Fibrinogenolytic and Fibrinolytic Activity of Cell-Associated Plasmin

John E. Humphries, Jayanand Vasudevan, and Steven L. Gonias

Binding of plasmin(ogen) to rat C6 glioma cells is saturable and kringle-domain dependent. This interaction was studied as a model of plasmin(ogen) receptor interactions in nucleated mammalian cells. Apparent \(^{125}\)I-plasmin dissociation from C6 cell binding sites was slow; however, the dissociation rate was increased when the solution contained diisopropyl phosphoryl-plasmin (0.3 \(\mu\)M), fibrinogen (0.16 or 0.8 mg/ml), 1.08 mM D-Val-L-Leu-L-Lys-p-nitroanilide-HCl (S-2251), or e-amino-n-caproic acid (EACA, 5.0 mM). EACA promoted the most rapid dissociation of plasmin. C6 cell-associated plasmin and plasmin in solution demonstrated similar amidase activity. Only specifically bound plasmin (75% of total binding) was active against S-2251. Plasmin that was initially bound to C6 cells digested fibrinogen in a time- and plasmin concentration-dependent manner. \(\alpha\)-Antiplasmin (\(\alpha\)AP, 0.1 \(\mu\)M) completely inhibited fibrinogenolysis by plasmin that was initially C6- or human umbilical vein endothelial-cell associated. Since \(\alpha\)AP reacts selectively with plasmin in solution (minimally with plasmin bound to cells), fibrinogen digestion by cell-associated plasmin probably occurred only after the plasmin dissociated into solution. Crosslinked fibrin clots were formed in uniform layers over C6 cells. If the cells were incubated with plasmin before addition of fibrinogen and thrombin, the clots were rapidly lysed. \(\alpha\)AP incompletely inhibited fibrinolysis when added after fibrin polymerization (44% inhibition with 0.1 \(\mu\)M \(\alpha\)AP). Fibrinolysis was completely inhibited when \(\alpha\)AP was added before fibrin polymerization. These studies suggest that plasmin must first dissociate from cellular binding sites to mediate fibrinogenolysis or fibrinolysis. After dissociation, plasmin activity is modulated by antiplasmins, which are ineffective at the cell surface. (Arteriosclerosis and Thrombosis 1993;13:48-55)

**KEY WORDS** • plasmin • fibrin • fibrinogen • \(\alpha\)-antiplasmin • e-amino-n-caproic acid • receptors • glioma cells • endothelial cells

The function of plasmin in the lysis of fibrin clots is well characterized; however, plasmin has many other physiologically significant substrates, including metalloproteinase zymogens, extracellular matrix proteins, and cytokines. The complete structure of [Glu\(^{\prime}\)]-plasminogen includes 791 amino acids and either one or two oligosaccharide chains. Part of the plasmin(ogen) sequence forms five homologous kringle domains, each with about 80 amino acids. The kringle domains contain lysine-binding sites, which allow plasmin(ogen) to associate noncovalently with diverse macromolecules. Kringle-mediated interactions promote substrate cleavage, increase plasminogen activation, and protect plasmin from the rapidly acting antiplasmins, \(\alpha\)-antiplasmin \((\alpha_2\)AP\) and \(\alpha_2\)-macroglobulin \((\alpha_2\)M). Most nucleated cells bind plasminogen and plasmin with similar affinity \((K_d = 0.3-3.0 \mu\text{M})\). There is some variability in the number of binding sites per cell; however, in general, the number is high \((10^4-10^5\) per cell). The biochemical nature of the cellular plasmin(ogen) binding sites remains incompletely understood. Different membrane-associated macromolecules may bind plasmin(ogen) with comparable affinity, affecting the activity of plasmin similarly. Since more than one plasmin(ogen) domain may be involved in cellular receptor interactions, the possibility of cooperativity in binding cannot be excluded.

