Bound plasminogen is rate-limiting for cell-surface-mediated activation of plasminogen by urokinase

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The ability of U937 monocyte-like cells and KATO III cells (a human gastric carcinoma line) to potentiate activation of plasminogen by single-chain urokinase-type plasminogen activator (scu-PA), as mediated by the cell receptor for urokinase (u-PAR), was compared. It was observed that, although the concentration of u-PAR on these cell lines differed considerably (U937 cells: 5000 receptors/cell, K_d 0.35 nM; KATO III cells: 400 receptors/cell, K_d 0.85 nM), the rate of activation of plasminogen by scu-PA in the presence of the same density of each cell line was equivalent. From data generated in the presence of increasing concentrations of scu-PA, the $k_{cat.}$ for plasminogen activation in the presence of each cell line was calculated and found to differ by 26-fold (0.36 s^{-1} on U937 cells; 9.25 s^{-1} on KATO III cells). However, the K_m for plasminogen with respect to the rate of formation of plasmin was lower than the K_d for binding (0.2 μM compared with 0.5 μM on U937 cells; 0.34 μM compared with 1.6 μM on KATO III cells). A rapid transformation from Glu-plasminogen (native plasminogen with N-terminal Glu) to Lys-plasminogen (plasmin-degraded plasminogen with primarily N-terminal Lys-77) occurred on the surface of U937 cells (unlike KATO III cells), but this transition did not coincide with faster rates of plasminogen activation. From this evidence it is concluded that the accessibility of bound plasminogen acts to limit the rate of activation by cell-bound urokinase. The significance of this proposal is that the proteolytic potential of the cell-mediated activation of plasminogen would be controlled by the accessibility of plasminogen for activation rather than by the concentration of u-PAR (the latter may act to localize proteolysis to appropriate domains on the surface of the cell).

INTRODUCTION

The urokinase-type plasminogen activator (u-PA) is considered to play an important role in the dissolution of the extracellular matrix, which is necessary for the passage of cells within and through tissue barriers in a diversity of biological processes [1–3]. u-PA, which has been shown to be produced by a variety of cultured cells as a single-chain glycoprotein (scu-PA) of 54 kDa [1,4–6], can be converted into a two-chain form (tcu-PA) which is distinguished kinetically by an increase in the initial rate of plasminogen activation. Unlike tcu-PA, scu-PA is unavailable for inhibition by proteinase inhibitors such as plasminogen activator inhibitor type 1 [5,7,8]. This property is probably of importance in the pericellular environment, where the catalytic activity is also controlled by a specific cell receptor for u-PA (u-PAR) [9–12] which binds the activator tightly through an epidermal growth factor-like domain towards the N-terminus of the molecule [10,13].

u-PAR has been described on a number of cell types and has been shown to enhance the rate of activation of plasminogen by scu-PA [14]. A number of reasons have been identified for this enhanced rate. Firstly, the simultaneous binding of plasminogen and urokinase to the same cell surface increases the possibility of interaction, observed as a decrease in the apparent Michaelis constant [15]. Secondly, the plasmin generated on the surface is less available for inhibition by α_2-antiplasmin inhibitor [15–17]. Surface-bound plasmin is probably responsible for a rapid conversion of scu-PA to the more active tcu-PA. The cellular binding of plasminogen is therefore essential to the cell-mediated acceleration of the rate of plasminogen activation by scu-PA. Specific binding of plasminogen has been reported on a number of cell types [16,18–20], and in the case of most cultured cells is characterized by binding of the ligand at very high levels (10^7 sites/cell) and at low affinity ($K_d \sim 10^{-6}$ M), in contrast to the binding of u-PA to u-PAR (up to 10^5 receptors/cell; $K_d \sim 10^{-10}$–10^{-9} M) [21].

In the present study, we have compared the ability of two cell lines to enhance plasminogen activation by scu-PA. We observed that the activity was not related to the number of u-PARs on the surface, as simple kinetic theory might suggest. From a mechanistic investigation, we propose that the rate-controlling component of plasminogen activation by urokinase on the cell surface is the accessibility of the plasminogen. These observations could have implications in the consideration of the physiological functioning of cell-mediated plasminogen activation by urokinase.

MATERIALS AND METHODS

Human scu-PA was provided by Sandoz Forschung-Institute (Vienna, Austria) and was greater than 99.8 % single-chain form. Human tcu-PA was prepared by incubation with plasmin as described previously [22]. The native and partially degraded forms of human plasminogen [N-terminal Glu- and Lys-plasminogen respectively) were obtained from KabiVitrum (Stockholm, Sweden) and Enzyme Research Laboratories (Swansea, U.K.) respectively. The N-terminal fragment (ATF) of u-PA (residues 1–135) was a gift from Dr. J. Henkin (Abbott Laboratories, Abbott Park, IL, U.S.A.). Carrier-free ^{125}I was

Abbreviations used: u-PA, urokinase-type plasminogen activator; scu-PA, single-chain u-PA; tcu-PA, two-chain u-PA; u-PAR, u-PA receptor; ATF, N-terminal fragment of urokinase; Glu-plasminogen, native plasminogen with N-terminal Glu; Lys-plasminogen, plasmin-degraded plasminogen with primarily N-terminal Lys-77; TBS, Tris-buffered saline.

