer, pH7.4. The activated protein was gel-filtered on a Sephadex G-25 column (1cm × 6cm), with 0.1M-sodium phosphate buffer, pH7.4, as eluent. The specific activity of the final product was 7.8units/mg of protein [one proteolytic unit was arbitrarily taken as the amount of enzyme producing an increase of 450μg of acid-soluble tyrosine in a medium of 4% casein in 1h (Remmert & Cohen, 1949)]. The plasmin activity was measured as described previously with Bz-Arg-OEt or S-2251 as substrates (Machovich *et al.*, 1981*a*,*b*).

Active-site-blocked plasmin or thrombin was prepared by the successive addition of PMSF at 23°C until it lost amidolytic enzyme activity. Pyridoxal-*P*-modified plasmin was obtained by the incubation of the enzyme with the modifier, followed by reduction with $NaBH_4$ as described previously (Machovich *et al.*, 1981*b*). The final product contained 3–4mol of pyridoxal-*P*/mol of plasmin.

Plasmin and other proteins were labelled with ^{125}I by using the chloramine-T method and had specific radioactivities of (2.4–3.4) × 10^9d.p.m./mg of protein.

α-Thrombin was purified from Cohn fraction III by the method of Fenton *et al.* (1977). Antithrombin III, purified from human plasma (Wickerhauser *et al.*, 1979), was obtained from the American Red Cross Fractionation Center, Bethesda, MD, U.S.A. Fibrinogen (human, grade L) was the product of Kabi Diagnostica, Stockholm, Sweden.

Mini-pig aortic endothelial cells were cultured as described elsewhere (Csonka *et al.*, 1975), using Dulbecco's modified Eagle medium supplemented with 20% (v/v) foetal-calf serum. The cells proved to be endothelial cells on the basis of the following criteria: (1) the cells showed 'cobblestone' growing character and the other morphological characters of endothelial cells [judged by microscopic examinations described previously (Csonka *et al.*, 1980)]; (2) the cells produced PGI_2, and thrombin increased the amount of prostacyclin released; (3) the cells contained the angiotensin-converting enzyme, and this enzyme activity could be activated by Ca^{2+} and was inhibited by SQ 20881 (<Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro) as described by Hayes *et al.* (1978).

For the binding studies, confluent cultures of endothelial cells were used. The cells, after 3–12 passage, were grown in the bottom of scintillation vials for 5–6 days. The cells were counted after trypsin treatment of parallel samples. Before the experiments, cells were washed three times with phosphate-buffered saline containing 2mg of human serum albumin/ml. The labelled compounds were added in 0.5ml of DMEM containing 5mg of albumin/ml and 10mM-Hepes, pH7.4. The incubation was performed at room temperature. After incubation the cells were washed three times with phosphate-buffered saline containing 2mg of albumin/ml. The washing process lasted 5 ± 1s, during which only a minute amount of bound radioactivity was lost (<5%). After the final washing, 5ml of liquid-scintillation cocktail {333ml of Triton X-100 and 666ml of toluene containing 4.2g of PPO (2,5-diphenyloxazole) and 0.05 of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene]} was added and the radioactivity of the samples was determined in a Beckman LS 350 liquid-scintillation spectrometer. Binding-experiment results were subjected to Scatchard (1949) analysis.

[^{3}H]Δ_4Ach release was measured as follows: cells were incubated with 1μCi of [^{3}H]ΔAch/ml at 37°C overnight. After washing with prewarmed phosphate-buffered saline containing 2mg of albumin/ml, 0.5ml of medium (DMEM supplemented with 10mM-Hepes, pH7.4, and 2mg of albumin/ml) was added which contained various amounts of plasmin. At the times indicated, 50μl samples

were withdrawn and their radioactivity was determined.