Plasmin that is activated on the cell surface dissociates relatively slowly. Therefore, plasmin may demonstrate significant activity toward various substrates while bound to cells. One physiologically significant substrate for cell-associated plasmin is high-molecular-weight single-chain urokinase \((\text{scu-PA})\). Scu-PA, which is bound to urokinase plasminogen activator receptor \((\text{uPAR})\), is converted into the active two-chain enzyme at the cell surface. Cell-associated plasmin also cleaves small chromogenic and fluorogenic substrates. By contrast, the large proteinase inhibitors \(\alpha\)AP and \(\alpha\)M react poorly with cell-associated plasmin. \(\alpha\)M \((\alpha_2\)M \((M = 718,000\), which is the largest of the proteinase inhibitors, may be totally nonreactive with plasmin that is bound to cells. The purpose of the present investigation was to determine whether fibrinogen or fibrin is digested by cell-associated plasmin. Such activity could be highly important because of the ineffectiveness of antiplasmins...
at the cell surface. Most of the reported experiments were performed with the C6 glioma tumor-cell line, a well-characterized model for plasminogen-receptor interactions in nucleated mammalian cells.\textsuperscript{15,22-24} Fibrinogenolyis experiments were also performed with cultures of human umbilical vein endothelial cells (HUVECs). The results demonstrate that the large substrates for solution-phase plasmin, fibrinogen and fibrin, are not significant substrates for cell-associated plasmin.

**Methods**

\textbf{Reagents}

Earle's balanced salt solution (EBSS), minimum essential medium with EBSS (MEM), and bovine crystalline insulin were from Gibco Laboratories, St. Lawrence, Mass. Fetal calf serum, p-nitrophenyl p'-guanidinobenzoate hydrochloride (PNPGB), bovine serum albumin (BSA, 98–99% pure, fatty acid free), bovine thrombin, and diisopropylphosphorofluoridate were from Sigma Chemical Co., St. Louis, Mo. Fibrinogen and D-Val-L-Leu-L-Lys-p-nitroanilide-HCl (S-2251) were from KabiVitrum AB, Stockholm, Sweden. Na\textsuperscript{125}I was from Amersham, Arlington Heights, Ill., and Iodobeads were from Pierce Chemical, Rockford, Ill. Low-molecular-weight urokinase (u-PA) was from Calbiochem Corporation, La Jolla, Calif. Spli-Prest kits for determining fibrinogen digestion products were from Diagnostica Stago, Asniere-Sur-Seine, France.

\textbf{Proteins}

[Gluc]-plasminogen was purified from human plasma as previously described.\textsuperscript{19} Each preparation included a mixture of carbohydrate variants I and II (60–70% type II).\textsuperscript{24} Plasminogen (100–200 \(\mu\)g) was activated with 50 units of u-PA for 15 minutes at 37°C. Streptokinase was purified from KabiVitrum AB, Stockholm, Sweden. Na\textsuperscript{125}I was from Amersham, Arlington Heights, Ill., and Iodobeads were from Pierce Chemical, Rockford, Ill. Low-molecular-weight urokinase (u-PA) was from Calbiochem Corporation, La Jolla, Calif. Spli-Prest kits for determining fibrinogen digestion products were from Diagnostica Stago, Asniere-Sur-Seine, France.

\textbf{Radioiodination}

Fibrinogen, plasminogen, and \(\alpha_2\)AP were radioiodinated with Iodobeads. Specific activities of \(^{125}\)I-fibrinogen, \(^{125}\)I-plasminogen, and \(^{125}\)I-\(\alpha_2\)AP were 0.5–1.5 \(\mu\)Ci/\(\mu\)g. \(^{125}\)I-Plasminogen was activated with u-PA as described above. \(^{125}\)I-Plasmin activity was decreased by less than 5% compared with nonradiolabeled enzyme, as determined by S-2251 hydrolysis. By sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), \(^{125}\)I-plasminogen activation resulted in complete conversion of the single-chain zymogen into the two-chain active-enzyme form.

