Activation of Pro-Urokinase and Plasminogen on Human Sarcoma Cells: A Proteolytic System with Surface-bound Reactants

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Abstract. Human HT-1080 fibrosarcoma cells produce urokinase-type plasminogen activator (u-PA) and type 1 plasminogen activator inhibitor (PAI-1). We found that after incubation of monolayer cultures with purified native human plasminogen in serum-containing medium, bound plasmin activity could be eluted from the cells with tranexamic acid, an analogue of lysine. The bound plasmin was the result of plasminogen activation on the cell surface; plasmin activity was not taken up onto cells after deliberate addition of plasmin to the serum-containing medium. The cell surface plasmin formation was inhibited by an anticatalytic monoclonal antibody to u-PA, indicating that this enzyme was responsible for the activation. Preincubation of the cells with diisopropyl fluorophosphate–inhibited u-PA led to a decrease in surface-bound plasmin, indicating that a large part, if not all, of the cell surface plasminogen activation was catalyzed by surface-bound u-PA. In the absence of plasminogen, most of the cell surface u-PA was present in its single-chain proenzyme form, while addition of plasminogen led to formation of cell-bound two-chain u-PA. The latter reaction was catalyzed by cell-bound plasmin. Cell-bound u-PA was accessible to inhibition by endogenous PAI-1 and by added PAI-2, while the cell-bound plasmin was inaccessible to serum inhibitors, but accessible to added aprotinin and an anticatalytic monoclonal antibody.

A model for cell surface plasminogen activation is proposed in which plasminogen binding to cells from serum medium is followed by plasminogen activation by trace amounts of bound active u-PA, to form bound plasmin, which in turn serves to produce more active u-PA from bound pro-u-PA. This exponential process is subject to regulation by endogenous PAI-1 and limited to the pericellular space.

HUMAN tumor cells are very commonly found to secrete plasminogen activator of the urokinase type (u-PA)[1] (7). By this means they are able to recruit the proteolytic potential available in the high concentration of plasminogen in plasma and other body fluids. Several lines of evidence now strongly suggest that the invasive properties of tumor cells may be at least partly dependent on their proteolytic capability (2, 13, 22, 31, 45) mediated through the broad spectrum of activity of plasmin (20), and including its indirect actions in activating other latent proteases, such as collagenases (30, 47, 49). The expression of protease activity by tumor cells is proposed to facilitate their penetration of basement membranes, capillary walls, and interstitial connective tissues, allowing them to spread to other sites and establish metastases (7).

One aspect of the tumor plasminogen activator/plasmin system has been problematic for some time: how is the proteolytic activity expressed in a plasma or interstitial tissue fluid environment dominated by very large excesses of antiplasmin inhibitors? The fibrinolytic system, which uses tissue-type plasminogen activator (t-PA), has been shown to escape these antiplasmins by focusing the participating t-PA and plasminogen on the fibrin substrate. Plasmin formed in situ is bound to fibrin through the lysine affinity sites located in the kringle structures of the plasmin heavy chain, and is thereby protected from plasmin inhibitors (5). The plasmin may be released and its enzyme activity assayed after treatment with agents which compete for the lysine affinity sites, such as 6-aminocaproic acid (19). Furthermore, the t-PA, by its interaction with fibrin, is protected from one of the specific plasminogen activator inhibitors (PAIs), PAI-2 (19).

Recent evidence has shown that u-PA binds to specific receptor sites on many types of cells (3, 4, 43, 48), so that

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1. *Abbreviations used in this paper*: DFP, diisopropyl fluorophosphate; NPBG, *p*-nitrophenyl guanidinobenzoate; PAI, plasminogen activator inhibitor; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator.

upon secretion of the enzyme in its inactive proenzyme form, it may subsequently bind to the cell membrane and permit expression of enzyme activity at the site where it will have the maximum effect on the cell surface/cell matrix interface (34). It is now appropriate to consider whether this location is functionally analogous to that for t-PA (i.e., fibrin), and to determine if receptor-bound u-PA may function to generate surface protease activity while surrounded by plasma protease inhibitors. Also, recent work (12, 23, 24, 33) has demonstrated the existence of cell surface plasminogen/plasmin binding sites, and the resistance of bound plasmin to alpha-2-antiplasmin (33). It is therefore possible that receptor-bound u-PA may interact with a bound form of plasminogen to produce functionally active plasmin in spite of the presence of plasma inhibitors of both u-PA and plasmin.

In this report we explore the ability of human fibrosarcoma cells to produce plasmin on their surface while growing in serum-containing medium. We also investigated the effect of a range of plasmin and u-PA inhibitors, including anticatalytic monoclonal antibodies. The results establish that functionally active plasmin can be generated on the surface of cells growing in serum-containing medium. Plasmin formation is dependent on an interaction between bound active u-PA and bound plasminogen. We demonstrate that the availability of bound active u-PA is itself dependent on the activation of pro-u-PA by bound plasmin.

Materials and Methods

Cell Cultures

Human fibrosarcoma cells (HT-1080 and CCL 121) were obtained from the American Type Culture Collection (Rockville, MD). Confluent cell layers were grown in plastic Linbro wells (2 cm^2; Flow Laboratories, Inc., McLean, VA) in MEM supplemented with 10% heat-inactivated (56°C for 60 min) FCS (Gibco Laboratories, Grand Island, NY), 100 IU/ml penicillin, and 50 μg/ml streptomycin. After reaching confluence, cells were rinsed three times with MEM containing 0.2% BSA, and then changed to either serum-free medium (0.5 ml) or medium containing 10% heat-inactivated and plasminogen-depleted (i.e., absorbed with lysine-Sepharose; Pharmacia Fine Chemicals, Uppsala, Sweden) FCS as indicated in the experiments.