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obtained from Amersham U.K. Bolton–Hunter reagent and Iodo-Gen were purchased from Sigma Chemical Co. Chromogenic substrate H-D-Val-Leu-Lys-*p*-nitroanilide (S2251) was obtained from Quadratech. Pre-packed chromatography columns were obtained from Pharmacia (PD-10) and Bio-Rad (P6-DG). Cell culture flasks and reagents were obtained from Costar and Gibco Ltd. respectively.

Cell culture

Two cell lines, KATO III and U937 monocyte-like cells, were cultured. The KATO III cell line is derived from the metastatic pleural effusion of a 55-year-old male with scirrhous carcinoma of the stomach [23]. The human monocytoid cell line U937 was obtained and cultured as described previously [14]. The cell lines were grown in RPMI 1640 medium (Gibco) supplemented with L-glutamine, 25 mM Hepes buffer, 2.5 μg/ml Fungizone, 100 i.u./ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum at 37 °C in 5% CO_2.

Quantification of ligand binding to cell receptors

ATF and scu-PA were radiolabelled with ^{125}I to a specific radioactivity of between 10 and 30 μCi/μg by the method of Bolton and Hunter [24]. After labelling, free iodide was separated by passing the mixture over a PD-10 column (scu-PA) or a P6-DG column (ATF) equilibrated with 0.05 M sodium phosphate buffer, pH 7.5, containing 0.25% gelatin. The labelled proteins were stored frozen at −70 °C in 0.05 M sodium phosphate buffer, pH 7.5, containing 0.25% (w/v) gelatin and 10 mg/ml BSA. Glu-plasminogen was radiolabelled with ^{125}I using Iodo-Gen [25], and free iodide was removed using a PD-10 column as described above. Prior to binding studies, the cells were acid-washed (removing endogenous ligand bound to u-PAR) by suspension in buffer A (0.05 M glycine/HCl, pH 3.0, 0.1 M NaCl) for 3 min at room temperature, followed by immediate neutralization with 0.5 M Hepes, pH 7.5, and 0.1 M NaCl (buffer B) [26]. The cells were then collected by centrifugation (200 *g* at 4 °C) and washed (twice) in RPMI 1640 before finally resuspending into binding buffer [RPMI 1640 containing 25 mM Hepes and 0.1% (w/v) BSA]. Binding saturation curves for ATF, scu-PA and plasminogen were obtained by incubating the acid-washed cells in 200 μl of binding buffer containing various amounts of unlabelled ligand and a single concentration of ^{125}I-labelled ligand at 4 °C for 2 h (ATF and scu-PA) or at 37 °C for 1 h (plasminogen). The cells were then pelleted by centrifugation and washed twice in PBS containing 0.1% (w/v) BSA before the radioactivity of the pellet was counted in a γ-radiation counter. The binding data were analysed according to the method of Scatchard using the Enzfitter program [27].

Measurement of plasminogen activation by cell-bound uPA

Suspended monolayer U937 and KATO III cells were each harvested by centrifugation (200 *g*), washed in RPMI 1640 containing 1000 units/ml aprotinin (Trasylol; Bayer) and then subjected to a further wash in buffer A to remove bound u-PA, followed by neutralization with buffer B (as described above). The cells were washed twice with RPMI 1640 containing 25 mM Hepes. Immediately prior to use, the cells were centrifuged and resuspended in 0.05 M Tris/HCl, pH 7.4, 0.1 M NaCl containing 0.1% (w/v) BSA (TBS/BSA). Cells were preincubated with scu-PA for 5 min at 37 °C as described by Ellis et al. [14] prior to the addition of either Glu-plasminogen (final concentration 0.4 μM) or Lys-plasminogen (final concentration 0.1 μM) followed by the plasmin substrate, H-D-Val-Leu-Lys-*p*-nitroanilide (final concentration 0.36 mM). The rate of formation of plasmin was monitored in an Abbott ABA100 Biochromatic Analyzer fitted with a 380/450 nm filter. The change in absorbance per min was related to a curve prepared with known concentrations of plasmin in order to calculate the concentration of plasmin and the initial rate calculated as previously described [14].

Conversion of cell-associated Glu-plasminogen to Lys-plasminogen

Acid-washed cells (final concentrations: U937 cells, 5×10^6 cells/ml; KATO III cells, 7.5×10^6 cells/ml) were incubated with 0.4 μM Glu-plasminogen in TBS/BSA for various times at 37 °C. The cells were washed three times in PBS/BSA and plasminogen was eluted by incubation in TBS containing 2 mM tranexamic acid for 10 min. The eluates were prepared and applied to a 15% polyacrylamide acid gel and run in 0.1 M acetic acid at 5 mA for 4.5 h [28]. The gels were washed free of acetic acid by soaking in 0.1 M Tris/HCl buffer, pH 8.45 (containing 0.15 M glycine and 0.1% SDS), for 15 h. Gels were blotted on to nitrocellulose and developed using anti-plasminogen (goat antibody) followed by anti-goat horseradish peroxidase conjugate. Blots were developed using enhanced chemiluminescence (ECL Reagent; Amersham).