Determination of PGI_2 of endothelial cells. Mini-pig aortic endothelial cells were incubated with 1 μCi of Δ_4Ach/ml overnight. The samples were washed three times with prewarmed phosphate-buffered saline, and 0.5ml of DMEM medium containing 10mM-Hepes, pH7.4, and various factors were added. The cells were incubated for 30min at 37°C. After incubation, the medium was collected and acetic acid was added (acid/medium 1:50, v/v), and the samples were extracted with 3 x 1ml of ethyl acetate. The samples were evaporated under a stream of N_2 and the remaining material was dissolved in 0.1ml of ethyl acetate. Portions (10μl) of the solutions were spotted on to Kieselgel thin layers. The chromatograms were developed with the solvent system ethyl acetate/acetic acid/iso-octane/water (11:2:5:10, by vol.) or with water/chloroform/methanol/acetic acid (5:200:40:4, by vol.). The chromatograms were sliced and radioactivity was determined by using a dioxan-based liquid-scintillation cocktail. Authentic Δ_4Ach and 6-oxo-$PGF_{1\alpha}$ (as a stable metabolite of prostacyclin) were run as unlabelled markers.

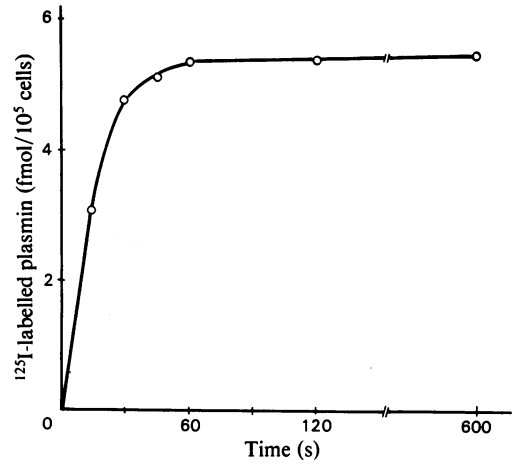

Fig. 1. *Time course of plasmin binding to endothelial cells*
Cells (2×10^5) were incubated in 0.5ml of buffer containing 2.6nM-^{125}I-labelled plasmin (for details, see the Experimental section). After various periods of time, medium was removed, the cells were washed three times with 2ml of buffer, dissolved and the radioactivity determined. Each point represents an average of triplicate determinations. Data are corrected by the non-specific binding of plasmin to endothelial cells.

Results

Binding of plasmin to endothelial cells

The maximum binding of plasmin by endothelial cells occurred within 60s when the enzyme concentration was approx. 3nM. After 10min incubation at 22°C, there was no more change in bound radioactivity (Fig. 1). When 5μg of unlabelled plasmin/ml was also present, the radioactivity bound to cells was decreased by approx. 80% (results not shown). The binding was reversible (Fig. 2). The rate of dissociation of plasmin was first-order, with an approx. k of 0.77min^{-1}. In the presence of unlabelled enzyme the rate of dissociation increased and a k of 1.38min^{-1} was measured.

The binding of plasmin to endothelial cells was also determined as a function of enzyme concentration. The binding curve is shown in Fig. 3. Scatchard analysis of the binding data revealed two distinct classes of binding sites. The results are summarized in Table 1.

Characterization of the interaction between plasmin and endothelial cells

Experiments were carried out to study the site of plasmin involved in binding to endothelial cells. For this purpose, plasmin was modified chemically. The active centre of the enzyme was blocked with PMSF, whereas the heparin-binding site of plasmin was modified with pyridoxal-*P*. Since heparin can bind to plasmin (Hatton & Regoeczi, 1977; Sturzebecher & Markwardt, 1977; Machovich *et al.*, 1981*a*; Smith & Sundboom, 1981) as well as to mini-pig aortic endothelial cells (Bauer *et al.*, 1983), the possible effect of this polysaccharide on the plasmin–endothelial-cell interaction was also examined. The results are collected in Table 2. As it can be seen, heparin did not influence binding, whereas modified plasmins showed competition with native enzyme.