In the experiments on plasmin binding to cells from medium, human plasmin (~18 caseinolytic U/mg; Kabi Diagnostica, Stockholm, Sweden) was added to the cultures at final concentrations of 0–5 μg/ml. The cells were incubated for 3 h at 37°C before assay of cell-bound and supernatant plasmin (see below). For plasmin release experiments, cells were loaded for 1 h at 37°C with 0–5 μg/ml plasmin in serum-free medium, and then rinsed three times with MEM.

Human plasminogen (with glutamic acid NH_2 terminal) was prepared by affinity chromatography on lysine-Sepharose (8) from freshly separated, unfrozen human plasma pretreated with 10 μM *p*-nitrophenyl guanidinobenzoate (NPGB), 1 mM PMSF, and 0.1 μg/ml of an anticatalytic murine monoclonal IgG antibody to human t-PA (ESP-2 in reference 21; American Diagnostica, Greenwich, CT).

Inhibition studies made use of the following reagents added to cell cultures: an anticatalytic murine monoclonal IgG antibody to human plasmin (anti-plg 1 in reference 38; 20 μg/ml); aprotinin (Trasylol, Bayer, Leverkusen, FRG; 200 kIU/ml); tranexamic acid (Cyclokapron, Kabi Vitrum, Stockholm; 10 μM and 100 μM); human PAI-2 minactivin (10), PAI-2 purified from cultures of human U-937 histiocytic lymphoma cells (19) (titration equivalent of 3.6 IU u-PA/ml); an anticatalytic murine monoclonal IgG antibody to human u-PA (clone 2 in reference 28; 10 μg/ml); the anticatalytic monoclonal antibody to human t-PA (10 μg/ml); a neutralizing murine monoclonal IgG antibody to human PAI-1 (27) (10 μg/ml); and diisopropyl fluorophosphate (DFP)-inactivated u-PA (0–10 μg/ml).

DFP-inactivated u-PA for Competition Studies

Active two-chain u-PA (Ukidan, Serono) was dissolved in 0.1 M Tris-HCl (pH 8.1), 0.1% Tween 80 (Tris/Tween). A freshly prepared solution of 500 mM DFP (Sigma Chemical Co., St. Louis, MO) in isopropanol was added to yield a final DFP concentration of 5 mM. After thorough mixing, the sample was incubated for 2 h at 37°C, after which period addition of DFP was repeated as above. After renewed incubation for 2 h at 37°C, the reaction was terminated by thorough dialysis at 0°C against Tris/Tween. No residual DFP inhibitor could be detected when the preparation was tested in an activity assay of soluble urokinase.

Metabolic Labeling of Cell-bound u-PA

Confluent layers of HT-1080 cells were rinsed three times with methionine-free MEM containing 0.2% BSA, and then prelabeled for 5 h at 37°C with 170 μCi/ml [^{35}S]methionine (800 Ci/mmol; Amersham Corp., Arlington Heights, IL). Human plasminogen (50 μg/ml) and the neutralizing monoclonal antibody to human PAI-1 (10 μg/ml) were added to one of two cultures, and the incubations continued for another 3 h. Aprotinin (200 kIU/ml) was added to both cultures before the medium was removed, after which the cells were rinsed three times with Dulbecco's medium containing 0.2% BSA. The cell-bound u-PA was then eluted with 50 mM glycine-HCl (pH 3.0) containing 0.1 M NaCl for 3 min at 23°C (44). The acid eluate was neutralized with 0.5 M Tris-HCl (pH 7.8), before immunoprecipitation for 2 h at 23°C with 3 μg/ml goat IgG antibodies to human u-PA (American Diagnostica) or 3 μg/ml goat IgG antibodies to human t-PA (American Diagnostica) as a control. Immune complexes were collected by adsorption to protein A–Sepharose in an end-over mixer for 1 h. Immunoprecipitates were washed several times with immunoprecipitation buffer of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5% sodium deoxycholate, 0.5% NP-40, 0.1% SDS containing 100 kIU/ml aprotinin, twice with PBS, and finally with 20 mM Tris-HCl (pH 7.5). Immunocomplexes were solubilized by boiling in Laemmli's (17) sample buffer under reducing conditions (10% beta-mercaptoethanol), and electrophoresed in 10% SDS-polyacrylamide gels. Fixed gels were treated with Amplify^R (Amersham Corp.) and exposed to Kodak XAR-5 film at −70°C.

u-PA Assays

Cell culture supernatants were assayed for pro-u-PA and active u-PA by the following modification of an immunocapture method (41, 42). Microtitre wells of polystyrene immunoplates (type 269620; A/S Nunc, Roskilde, Denmark) were coated overnight at 37°C with 50 μl of a solution of goat IgG antibodies to human u-PA (catalog No. 398; American Diagnostica). The coating solution contained 2.5 μg IgG/ml of 0.1 M sodium carbonate (pH 9.8). After rinsing, the wells were treated with conditioned medium (50 μl) for 2 h at 23°C, then rinsed again. Half the wells were then treated with 50 μl of freshly prepared 2 μM NPGB (Sigma Chemical Co.) (6) for 20 min at 37°C. The other half (controls) received 50 μl rinsing buffer (0.05% Tween 20 in PBS). After rinsing, u-PA was assayed in all the wells by addition of 40 μl of a solution containing plasminogen (100 μg/ml) and plasmin (10 ng/ml) in assay buffer (50 mM sodium glycinate [pH 7.8], 0.1% Triton X-100, 0.1% gelatin, and 10 mM 6-aminocaproic acid). The plates were incubated for 30 min at 37°C. This concentration of plasmin in the plasminogen incubation was sufficient to enable full realization of the potential activity of pro-u-PA (32). The plasmin produced by this incubation was assayed by its thioesterase activity (11) by the addition of 200 μl of a solution containing 200 mM potassium phosphate (pH 7.5), 200 mM KCl, 0.1% Triton X-100, 220 μM Z-lysine thiobenzyl ester (Peninsula Laboratories, Inc., Belmont, CA), and 220 μM 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma Chemical Co.). This mixture was incubated for 30 min at 37°C, and the absorbancies of the wells read at 405 nm. Active u-PA (60,000 IU/mg) was purchased from Calbiochem-Behring Corp. (La Jolla, CA) and pro-u-PA (potential activity 90,000 IU/mg) was obtained from American Diagnostica.