RESULTS

Characteristics of binding of ^{125}I-labelled ATF to cultured human cell lines

Cells were incubated for 2 h at 4 °C with ^{125}I-labelled ATF in the presence of different concentrations of unlabelled ligand and, from the amount of radioactivity bound, ATF-specific binding curves were prepared and analysed by Scatchard plots. The data obtained for binding by U937 and KATO III cells are shown in Figure 1. A dissociation constant (K_d) was calculated of 0.35 ± 0.1 nM for U937 cells and the number of ATF binding

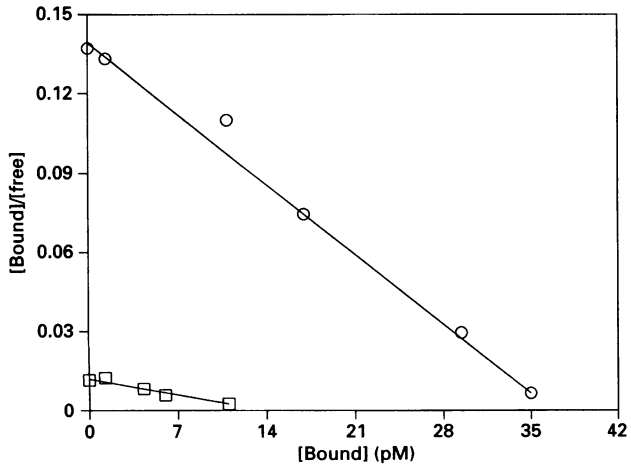

Figure 1 Characteristics of binding of ^{125}I-ATF to U937 and KATO III cells

Acid-washed cells suspended in RPMI 1640 containing 25 mM Hepes and 0.1% (w/v) BSA were incubated with various concentrations of unlabelled ATF and a constant trace amount of ^{125}I-labelled ATF for 2 h at 4 °C. The results shown were from experiments with U937 cells (○) and KATO III cells (□) at concentrations of 5×10^6 and 1×10^7 cells/ml respectively. After incubation with ligand, cells were centrifuged, washed twice and the residual radioactivity counted. The binding data were analysed according to the method of Scatchard by plotting the ratio of bound over free ligand concentration against bound.

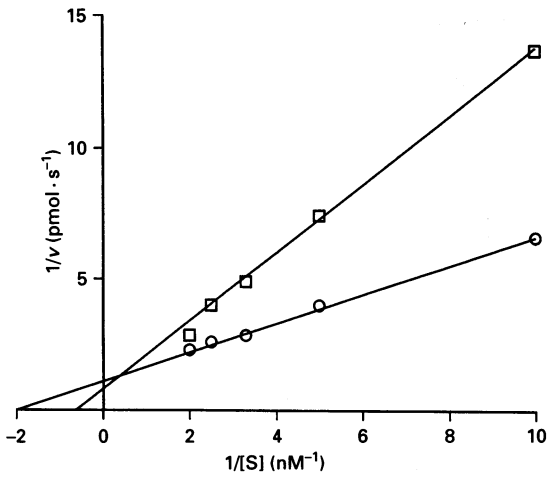

Figure 2 Effects of various concentrations of scu-PA on plasmin formation in the presence of the two cell lines

Reciprocal plots are shown relating the rate of plasmin generation to the concentration of scu-PA. Acid-washed U937 (○) and KATO III (□) cells (final concentration of both cell lines, 3×10^5 cells/ml) were preincubated with various concentrations of scu-PA (0.1–0.5 nM) for 5 min prior to the addition of 0.4 μM Glu-plasminogen and the chromogenic substrate H-D-Val-Leu-Lys-*p*-nitroanilide (0.36 mM). Plasmin generation was measured continuously as ΔA at 380/450 nm. The absorbance change per min was related to a plasmin calibration curve in order to calculate the concentration of plasmin at timed intervals. The initial rate of plasmin generation was calculated as previously described [14]. The results are the means of three experiments measured in duplicate.

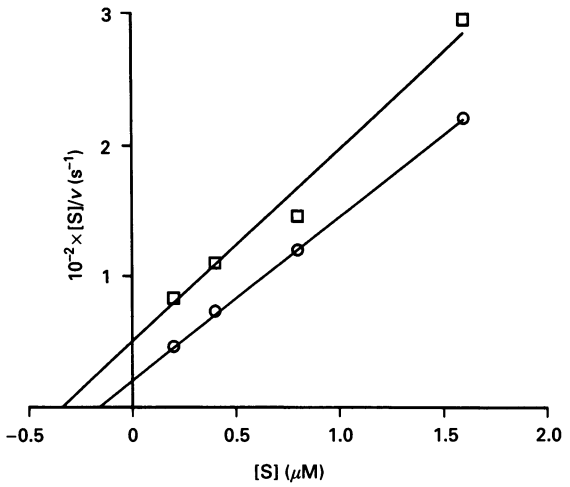

Figure 3 Effects of various concentrations of plasminogen upon plasmin formation in the presence of the two cell lines

Hanes plot of data relating the rate of plasmin generation to the plasminogen concentration. Acid-washed U937 (○) and KATO III (□) cells (each at 3×10^5 cells/ml) were suspended in TBS/BSA and preincubated with scu-PA (0.1 nM) for 5 min prior to the addition of Glu-plasminogen at various concentrations (0.2–1.6 μM) together with H-D-Val-Leu-Lys-*p*-nitroanilide (0.36 mM). The initial rate of plasmin generation is shown plotted according to the concentration of Glu-plasminogen.

molecules/cell was 5000 ± 960 ($n = 4$). For KATO cells values for the K_d of 0.85 ± 0.15 nM and 400 ± 135 ATF binding sites per cell ($n = 2$) were calculated. The values obtained for the K_d and the number of receptors per cell are within the ranges reported previously for a number of other cultured cell lines by Blasi [29]. Similar values were obtained with U937 and KATO III cells when binding curves were prepared using ^{125}I-scu-PA (results not shown).