The binding sites of cells for plasmin were also investigated. Binding of plasmin to endothelial cells was measured in the presence of PMSF-modified thrombin. The results show that thrombin did not compete with plasmin (Table 2). Similar results were obtained when thrombin binding was measured in the presence of PMSF-modified plasmin; namely, that thrombin binding to endothelial cells was not inhibited by the presence of plasmin (results not shown).

Pretreatment of cells with neuraminidase or chondroitin ABC lyase resulted in approx. 50% decrease of the binding of thrombin or plasmin respectively. Hyaluronidase or chondroitin AC lyase treatment, on the other hand, did not influence either thrombin or plasmin binding (results not shown).

These findings indicate that the receptors are different for plasmin and thrombin.

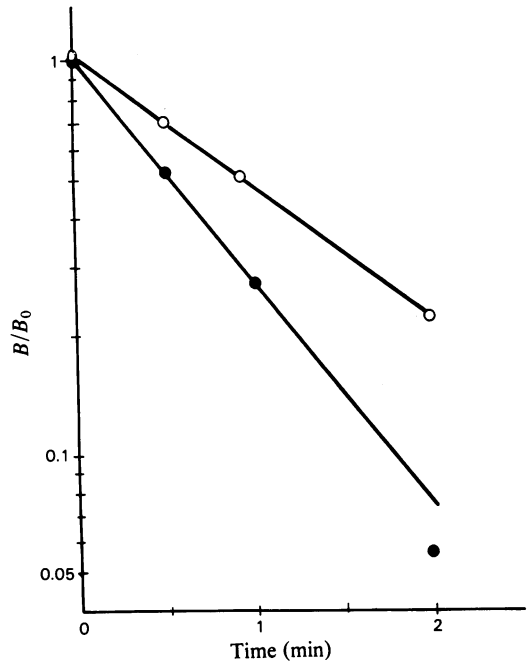

Fig. 2. *Dissociation of plasmin bound to endothelial cells*
Cells (2×10^5/vial) were incubated with ^{125}I-labelled plasmin (2.6nM) for 2min and washed three times under the conditions described in the Experimental section. Thereafter, 0.5ml of dissociation medium (Dulbecco medium supplemented with 10mM-Hepes, pH7.4, and 2.5mg of albumin/ml) was added to each vial. At various intervals, medium was removed and the bound radioactivity was determined. Results represent the average of triplicate measurements, and the errors are less than 20%. Data are corrected with the non-specific binding of plasmin to endothelial cells. B/B_0 is the ratio of the remaining radioactivity after dissociation and the dissociation value at zero time without (○) or with (●) 1.3nM-unlabelled plasmin as competing ligand.

Table 1. *Binding parameters of plasmin*
Binding parameters were calculated from Scatchard (1949) plots of data in Fig. 3.

	$10^{-4}\times$ Binding sites/cell	K_d (M)
High affinity	1.24	1.4×10^{-9}M
Low affinity	7.20	2.0×10^{-8}M

Table 2. *Effect of heparin and modified plasmin on the binding of the enzyme to endothelial cells*
Endothelial cells (2×10^5) were incubated with ^{125}I-labelled plasmin (3.2nM) for 2min in the presence of heparin or pyridoxal-*P*-modified plasmin (pyridoxal-*P*-plasmin) or PMSF-modified plasmin (PMSF-plasmin) or PMSF-thrombin or unlabelled plasmin as detailed in the Experimental section. The modification of plasmin with pyridoxal-*P* resulted in approx. 3–4mol of pyridoxal-*P*/mol of plasmin.