Pro-u-PA and active u-PA bound to the cell layer were recovered for immunocapture assays by the same method as used in the metabolic labeling (above). Each culture well (2 cm^2) was eluted with 150 μl of acid glycine at pH 3 (44). For conditioned medium and cell-bound u-PA, the u-PA activity assayed after NPGB treatment was expressed as a percentage of the total activity obtained without NPGB treatment, and this percentage was used as an index of pro-u-PA content (pro-u-PA index). The conditions used for the NPGB treatment were previously established (42) to allow selective inactivation of active u-PA, while leaving the pro-u-PA unchanged and still able to be activated by the added plasmin to the same extent as untreated pro-u-PA.

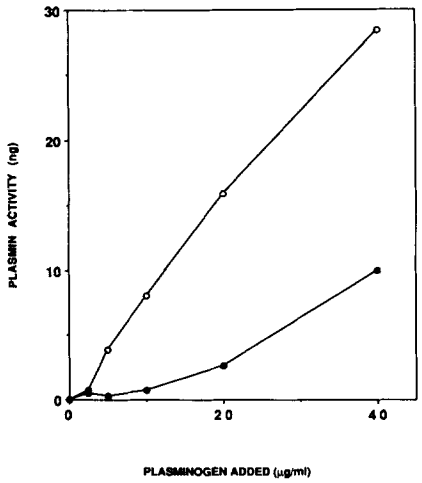

Figure 1. Dependence of plasmin formation in serum medium on the concentration of added native human plasminogen. Confluent layers of HT-1080 cells were incubated for 3 h in MEM (0.5 ml) containing 10% heat-inactivated and plasminogen-depleted FCS, with the addition of native human plasminogen to the concentrations shown. The conditioned media were harvested and the cells rinsed three times with PBS. Cells were treated with 1 mM tranexamic acid in PBS to obtain the bound fraction of plasmin. Plasmin was assayed in the cell-bound fraction (○) and the medium (●) as thioesterase activity.

Plasmin Assays

The plasmin activity of culture supernatant samples (50 μl) was assayed directly by incubation with the thioester substrate solution above (200 μl) for 30 min (serum-free supernatants) or 3 h (serum-containing supernatants) at 37°C. An estimate of the amount of active plasmin present was made from calibration curves using human plasmin dilutions in serum-free medium covering the appropriate ranges of activity.

Plasmin bound to the cell layer was recovered and assayed as follows. After harvest of culture medium, the cells were rinsed three times with PBS (plasmin assays of furuther rinses were negative), and then the bound plasmin was specifically eluted (24) with a solution of 1 mM tranexamic acid in the same rinsing solution (150 μl/well). Plasmin activity was assayed in eluate samples (50 μl) as above with an incubation time of 3 h at 37°C. Tranexamic acid at 1 mM had no effect on the thioesterase activity of plasmin in these assays.

Results

Plasminogen Is Activated on the Cell Surface

After addition of purified preparations of human plasminogen to cultures of human fibrosarcoma cells (HT-1080) growing in a medium with 10% plasminogen-depleted FCS, plasmin activity could be recovered as a bound fraction from the cell layer. Upon varying the concentration of added plasminogen, the bound plasmin activity increased in a dose-dependent manner (Fig. 1). The binding was specific, so that after rinsing of the cells with isotonic buffer, the plasmin could be released by 1 mM tranexamic acid. This agent disrupts interactions with plasminogen or plasmin which involve the lysine-affinity sites of the heavy-chain kringles (23). The plasmin released from HT-1080 cell surfaces was conveniently measured by its thioesterase activity, a method which was unaffected by the presence of tranexamic acid. Some plasmin activity was also detected in the medium. At a concentration of 40 μg/ml human plasminogen added to 0.5 ml medium above a confluent 2-cm² cell layer, activity corresponding to 28 ng plasmin could be recovered from the cell layer with tranexamic acid, while 10 ng was measurable in the medium after a 3-h incubation at 37°C. This concentration of plasminogen is well below the 200 μg/ml present in normal human plasma.

To test whether the cell surface plasmin might have been derived from either preformed plasmin (added as a trace contaminant with the plasminogen preparation) or from plasmin formed in the medium and subsequently bound to the cells, we added plasmin to the culture medium of HT-1080 cells. As shown in Fig. 2, virtually no plasmin activity was detected on the cell surface when the medium contained 10% FCS, while there was a considerable dose-dependent plasmin binding in the absence of serum. These findings indicated that the cell-bound plasmin activity found in the experiment shown in Fig. 1 was formed by activation of plasminogen on the surface of the cells.