Potentiation of scu-PA-mediated plasminogen activation by U937 and KATO III cell lines

Initial experiments were carried out as described previously [14]. Acid-washed cells were incubated with Glu-plasminogen (0.4 μM) and a single concentration of scu-PA (0.1 nM), and the rates of formation of plasmin were compared for the two cell lines. The rate of activation in the presence of each cell line at an equal density (3×10^5 cells/ml) was similar, in spite of the marked difference in the number of urokinase binding sites (results not shown). Furthermore, this equivalence in the rate of cell-dependent plasminogen activation was also observed when measured in the presence of tcu-PA or in the presence of the more readily activatable form of plasminogen, Lys-plasminogen.

In order to investigate these observations further, the catalytic efficiency of urokinase when bound to u-PAR on each of the two cell lines was estimated by measuring the rate of plasmin generation after preincubating acid-washed cells with different concentrations of scu-PA for 5 min before the addition of Glu-plasminogen and chromogenic substrate. Upon expressing the reciprocal of the rate of plasmin formation against the reciprocal of scu-PA concentration, a linear relationship was found from which an apparent K_d ($K_{d,app.}$, the binding affinity of scu-PA for u-PAR measured in respect of plasminogen activation) and a catalytic rate could be calculated (Figure 2). These two values were determined for U937 cells as 0.5 nM and 0.36 s^{-1} respectively, and for KATO III cells as 1.6 nM and 9.25 s^{-1} respectively. The turnover rate for scu-PA when bound to u-PAR on the surface of KATO III cells was therefore 25.7-fold higher than that of scu-PA bound to u-PAR on U937 cells.

The rate of plasmin generation was also measured after preincubation of cells at a single concentration of scu-PA (0.1 nM) for 5 min before the addition of increasing concentrations of Glu-plasminogen. A Hanes plot of this data (Figure 3) permitted calculation of an apparent K_m for plasminogen activation of 0.16 μM and a maximum velocity ($V_{max.}$) of 0.22 pM · s^{-1} for U937 cells, and corresponding values of 0.34 μM and 0.19 pM · s^{-1} for KATO III cells. At a concentration of scu-PA of 0.1 nM, the u-PAR population will be only partially saturated at equilibrium. Using the values obtained from Figure 1 for the K_d and $B_{max.}$, the amount of bound enzyme at a density of 3×10^5 cells/ml is calculated as 0.55 pmol for U937 cells and 0.02 pmol for KATO III cells, from which catalytic rates of 0.4 s^{-1} (U937 cells) and 9.4 s^{-1} (KATO III cells) were calculated.

Binding and partial digestion of Glu-plasminogen on the surface of U937 and KATO III cells

As noted previously, the apparent binding affinity of scu-PA for u-PAR as calculated from the rate of plasmin generation observed with increasing concentrations of scu-PA ($K_{d,app.}$) coincided with the binding affinity determined using ^{125}I-labelled ATF as a specific probe for the receptor (K_d). Thus for U937 cells, $K_{d,app.} = 0.5$ nM and $K_d = 0.35$ nM, and for KATO cells $K_{d,app.} = 1.6$ nM and $K_d = 0.85$ nM. However, when the binding of plasminogen to the cells surface was compared with the affinity of the protein for cell-bound u-PA, differences were observed. The binding properties of ^{125}I-labelled plasminogen with these two cell types were measured by incubating acid-washed cells with increasing concentrations of non-labelled plasminogen (0.1–2.0 μM) in the presence of a constant amount of ^{125}I-

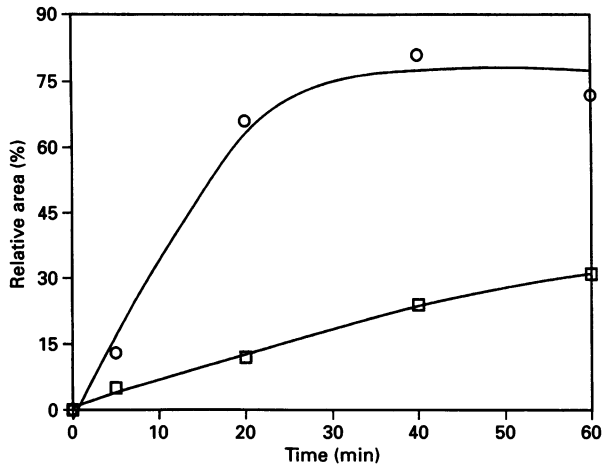

Figure 4 Conversion of cell-associated Glu-plasminogen to Lys-plasminogen

Acid-washed cells were incubated with Glu-plasminogen (0.4 μM) at 37 °C for various times and washed twice in PBS/BSA, and the cell-associated plasminogen was eluted from the KATO III cells (□) and U937 cells (○) by the addition of TBS containing 2 mM tranexamic acid. The formation of Lys-plasminogen was monitored by gel electrophoresis by the method of Panyim and Chalkley [28]. After blotting, prints of gels were developed by chemiluminescence using goat anti-plasminogen as described in the Materials and methods section. The percentage of Lys-plasminogen generation in samples taken at the four timed intervals was estimated by scanning densitometry.