Additions	Concn. (nM)	Bound plasmin (fmol/10^5 cells)
None	–	4.75
Unlabelled plasmin	131	1.60
Pyridoxal-*P*-plasmin	13	2.65
	65	1.55
PMSF-plasmin	13	3.40
	65	1.60
PMSF-thrombin	111	4.60
	555	3.85
Heparin	5454	4.65

Recognition of plasmin and plasminogen by endothelial cells

Under conditions *in vivo* there are many plasma constituents which may interfere with plasmin binding to endothelial cells. For example, the precursor form of the enzyme, plasminogen, circulates in blood at relatively high levels. It is also well-documented that thrombin binds tightly to endothelial cells, and antithrombin III may influence this interaction (Bauer *et al.*, 1983). Therefore we examined the relative binding nature of these components. Cells were incubated with ^{125}I-proteins, -plasmin, -plasminogen, -thrombin or -antithrombin III, at the same molar concentrations, and the amount of proteins bound to the cells was determined as detailed in the Experimental section. As a control, the binding of streptokinase was also measured. It can be seen from Table 3 that only thrombin and plasmin bind tightly to cells, whereas binding of plasminogen is less, by more than two orders of magnitude. Antithrombin III, on the other hand, shows a tendency to bind, but the exact description of this association needs further experimentation.

In another series of experiments, ^{125}I-labelled plasminogen was incubated with streptokinase, and at various intervals samples were taken and determined for endothelial-cell binding. During a 30-min incubation period an approx. 160-fold increase in cell-bound radioactivity was measured, indicating that it is plasmin which binds primarily to cells, not plasminogen (results not shown).

Δ_4Ach metabolism of endothelial cells in the presence of plasmin

Incubation of mini-pig aortic endothelial cells with [^{3}H]Δ_4Ach resulted in its rapid uptake and incorporation into the cell's phospholipid fraction. This process was not influenced by plasmin (results not shown). On the other hand, plasmin increased the release of radioactivity from the cells into the incubation medium in a concentration-dependent manner. The rate of release was always higher in

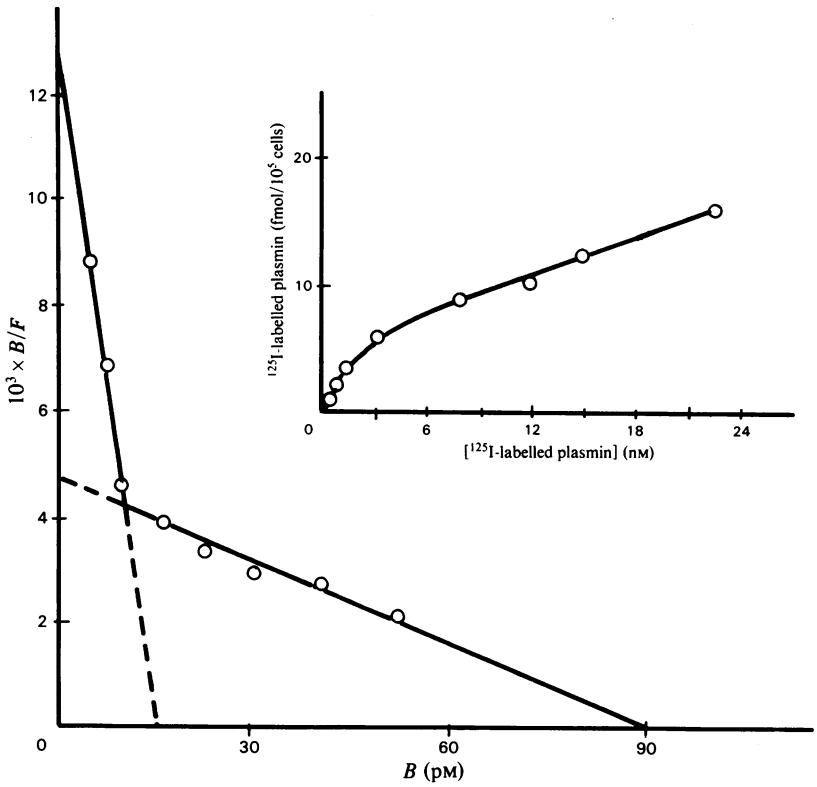

Fig. 3. *Dependence of plasmin binding to endothelial cells on the concentration of enzyme*
Cells (4×10^5) were incubated in the presence of various concentrations of plasmin (between 0.3 and 23 nM) under the conditions described in the Experimental section for 2 min. Thereafter bound (*B*) and free (*F*) enzyme was determined. The inset shows the amount of plasmin bound versus the amount of plasmin added.