Incubation of cells carrying plasmin with fresh serum-free medium showed that ~40% of the activity remained bound after 2 h at 37°C (Fig. 3, *a* and *b*). When the cells were incubated in 10% serum-containing medium, the same fraction (40%) of this activity could be recovered from the cells:

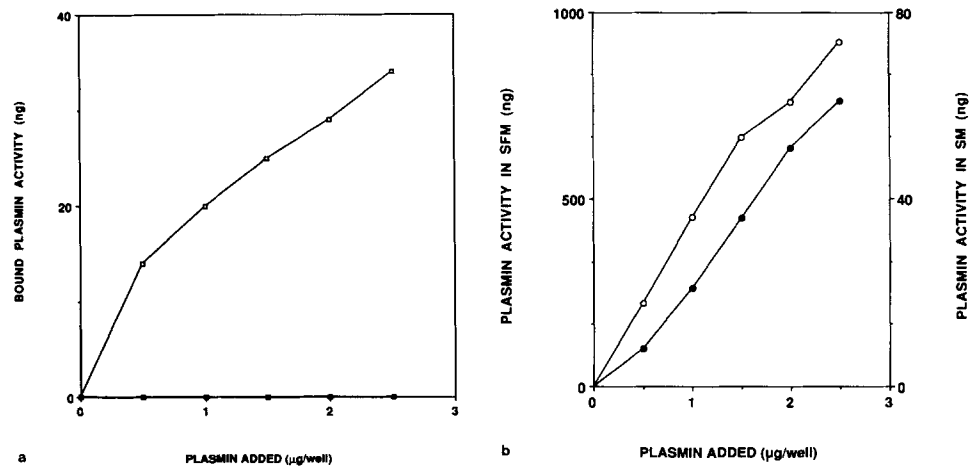

Figure 2. Plasmin uptake onto HT-1080 cells from serum-free and serum-containing media. Human plasmin (0–5 μg/ml) was added to HT-1080 cell layers growing in either serum-free MEM (*SFM*, 0.5 ml) (○) or MEM with 10% heat-inactivated FCS (*SM*) (●). After a 3-h incubation at 37°C the plasmin in the bound fraction (*a*) and the conditioned media (*b*) were assayed as thioesterase activity. Note the different scales used in Fig. 2 *b* for activity in SFM and SM.

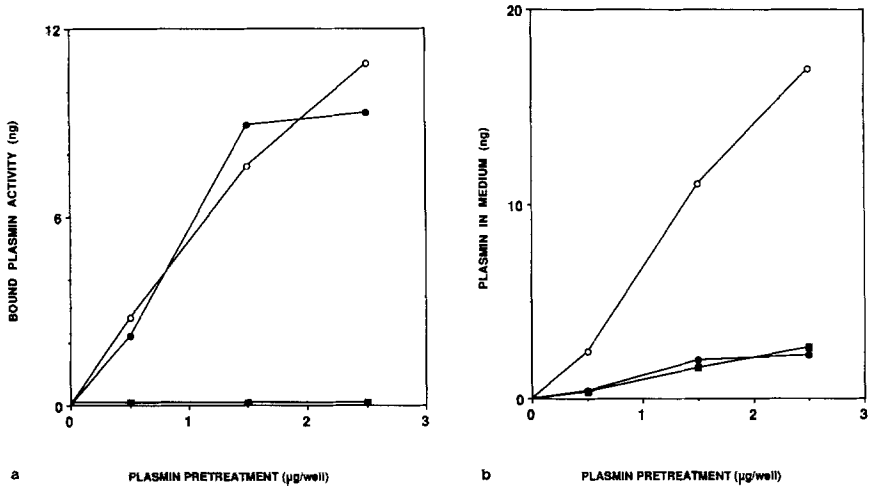

Figure 3. Plasmin release from HT-1080 cells into serum-free and serum-containing media. Confluent layers of HT-1080 cells were first loaded with plasmin by incubation for 1 h at 37°C in serum-free MEM (0.5 ml) containing human plasmin (0–5 μg/ml). After rinsing the cell layers three times, they were incubated for 2 h at 37°C with either serum-free medium (○), medium containing 10% heat-inactivated and plasminogen-depleted FCS (●), or the latter with tranexamic acid (100 μM) (■). Plasmin was then assayed in the cell-bound fraction (*a*) and the media (*b*). At the time of transfer to new media there was ~28 ng of plasmin bound to the cells from the pretreatment with 2.5 μg plasmin/well.

the bound plasmin was not inactivated by the serum. However, only ~11% (compared to 60% for serum-free medium) could be detected in the serum-containing medium (Fig. 3 *b*). When 1 mM tranexamic acid was added to the serum-containing medium, no plasmin activity could be recovered from the cells (Fig. 3 *a*).

Cell Surface Plasminogen Activation Is Catalyzed by Cell-bound u-PA

HT-1080 cells are prolific producers of u-PA (36) but, although they synthesize some t-PA, this does not appear to be secreted (Stephens, R., unpublished observations). To test which of the activators were responsible for the cell surface plasminogen activation, the cells were incubated with plasminogen in the presence of monoclonal antibodies that inhibit each of the activators. The results in Table I show that inhibition of the enzymatic activity of u-PA resulted in virtually no plasmin activity being detected on the cell surface, while inhibition of t-PA did not decrease the amount of plasmin activity, indicating that the cell surface plasminogen activation was catalyzed by u-PA. Bound plasmin activity was also reduced in cultures containing PAI-2 (10), aprotinin, or an anticatalytic monoclonal antibody to human plasmin (38).

In HT-1080 cell cultures, u-PA is present both in the medium and bound to the cell surface (29). To test whether the surface-bound u-PA was involved in the cell surface plasminogen activation in serum cultures, we preincubated the cells with either the anti-catalytic u-PA antibody or PAI-2, then washed the cells thoroughly before they were incubated with plasminogen in serum medium. Both inhibitors caused a significant decrease in the cell-bound plasmin activity, while no inhibition of the u-PA activity in the medium was detected (Table II).