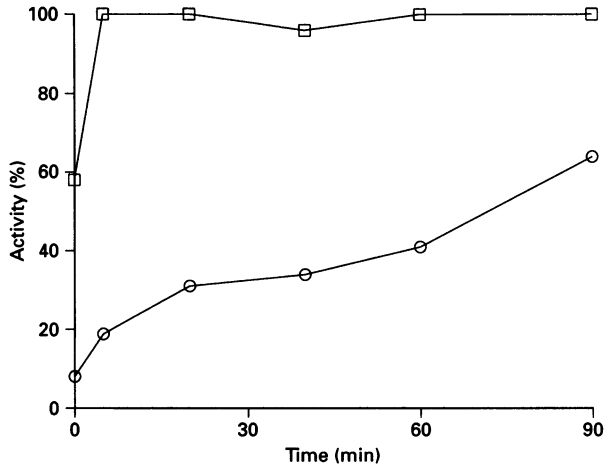

Figure 5 Rate of activation of plasminogen by scu-PA on the surface of U937 cells according to the period of preincubation

Acid-washed cells were incubated with Glu-plasminogen (○) or Lys-plasminogen (□) at a concentration of 0.4 μM at 37 °C. At timed intervals scu-PA was added (final concentration 0.1 nM) together with the chromogenic substrate H-D-Val-Leu-Lys-*p*-nitroanilide (0.36 nM). Plasmin generation was measured as described in the legend to Figure 2 and is shown here expressed as a percentage of the rate observed in the reaction mixtures after 5 min of preincubation with Lys-plasminogen.

labelled plasminogen at 37 °C for 60 min. K_d values for U937 and KATO III cells were calculated by Scatchard analysis as 0.5 μM and 1.6 μM respectively (results not shown). The number of plasminogen binding sites was estimated as approx. 3.7×10^7 sites/cell for U937 cells and 4×10^7 sites/cell for KATO III cells. These values are similar to those reported previously for binding of plasminogen to cultured cells [21]. The K_m values for activation of plasminogen by cell-bound scu-PA (Figure 3) are 3–5-fold lower, therefore, than the binding affinities for plasminogen (U937 cells, K_m 0.16 μM; KATO III cells, K_m 0.34 μM).

Lys-plasminogen is activated considerably more quickly than is Glu-plasminogen by both scu-PA and tcu-PA. Glu-plasminogen bound to the surface of U937 cells has been shown previously to undergo partial digestion to the more activatable form by an as yet unknown mechanism [15]. When the digestion of Glu-plasminogen was compared upon the surface of the two cell types, the formation of Lys-plasminogen was observed to occur much faster on the surface of U937 cells than on KATO III cells (Figure 4), being essentially complete within 20 min. Considering that this conversion should result in a more rapid activation of plasminogen upon addition of scu-PA, U937 cells were incubated with Glu- or Lys-plasminogen for various periods of time before the addition of 0.1 nM scu-PA to initiate plasmin generation. The rate of plasmin generation in incubation mixtures containing Lys-plasminogen was constant at each time point (Figure 5). In mixtures containing Glu-plasminogen a progressive increase in the rate of plasmin generation was observed, but the rate did not reach that seen in the presence of Lys-plasminogen when tested up to 1.5 h. Thus, although the transition of the bulk of the cell-bound Glu-plasminogen to Lys-plasminogen is completed within 20 min (Figure 4), the transition to a more activatable form as regards cell-bound u-PA occurs much more slowly. For this reason it is possible to discriminate differences between the rates of activation of Glu-plasminogen and Lys-plasminogen on the surface of U937 cells [14]. These observations suggest that sites which bind plasminogen in respect of u-PAR may be distinct from sites responsible for the bulk of binding to the surface of these cells.

DISCUSSION

During preliminary work to this present study we used our previously described methodology [14] to measure plasminogen (0.4 μM) activation by scu-PA (0.1 nM) in the presence of five different cell lines (U937, KATO III, human skin fibroblasts, human umbilical vein endothelial cells and a gastric carcinoma cell line, MKN28). The rates of activation were observed to be similar in spite of a wide variation in the number of ATF binding sites per cell (from 400 per cell on KATO III cells to 3×10^5/cell on fibroblasts). To investigate these findings further, more detailed investigations of the cell-mediated activation of plasminogen by u-PA were carried out with the U937 and KATO III cells. The characteristics of the observed potentiation by each cell line were investigated thoroughly and found to be entirely attributable to a mechanism requiring coincident binding of scu-PA and plasminogen to a specific cell receptor (u-PAR). Although a difference in the binding affinity for ATF was observed, the principal difference between the two cell lines was in the number of u-PARs per cell, which varied over an order of magnitude. Using the values obtained for the number of receptors per cell, the concentration of the scu-PA–u-PAR complex at saturation was calculated, and from this the apparent catalytic rate, for cell-bound u-PA. A 25-fold variation in the specific activity was observed when KATO III and U937 cells were compared (Figure 2).