Table 3. *Interaction of some proteins with endothelial cells*
Endothelial cells (2×10^5) were incubated with 10 nM-^{125}I-plasminogen, -plasmin, -thrombin, -antithrombin III or -streptokinase for 7 min at room temperature. Thereafter the amount of bound proteins was determined as detailed in the Experimental section. The results are averages of triplicate determinations.

Additives	Bound protein (fmol/10^5 cells)
Plasminogen	0.05
Plasmin	8.2
Thrombin	21.5
Antithrombin III	2.6
Streptokinase	0.025

the presence of albumin than in its absence (results not shown). Analysis of the radioactivity measured in the medium revealed that only [^{3}H]Δ_4Ach was released and there was no change in PGI_2 synthesis in the presence of plasmin, as detected with Kieselgel t.l.c. using two different solvent systems (Table 4). At the same time, cells were able to synthesize prostacyclin induced by thrombin or ionophore A23187 (Table 4).

Table 4. *Δ_4Ach and PGI_2 release by endothelial cells in the presence of plasmin*
Endothelial cells (2×10^5 cells/vial in 2 ml of medium) were incubated overnight in the presence of [^{3}H]Δ_4Ach (1 μCi/ml) under the conditions described in the Experimental section. Thereafter the cells were washed three times with 2 ml of phosphate-buffered saline containing 2 mg of albumin/ml, and finally 0.5 ml of medium was added to the cells. After 30 min incubation at 37°C, 50 μl aliquots of medium were withdrawn and measured for radioactivity. For PGI_2 (6-oxo-PGF_α) determinations, media from four parallel samples were collected, extracted and chromatographed, as detailed in the Experimental section.

	Radioactivity (d.p.m./10^5 cells)	
Additives	Δ_4Ach	6-Oxo-$PGF_{1\alpha}$
None	21000	5250
Plasmin (nM)		
16	29500	
79	93000	
158	54000	4816
395	55000	
Thrombin (42 nM)		11883
A23187 (10 μM)		24166

Discussion

There is quite a lot of data in the literature concerning the interaction of thrombin with endothelial cells. On the other hand, the binding, and especially the role, of plasmin in endothelial-cell biology is not clear at present. This question seems to be interesting, since endothelial cells produce plasminogen activator (Loskutoff & Edington, 1977; Loskutoff, 1979), resulting in an increased plasmin level in circulating blood.

The present findings demonstrate that plasmin binds tightly to endothelial cells with a K_d of approx. 10^{-9}M. The binding is saturable, and the rapid dissociation suggests that there is no uptake of plasmin by cells.

Experiments with chemically modified plasmins revealed that neither the active centre nor the heparin-binding site of the enzyme is involved in the reaction with endothelial cells. The latter is further supported by the results with heparin, i.e. the polysaccharide did not interfere with the plasmin–endothelial-cell interaction.

In the terms of endothelial-cell receptors, our data suggest that the receptors are different for plasmin and thrombin. Namely, thrombin does not compete with plasmin for binding, and neuraminidase treatment of cells results in a decrease of thrombin binding, whereas chondroitin ABC lyase treatment causes less plasmin binding to the cells.

Regarding the specificity of plasmin binding to cells, comparative studies reveal that plasminogen does not bind to endothelial cells, indicating that these cells recognize primarily plasmin and not its zymogen, the plasminogen.

The change in Δ_4Ach metabolism induced by plasmin binding to endothelial cells is unknown at present. Although plasmin increases arachidonic acid release from the cells, this process is not combined with prostaglandin synthesis. This effect of plasmin, however, seems to be non-specific, since thrombin and trypsin also produce aspecific Δ_4Ach release on cultured endothelial cells in the presence of albumin (Lollar & Owen, 1980).

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