An alternative method of studying the role of cell-bound vs. free u-PA is illustrated in Fig. 4. u-PA is bound to its receptor at the surface of HT-1080 cells (29). This binding does not involve the active site of u-PA (3), therefore, the u-PA receptor also binds u-PA that has been treated with the irreversible active-site titrant, DFP (29). To decrease the

Table I. Effects of Inhibitors of Plasmin and u-PA on Formation of Bound Plasmin on HT-1080 Cells in Serum Culture

	Bound plasmin activity		u-PA activity in medium	
Incubation	3 h	17 h	3 h	17 h
	%	%	%	%
Control	3.5	7.1	100.0	100.0
Plg	100.0	100.0	111.0	83.0
Plg + anti-u-PA	6.2	11.0	9.9	29.0
Plg + anti-t-PA	101.0	123.0	102.0	81.0
Plg + PAI-2	48.0	19.0	89.0	77.0
Plg + aprotinin	13.2	7.8	93.0	99.0
Plg + anti-Plg-1	2.8	6.4	90.0	88.0
Plg + TA (10 μM)	9.7	34.0	116.0	87.0
Plg + TA (100 μM)	2.2	13.0	92.0	95.0

The following additions were made to cell layers growing in MEM (0.5 ml) containing 10% heat-inactivated and plasminogen-depleted FCS: native human plasminogen (Plg, 40 μg/ml); anticatalytic monoclonal antibody to human u-PA (10 μg/ml); anticatalytic monoclonal antibody to human t-PA (10 μg/ml); PAI-2 (titration equivalent of 3.6 IU u-PA/ml); anticatalytic monoclonal antibody to human plasmin (20 μg/ml); aprotinin (200 kIU/ml); and tranexamic acid (TA). The cultures were incubated for the times shown before assay of cell-bound plasmin. The incubation with plasminogen was used as the 100% control for bound plasmin.

Table II. Effect of Pretreatment of HT-1080 Cells with u-PA Inhibitors on Subsequent Ability To Produce Bound Plasmin in Serum Culture

Preincubation	Plasminogen	Bound plasmin activity	u-PA activity in medium
		%	%
Control	−	2.2	100.0
Control	+	100.0	86.0
Anti-u-PA	+	32.0	96.0
PAI-2	+	54.0	86.0

Confluent cell layers in serum medium (0.5 ml) were preincubated for 1 h at 37°C with an anticatalytic monoclonal antibody to human u-PA (10 μg/ml) or PAI-2 (tritration equivalent of 3.6 IU u-PA/ml). The cells were then rinsed three times with serum-free MEM, before incubation for 3 h at 37°C with MEM containing 10% heat-inactivated and plasminogen-depleted FCS and native human plasminogen (40 μg/ml). The incubation with plasminogen was used as the 100% control for bound plasmin, while the control for u-PA was the incubation without plasminogen.

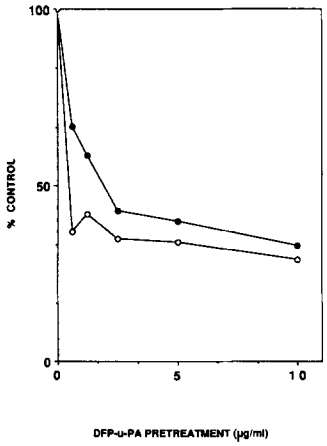

Figure 4. Effect of pretreatment of HT-1080 cells with DFP-u-PA on bound u-PA activity and ability to produce bound plasmin in serum medium. Confluent cell layers of HT-1080 cells were preincubated for 18 h at 37°C with the concentrations shown of DFP-u-PA in serum-containing medium (0.5 ml). After rinsing three times, the cells were incubated for 1 h at 37°C with MEM containing 10% heat-inactivated and plasminogen-depleted FCS, with addition of native human plasminogen (40 μg/ml). After incubation, half the replicate wells were rinsed and treated with acid-glycine to recover the total bound u-PA (○) which now included DFP-u-PA, pro-u-PA, and active u-PA. The other wells were used to recover bound plasmin (●) by elution with tranexamic acid.

amount of receptor-bound, catalytically active u-PA, we preincubated the HT-1080 cells for 18 h with DFP-inactivated u-PA which, when present in a large molar excess, resulted in a decrease of ~70% in surface-bound u-PA that was released by acid treatment (44). Concomitantly, there was a comparable decrease in the amount of plasmin generated on the cell surface (Fig. 4). These results indicate that a large part, if not all, of the cell surface plasminogen activation in serum cultures was catalyzed by the surface-bound u-PA.

Surface-bound Plasmin Activates Pro-u-PA

u-PA is released into the medium of HT-1080 cells as a single-chain proenzyme, pro-u-PA, that can be converted to a two-chain active u-PA by plasmin (26, 38). The enzymatic activity of the proenzyme is at least 250-fold lower than that of the two-chain u-PA (32), and it does not react with PAI-1 (1) or PAI-2 (40, 50). With the use of metabolic labeling, recovery of receptor-bound u-PA by acid treatment, immunoprecipitation, SDS-PAGE under reducing conditions, and fluorography, we found (Fig. 5) that the receptor-bound u-PA was almost exclusively present in the single-chain form when the cells were incubated in serum medium without added plasminogen. By contrast, virtually all the receptor-bound u-PA was in the two-chain form when the cells were incubated with 50 μg/ml human plasminogen in serum medium for 3 h.

As an alternative way of distinguishing between pro-u-PA and active u-PA, we took advantage of the fact that low molecular weight active-site reagents for u-PA do not bind to pro-u-PA (26). One of these, NPGB, was used to distinguish between the two u-PA forms in an immunocapture assay (41, 42). The u-PA present in samples was first absorbed by u-PA antibodies bound to microtitre wells. Half the wells for each sample were treated with NPGB, the other half were control treated. The total u-PA activity (pro-u-PA plus active u-PA) in the untreated wells and the pro-u-PA in the treated wells were then measured by a coupled plasminogen activator assay in the presence of an initial concentration of 10 ng/ml plasmin, and the results expressed as a pro-u-PA index (see