From our current understanding, the observed rate of activation of plasminogen by scu-PA in the presence of cells can be described by the following series of binding equilibria and enzymic reactions:

$$\text{scu-PA} + \text{CR} \leftrightharpoons \text{scu-PA–CR} \quad (1)$$

$$\text{Plg} + \text{CR} \rightleftharpoons \text{Plg–CR} \quad (2)$$

$$\text{G-Plg–CR} \xrightarrow[\text{scu-PA–CR}]{} \text{Pln} \quad (3)$$

$$\text{L-Plg–CR} \xrightarrow[\text{scu-PA–CR}]{} \text{Pln} \quad (4)$$

$$\text{G-Plg–CR} \xrightarrow[\text{Pln–CR}]{} \text{L-Plg–CR} \quad (5)$$

$$\text{scu-PA–CR} \xrightarrow[\text{Pln–CR}]{} \text{tcu-PA–CR} \quad (6)$$

$$\text{G-Plg–CR} \xrightarrow[\text{tcu-PA–CR}]{} \text{Pln} \quad (7)$$

$$\text{L-Plg–CR} \xrightarrow[\text{tcu-PA–CR}]{} \text{Pln} \quad (8)$$

where Plg is plasminogen, G-Plg is Glu-plasminogen, L-Plg is Lys-plasminogen, Pln is plasmin and CR is the respective cell receptor.

The higher rate of plasmin formation from Glu-plasminogen by scu-PA per mol of u-PAR on KATO III cells, as opposed to U937 cells, could not be ascribed to a significantly higher affinity of the receptor for scu-PA (as demonstrated in Figures 1 and 2), nor to a higher affinity or higher levels of plasminogen receptor as measured from binding of ^{125}I-labelled plasminogen (10^7 receptors on both cells). The relative differences in activity were seen when using Glu-plasminogen or Lys-plasminogen, and also in the presence of tcu-PA (reactions 7 and 8). The latter observation also ruled out the influence of reaction (6), since the concentration of tcu-PA bound to u-PAR in the reaction mixture would be considerably less on KATO III cells at an equivalent concentration of the two cell lines. Likewise, there was a slower transition of Glu-plasminogen to Lys-plasminogen on KATO III cells than on U937 cells (Figure 4). Thus no advantage from reaction (5) could not be considered. Although a direct effect of u-PAR on the catalytic efficiency of scu-PA cannot be entirely ruled out, there is no evidence from studies with isolated receptors to indicate that u-PAR can affect urokinase activity in this way [15].

Internalization of a number of u-PAR receptors on the surface of U937 cells, but not on KATO cells, is another possible explanation for the difference in catalytic activity. This down-regulation would need to be rapid to influence the kinetic rates. There was no evidence of unusual kinetics which a rapid loss of receptor-bound urokinase would cause, nor has such a loss been reported by other investigators. Furthermore, glycolipid-anchored receptors such as u-PAR [30] are not internalized by the clathrin-dependent endocytic mechanism [31]. When internalization of u-PAR has been observed, it occurs after the receptor-bound u-PA has been inhibited by plasminogen activator inhibitor 1 [32]. The inhibitor complex with the serpin is slowly cleared by the α_2-macroglobulin/low-density-lipoprotein receptor [33]. Such a mechanism is unlikely to occur under the experimental conditions used herein.

Taking all the above considerations into account, we are led to conclude that the difference in the catalytic activity of scu-PA bound to u-PAR on these two cell lines arises from a property of these reactions which acts independently of the concentration of u-PAR. We propose that this property arises from the nature of plasminogen binding which limits its accessibility as a substrate for receptor-bound urokinase.

The binding of plasminogen is obligatory for potentiation of the rate of activation by the cell surface principally, it is thought, because the approximation of enzyme and substrate on the cell surface favours the formation of a Michaelis complex. The simplicity of such a mechanism has been highlighted recently by a report of an antibody specific for urokinase which possesses a potentiating effect on the activation of plasminogen by scu-PA [34]. The antibody exerted this influence by coincident binding of urokinase to the variable region and of plasminogen at a C-terminal lysine. As noted above, studies with ^{125}I-labelled plasminogen report plasminogen binding to cultured cells to be characterized by high capacity and relatively low affinity. The high capacity makes it evident that the binding is mediated by a heterogeneous group of molecules, and a number of different candidates have been proposed [21,35]. The number of such receptors on cells is 4–5 orders of magnitude higher than that of u-PAR, making it unlikely that the mass of plasminogen bound is acting as a rate-limiting component. We propose instead that the rate is controlled by the accessibility of bound plasminogen for activation by the u-PA–u-PAR complex. Alternative explanations can be considered for such a reduced accessibility. The glycolipid anchor of u-PAR permits lateral mobility [30] and, at higher concentrations of receptor, patching may occur reducing the effective concentration of the receptor with respect to the surrounding cell-bound plasminogen. Secondly, the plasminogen receptors may exhibit some functional heterogeneity in that only a subpopulation may be favoured sterically for interaction with urokinase bound to u-PAR (shown schematically in Figure 6).