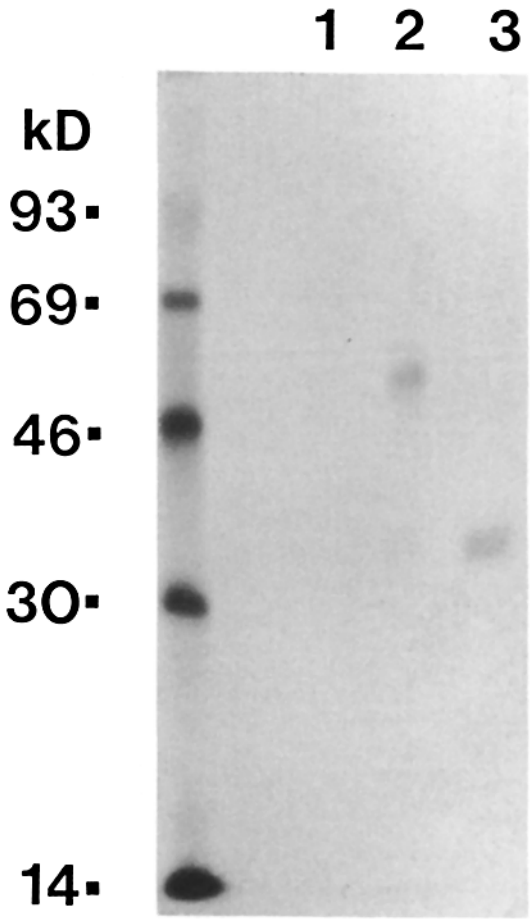

Figure 5. Activation of cell-bound u-PA proenzyme in serum medium after addition of plasminogen. Confluent layers of HT-1080 cells were prelabeled for 5 h at 37°C with [^{35}S]methionine. After restoring complete medium with 10% heat-inactivated and plasminogen-depleted FCS, native human plasminogen (50 μg/ml) was added and the incubation continued another 3 h. Aprotinin (200 kIU/ml) was added before harvest of medium, and the rinsed cells were treated with acid-glycine to recover the bound u-PA fraction. Acid eluates were neutralized and immunoprecipitated with goat antibodies to u-PA, before SDS-PAGE under reducing conditions. The fluorogram shows (lane *1*) control immunoprecipitate of culture without plasminogen with goat antibodies to human t-PA; (lane *2*) culture without plasminogen immunoprecipitated with goat anti-u-PA antibodies; and (lane *3*) culture with plasminogen immunoprecipitated with u-PA antibodies.

Materials and Methods). Fig. 6 *a* shows that the proportion of the total surface-bound u-PA that was present as pro-u-PA decreased dramatically when the cells were incubated with human plasminogen in serum medium, compared with plasminogen-free cultures (from ~90% to ~10%). These findings suggest that conversion of the surface-bound pro-u-PA to active u-PA by plasmin plays a role in cell surface plasminogen activation.

In the experiment described in Fig. 6, there was a markedly lower amount of total u-PA activity on the cells incubated with plasminogen (Fig. 6 *b*). We found that this difference was nearly abolished when a monoclonal antibody that neutralizes human PAI-1 was added during the incubation (Fig. 6 *b*). HT-1080 cells release large amounts of PAI-1 (27)

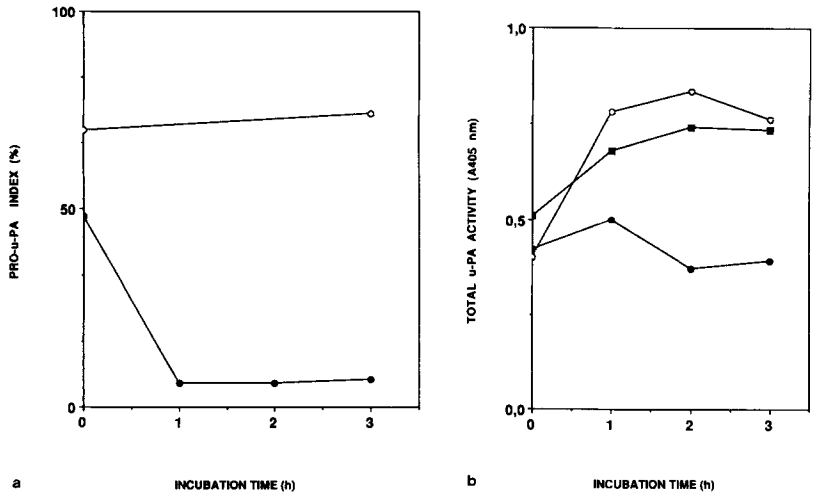

Figure 6. Activation of cell-bound u-PA proenzyme in serum medium after addition of plasminogen. Confluent layers of HT-1080 cells were incubated with MEM containing 10% heat-inactivated and plasminogen-depleted FCS and native human plasminogen (40 μg/ml). After the time intervals shown, aprotinin (200 kIU/ml) was added and the rinsed cells were treated with acid-glycine to recover the bound fraction of u-PA. The u-PA in the neutralized eluate was assayed by an immunocapture method, using an NPGB inactivation step to determine the pro-u-PA index (see Materials and Methods). *a* shows the pro-u-PA index for cultures without (○) and with (●) plasminogen. The zero-time sample with plasminogen shows some change already occurred during work-up of the cells. *b* shows the eluted u-PA activity from cultures without plasminogen (○), with plasminogen (●), and with plasminogen and a neutralizing monoclonal antibody to human PAI-1 (10 μg/ml) (■).

that binds to active u-PA, but not to pro-u-PA (1). The apparent decrease in total u-PA after incubation with added plasminogen (Fig. 6 *b*) can, therefore, be attributed to PAI-1 binding to active u-PA on the cell surface and inhibiting its activity in the subsequent assay.

To prevent the interference of PAI-1, we included the neutralizing PAI-1 antibody in the next experiment in which we studied the effect of the plasmin inhibitor aprotinin and of an anticatalytic monoclonal antibody to human plasmin on the conversion of pro-u-PA to active u-PA. As shown in Table III, both these inhibitors increased the relative amount of pro-u-PA, thus demonstrating that the activation of cell-bound pro-u-PA was catalyzed by plasmin. To study whether this was an effect of cell-bound plasmin, we also tested the effect of tranexamic acid in a concentration of 100 μM, which completely inhibits binding of plasmin to the cells, but does not affect the ability of plasmin to activate pro-u-PA in solution (Stephens, R., unpublished results). This treatment markedly decreased the relative amount of active u-PA, indicating that the activation of the cell surface pro-u-PA is catalyzed by the surface-bound plasmin.