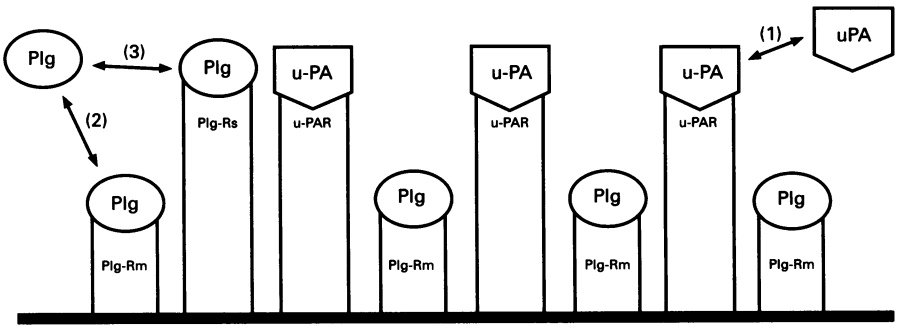

Figure 6 Schematic model for the cell surface activation of plasminogen by u-PA

u-PA binds to the u-PAR (1). Potentiation of plasminogen (Plg) activation occurs upon coincident binding of plasminogen to the cell surface. However, two types of plasminogen receptors are available. Plasminogen bound to the majority of the receptors (Plg-Rm) is unavailable for activation by u-PA for steric or other reasons (2). Plasminogen will be activated by u-PA–u-PAR complex when bound to a receptor subset (Plg-Rs) present at much lower concentrations than u-PAR (3).

Two experimental observations presented here tend to support, indirectly, the second of these explanations (direct evidence is difficult to obtain at this stage because of the promiscuous nature of the binding of plasminogen to the cell surface). The first of these is that the apparent K_m for plasminogen activation on the cell surface was found to be less than the K_d measured for the binding of ^{125}I-plasminogen to the cell surface. For example, in KATO III cells the apparent K_m is 0.34 μM as opposed to a value for the K_d of 1.6 μM by Scatchard analysis. Secondly, a rapid transition of Glu-plasminogen to the more readily activatable Lys-plasminogen was observed on the surface of U937 cells. However, this conversion was not demonstrable as regards the rate of activation of the plasminogen on the cell surface. For this reason it is possible to distinguish differences in the kinetics of activation of Glu- and Lys-plasminogen on U937 cells [14]. Preincubation of Glu-plasminogen with cells resulted in a transition to a more readily activatable form (Figure 5), but this occurred more slowly than the rapid conversion of the bulk of Glu-plasminogen on the cell surface (Figure 4). Our findings are in accord with a proposition by Ellis and Danø [36] that only a small proportion of plasminogen binding may be essential for functional activity. The existence of such a subpopulation would explain a previously described 5-fold decrease in catalytic rate observed upon treatment of U937 cells with phorbol 12-myristate 13-acetate [37]. The treatment with the phorbol causes a 5-fold increase in the number of u-PARs per cell. However, if the number of plasminogen receptors which are favoured for interaction with u-PAR remains constant, then a lower catalytic rate would be observed.

If, as our studies suggest, plasminogen receptors are functionally heterogeneous, this would have interesting implications for the role of the u-PAR-dependent system in initiating pericellular proteolysis, particularly as regards control. It has been shown previously that tcu-PA, unlike scu-PA, is liable to inhibition by plasminogen activator inhibitors (tcu-PA being generated from cell-bound scu-PA as plasmin is formed) [36]. However, plasmin bound to the surface is less available for inhibition by its inhibitor, α_2-antiplasmin, but only while it remains bound to the surface of the cell [16,17]. Since plasmin will leave the surface relatively rapidly because of the low affinity for the receptor, plasmin proteolysis will tend to occur at the cell surface. For urokinase-dependent pericellular proteolysis to be a normal feature of tissue physiology, for example during tissue remodelling, it is essential that the proteolytic events are locally induced, locally potentiated and locally controlled, ensuring that the digestion proceeds according to the needs of the tissue and within the pericellular microenvironment. A subpopulation of plasminogen receptors present at low concentrations and favoured for the interaction with the scu-PA–u-PAR complex would serve to localize the plasmin which is generated. This is a situation which contrasts markedly with one in which all plasminogen receptors are available for such an interaction and the potential to generate up to micromolar concentrations of plasmin would exist (since the glycolipid anchor of u-PAR permits the tightly bound u-PA to move within the plane of the cell membrane). Another consideration is that such a subset of plasminogen receptors would permit u-PAR to be present at high levels on the cell surface without causing excessive proteolysis. Immunological studies on fibroblasts and fibrosarcoma cells have demonstrated the presence of u-PA at sites of attachment of cells to the substratum, and its co-localization with vinculin [38,39]. Under the circumstances we have described, the localization of u-PAR would control the area in which proteolysis could occur, whereas the rate-limiting subset of plasminogen receptors would control the maximum proteolytic potential at that site.