Table III. Effectors of Pro-u-PA Activation and Plasmin on the Surface of HT-1080 Cells in Serum Medium

Incubation	Pro-u-PA index	Bound plasmin activity
	%	*ng*
Control	85	0.0
Plg	50	12.0
Plg + anti-PAI-1	21	33.0
Plg + anti-PAI-1 + aprotinin	93	3.0
Plg + anti-PAI-1 + anti-Plg-1	72	2.1
Plg + anti-PAI-1 + TA (100 μM)	88	0.0

Confluent cell layers were incubated for 2 h at 37°C with MEM (0.5 ml) containing 10% heat-inactivated and plasminogen-depleted FCS with the following additions: native human plasminogen (Plg, 40 μg/ml); neutralizing monoclonal antibody to human PAI-1 (10 μg/ml); aprotinin (200 kIU/ml); anticatalytic monoclonal antibody to human plasmin (20 μg/ml); and tranexamic acid (TA). Half the wells were then treated with aprotinin (200 kIU/ml) and used for assay of bound u-PA and its pro-u-PA index (see Materials and Methods). The other half were used for elution and assay of bound plasmin.

Discussion

The findings from these experiments provide functional evidence for the u-PA-catalyzed activation of plasminogen on the surface of human fibrosarcoma cells growing in serum-containing medium. The new findings include the requirement for binding of plasminogen, the ability of bound u-PA to activate plasminogen, the presence of pro-u-PA on the cells, the ability of bound plasmin to activate pro-u-PA, and the ability of endogenous PAI-1, as well as added PAI-2, to regulate the surface plasminogen activation. By these means tumor cells can acquire the broad-spectrum proteolytic activity of plasmin, bound to their surface in such a way that they are protected from inactivation by serum protease inhibitors and ideally situated to be used in the degradation of the pericellular matrix.

In this report the bound u-PA was shown to be the fraction most active in forming bound plasmin; however, it was not rigorously established if this u-PA was present on the previously identified and characterized specific receptors (3, 29), although this is very likely since (*a*) HT-1080 cells are known to possess specific u-PA receptors on their membranes (29); (*b*) the same acid elution method was used to recover bound u-PA as has been used in the receptor studies (44); and (*c*) a large proportion of the bound u-PA could be competed out by DFP-inactivated u-PA, which can bind to the u-PA receptor (29) but not to other known u-PA binding proteins which require the active site, such as PAI-1 and PAI-2 (1, 40, 50).

Before addition of native plasminogen to the serum cultures, it was clear that the vast majority of u-PA secreted by the fibrosarcoma cells, and present on the cell surface, was in the single-chain, proenzyme form. This is consistent with several previous reports, including our recent survey (42), indicating that many adherent cells secrete u-PA as its proenzyme. The proenzyme form has been characterized extensively by several groups, and recently finally established as being a true proenzyme, with little or no intrinsic activity (9, 32). After addition of native plasminogen, bound u-PA was found to be in the two-chain form, a reaction known to be catalyzed by plasmin (26, 38). However, under serum culture conditions, in the presence of a large excess of plasmin inhib-

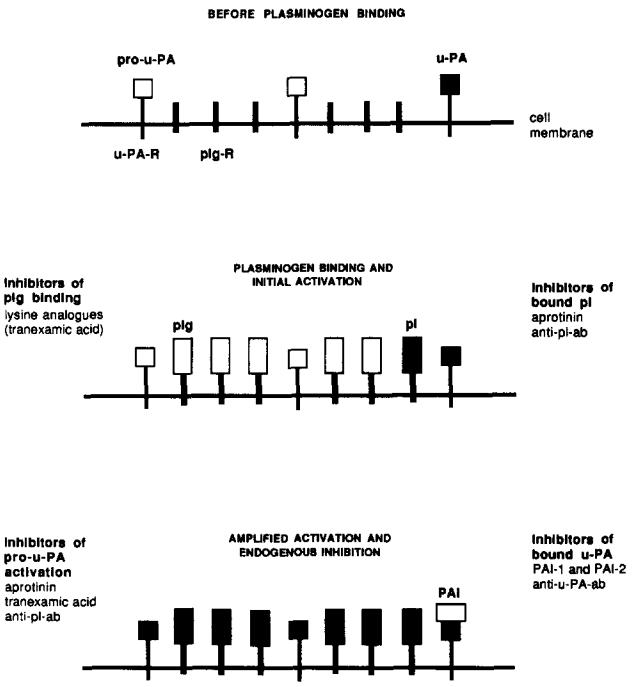

Figure 7. Model for cell surface plasminogen activation. In this proposed model, u-PA receptors (*u-PA-R*) and plasminogen receptors (*plg-R*) are depicted on the cell membrane. Before exposure to plasminogen (*plg*) virtually all the bound u-PA is present as pro-u-PA (*open squares*), but it is assumed that some active u-PA molecules exist (*solid squares*). On plasminogen (*open rectangles*) binding (which may be precluded by the presence of tranexamic acid), plasmin (*pl, solid rectangles*) is formed on the cell by the action of the bound active urokinase. This step may be inhibited by PAI-1 and PAI-2, and by an anticatalytic monoclonal antibody to u-PA (*anti-u-PA-ab*). The bound plasmin thus formed is resistant to inhibition by the alpha-2-antiplasmin present in the serum medium, but sensitive to inhibition by aprotinin and an anticatalytic monoclonal antibody to plasmin (*anti-pl-ab*). As active plasmin becomes available, it catalyzes the activation of more bound pro-u-PA to active u-PA, thus amplifying the proteolytic system. Activation of pro-u-PA is inhibited by tranexamic acid (which prevents plasminogen binding), aprotinin, and anticatalytic monoclonal antibody to plasmin.

itors, the binding of plasminogen and its activation product (plasmin) to the cell surface was a necessary prerequisite for formation of the two-chain u-PA. It appears likely that the activation of pro-u-PA occurs when it is actually surface bound. However this was not rigorously established by the present findings. It is conceivable that cell-bound plasmin activates pro-u-PA in the immediate environment of the cells and that the u-PA formed could subsequently exchange with bound pro-u-PA.

The binding and subsequent protection of plasmin was abolished by low concentrations of the lysine analogue, tranexamic acid, as reported by others (23). It is therefore likely that plasmin binding involves the lysine affinity sites situated in the heavy-chain kringles of plasmin. Plasmin released from the cells was partially inactivated in the serum medium. As long as the plasmin remained bound, it was protected from serum inhibitors (33) but could be inhibited by aprotinin or an anticatalytic monoclonal antibody.

This result provides a possible explanation for the effectiveness of aprotinin in certain therapeutic applications, such as the promotion of healing of corneal ulcers (37). Plasmin has been shown to be produced in this condition, yet one would expect that it would be inactivated by serum inhibitors. If a significant fraction is bound to cells, however, this may escape inhibition and retard development of healing tissue until an effective inhibitor is applied externally. The plasmin found on HT-1080 cells after addition of native plasminogen to serum cultures was demonstrated to be the product of plasminogen activation by bound u-PA. It was not taken up from serum medium; in fact, the experiments with plasmin uptake and release in serum medium clearly established the existence of a one-way movement of plasmin activity from the cells into the medium, and not vice versa. In keeping with this result, more plasmin was measurable in the fraction bound to cells than in the serum medium after addition of plasminogen. It is very likely that the plasmin activity, which could be measured in the serum medium, represented the thioesterase activity of plasmin complexes with serum inhibitors rather than free plasmin. These complexes retain activity towards small substrates, but their proteolytic activity is sterically hindered (46). Plasmin formation was not detected when cells were grown in serum medium without addition of a native preparation of plasminogen (Stephens, R., unpublished observation); the bovine plasminogen present in the FCS supplied commercially for cell cultures appeared to be denatured. A purified native preparation of bovine plasminogen was able to form cell surface plasmin activity; there was no apparent species specificity.

The model which we propose for plasminogen activation on cells growing in serum medium can be summarized in the following steps (Fig. 7). Plasminogen must first bind to the cell surface via its heavy-chain kringles. Formation of cell-bound plasmin from bound plasminogen is initiated by the action of trace amounts of active bound u-PA. This leads to rapid activation of u-PA proenzyme, which in turn enables more activation of bound plasminogen, formation of more plasmin, and so on. The availability of trace amounts of bound active u-PA to initiate plasminogen activation is clearly a critical requirement, but in the HT-1080 system we have been unable to demonstrate a plasmin-independent mechanism for proenzyme activation (e.g., by pulse-chase experiments). On the contrary, we have found that cultures of several human leukemia cell lines produce active urokinase without requiring added plasminogen (42). It is therefore possible that other nonplasmin serine proteases do exist which could be important in the initiation of plasminogen activation on the cell surface.

Once initiated, this exponential process was found to be susceptible to regulation by endogenous PAI-1 produced by the HT-1080 cells. Its control of bound u-PA was demonstrated by the use of a neutralizing antibody to PAI-1, which caused the formation of more active, bound u-PA and more plasmin than seen on control cells. Preparations of purified PAI-1 were not used in these experiments since this would have required pretreatment with denaturants (15), which could have adversely affected the cells. The observed production of cell surface plasmin, therefore, represents the net result of the pro-u-PA activation reaction, the activation of plasminogen, the partial inhibition of active u-PA by PAI-1, as well as the dissociation of plasmin from its binding sites.

PAI-1 can exist in both active and latent forms (15), and

the cell substratum has been shown to be an effective reservoir of the active form (18, 34). Thus a high concentration of bound PAI-1 exists in the immediate vicinity of the cell surface plasminogen activation system, while free PAI-1 in the liquid phase is known to more rapidly lose its capacity to inhibit activators after secretion (25). The cell surface activation of plasminogen was also inhibited by addition of exogenous PAI-2. This could have significance because PAI-2 is secreted by activated macrophages (10, 39); therefore, intratumoral macrophages could have a role in reducing the invasive proteolytic capacity of tumor cells.

The binding of the proenzyme and enzyme components of plasminogen activation to the cell surface, the accumulation of a regulatory inhibitor (PAI-1) in the cell substratum, and the abundance of plasmin inhibitors in the serum liquid phase, all serve to limit plasminogen activation and the action of plasmin to the immediate proximity of the cell surface, just as t-PA and plasmin are limited to fibrin in the course of fibrinolysis (5). Furthermore, in recent immunofluorescence and ultrastructural studies, u-PA was localized to the focal contact sites of human fibroblasts and HT-1080 fibrosarcoma cells, as well as to areas of cell to cell contacts and to cell extensions (34, 35). Extracellular u-PA antigen had a striking colocalization with the cytoskeletal protein vinculin at focal contacts (14, 35). Depletion of PAI-1 antigen in stria-like areas from an otherwise uniform deposit was also observed (34). Based on these distribution patterns, it is likely that cells express u-PA-mediated focal proteolysis. Limited plasmin activity may then serve to facilitate the detachment of cells from contacts with the substratum that are maintained by the cell adhesion molecules, such as integrins (16). Overexpression of the proteolytic activity of plasmin in the case of tumor cells may be the enabling condition for these sites to become the invasion front that penetrates adjoining normal tissue.

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