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REFERENCES

1 Danø, K., Andreasen, P. A., Grøndahl-Hansen, J., Kristensen, P., Nielsen, L. S. and Skriver, L. (1985) Adv. Cancer Res. **44**, 139–266
2 Saksela, O. and Rifkin, D. B. (1988) Annu. Rev. Cell Biol. **4**, 93–126
3 Vassalli, J.-D., Sappino, A.-P. and Belin, D. (1991) J. Clin. Invest. **88**, 1067–1072
4 Nielsen, L. S., Hansen, J. G., Skriver, L., Wilson, E. L., Kaltoft, K., Zeuthen, J. and Danø, K. (1982) Biochemistry **24**, 6410–6415
5 Vassalli, J.-D., Dayer, J.-M., Wohlwend, A. and Belin, D. (1984) J. Exp. Med. **159**, 1653–1668
6 Wun, T-C., Ossowski, L. and Reich, E. (1982) J. Biol. Chem. **257**, 7262–7268
7 Andreasen, P. A., Nielsen, L. S., Kristensen, P., Grøndahl-Hansen, J., Skriver, L. and Danø, K. (1986) J. Biol. Chem. **261**, 7644–7651
8 Eaton, D. L., Scott, R. W. and Baker, J. B. (1984) J. Biol. Chem. **259**, 6241–6247
9 Vassalli, J.-D., Baccino, D. and Belin, D. (1985) J. Cell Biol. **100**, 86–92
10 Stoppelli, M. P., Corti, A., Soffientini, A., Cassani, G., Blasi, F. and Assoian, R. K. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 4939–4943
11 Miles, L. A. and Plow, E. F. (1987) Thromb. Haemostasis **58**, 936–942
12 Bajpai, A. and Baker, J. B. (1985) Biochem. Biophys. Res. Commun. **133**, 994–1000
13 Appella, E., Robinson, E. A., Ullrich, S. J., Stoppelli, M. P., Corti, A., Cassani, G. and Blasi, F. (1987) J. Biol. Chem. **262**, 4437–4440
14 Ellis, V., Scully, M. F. and Kakkar, V. V. (1989) J. Biol. Chem. **264**, 2185–2188
15 Ellis, V., Behrendt, N. and Danø, K. (1991) J. Biol. Chem. **266**, 12752–12758
16 Plow, E. F., Freaney, D. E., Plescia, J. and Miles, L. A. (1986) J. Cell Biol. **103**, 2411–2420
17 Hall, S. W., Humphries, J. E. and Gonias, S. L. (1991) J. Biol. Chem. **266**, 12329–12336
18 Miles, L. A. and Plow, E. F. (1985) J. Biol. Chem. **260**, 4303–4311
19 Miles, L. A. and Plow, E. F. (1988) Fibrinolysis **2**, 61–71
20 Felez, J., Miles, L. A., Plescia, J. and Plow, E. F. (1990) J. Cell Biol. **111**, 1673–1683
21 Plow, E. F., Felez, J. and Miles, L. A. (1991) Thromb. Haemostasis **66**, 32–36
22 Scully, M. F., Ellis, V., Watahiki, Y. and Kakkar, V. V. (1989) Arch. Biochem. Biophys. **268**, 438–446
23 Sekiguchi, M., Sakakibara, K. and Fujii, G. (1978) Jpn. J. Exp. Med. **48**, 61–68
24 Bolton, A. E. and Hunter, W. M. (1973) Biochem. J. **133**, 529–539
25 Fraker, P. J. and Speck, J. C. (1978) Biochem. Biophys. Res. Commun. **80**, 849–857
26 Stoppelli, M. P., Tacchetti, C., Cubellis, M. V., Corti, A., Hearing, V. J., Cassani, G., Appella, E. and Blasi, F. (1986) Cell **45**, 675–684
27 Leatherbarrow, R. J. (1987) Enzfitter, Elsevier Science Publishing Co., New York
28 Panyim, S. and Chalkley, R. (1969) Arch. Biochem. Biophys. **130**, 337–346
29 Blasi, F. (1988) Fibrinolysis **2**, 73–84
30 Ploug, M., Ronne, E., Behrendt, N., Jensen, A. L., Blasi, F. and Danø, K. (1991) J. Biol. Chem. **266**, 1926–1933
31 Turner, A. J. (1994) Essays Biochem. **28**, 113–128
32 Cubellis, M. V., Wun, T. C. and Blasi, F. (1990) EMBO J. **9**, 1079–1085
33 Andreassen, P. A., Sottrup-Jensen, L., Kjoller, L., Nykjaer, A., Moestrup, S. K., Petersen, C. M. and Gliemann, J. (1994) FEBS Lett. **338**, 239–245
34 Ellis, V. and Danø, K. (1993) J. Biol. Chem. **268**, 4806–4813
35 Miles, L. A., Dahlberg, C. M., Plescia, J., Felez, J., Kato, K. and Plow, E. F. (1991) Biochemistry **30**, 1682–1691
36 Ellis, V. and Danø, K. (1992) Fibrinolysis **6** (Suppl. 4), 27–34
37 Ellis, V., Wun, T.-C., Behrendt, N., Rønne, E. and Danø, K. (1990) J. Biol. Chem. **265**, 9904–9908
38 Pollanen, J., Hedman, K., Nielsen, L. S., Danø, K. and Vaheri, A. (1988) J. Cell Biol. **106**, 87–95
39 Pollanen, J., Saksela, O., Salonen, E.-M., Andreasen, P., Nielsen, L., Danø, K. and Vaheri, A. (1987) J. Cell Biol. **104**, 1085–1096

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