\textbf{Cell Culture}

Rat C6 glioma cells were obtained from American Tissue Type Collection (ATCC), Rockville, Md., and cultured in MEM with 10% fetal calf serum as previously described.\textsuperscript{23} Experiments were performed using 90–95% confluent monolayers in 24-well cell-culture plates (GIBCO). HUVECs were also obtained from ATCC and cultured as previously described.\textsuperscript{28}

\textbf{Plasmin Binding Experiments}

\(^{125}\)I-Plasmin was incubated with C6 glioma cells in EBSS with 10 mM N-hydroxyethylpiperazine-N'-\(\alpha\)-ethanesulfonic acid (HEPES) and 10 mg/ml BSA, pH 7.4 (EBH buffer), for 2 hours at 4°C. \(\alpha\)-Amino-n-caproic acid (EACA) (5.0 mM) was included in some wells. EACA inhibits plasminogen binding to cells by interacting with the ligand (instead of the receptor); specific binding is equivalent when determined with or without EACA or a 50-fold molar excess of nonradiolabeled plasminogen.\textsuperscript{19,29} After washing the cells three times, cell-associated radioactivity was recovered in 0.1 M NaOH and 2% SDS. Cellular protein was determined by the method of Lowry, as reviewed by Peterson.\textsuperscript{30} Plasmin, at concentrations up to 0.1 \(\mu\)M, had no effect on the integrity of the cell layers, as determined by cell counts of the supernatants and phase-contrast microscopy, or on cellular viability as determined by trypan blue exclusion.

\textbf{Plasmin Dissociation Experiments}

Cultures of C6 glioma cells were incubated with 0.1 \(\mu\)M \(^{125}\)I-plasmin in EBH for 2 hours at 4°C. The cells were washed and then incubated for up to 15 minutes at 22°C in fresh medium supplemented with the following: 3.0 \(\mu\)M diisopropyl phosphoril-plasmin (DIP-plasmin); 1.0 \(\mu\)M DIP-plasmin; 1–10 mg/ml BSA; 5.0 mM EACA; 1.08 mM S-2251; fibrinogen (0.16 or 0.8 mg/ml); 5.0 mM EACA and 0.3 \(\mu\)M DIP-plasmin; or 1.08 mM S-2251 and 0.3 \(\mu\)M DIP-plasmin. At various times, the supernatants and cells were separated and the radioactivity in each was determined. The amount of \(^{125}\)I-plasmin that remained bound to the cells at each time was expressed as a percentage of that bound immediately after washing. With 0.3 \(\mu\)M DIP-plasmin, the maximum level of binding achievable at equilibrium is about 2.0% of total.\textsuperscript{15} Therefore, in the presence of DIP-plasmin, \(^{125}\)I-plasmin reassociation with cells after dissociation should be insignificant.

To examine how nonspecifically bound radioligand affects the results of plasmin dissociation experiments, \(^{125}\)I-plasmin was incubated with C6 cells in the presence of 5.0 mM EACA. The dissociation of this nonspecifically bound plasmin was then studied in the presence of 5.0 mM EACA, 0.3 \(\mu\)M DIP-plasmin, 1.08 mM S-2251, or 5.0 mM EACA and 0.3 \(\mu\)M DIP-plasmin.

\textbf{Amidase Activity}

Plasmin (0.1 \(\mu\)M) was incubated with C6 cells in EHB with or without 5.0 mM EACA for 2 hours at 4°C. The cells were washed and then incubated with EBSS, 10 mM HEPES, pH 7.4, containing different concentrations of S-2251. The absorbance at 405 nm was determined at 2.5-minute intervals with a Dynatech MR 580 MicroELISA reader. Final absorbance measurements were confirmed by a Hewlett-Packard 8450A spectrophotometer. The concentration of \(p\)-nitroanilide was determined with an extinction coefficient of 10\(^4\) M\(^{-1}\)·cm\(^{-1}\).

In some experiments, plasminogen (0.5 \(\mu\)M) was activated by u-PA (9.0 units) in the presence of C6 cells for 1 hour at 22°C. The cells were then washed so that free plasmin was removed. The amount of cell-associated plasmin ranged from 2.0 to 2.5 pmol/well in different studies. Washed cultures with bound plasmin
were incubated with S-2251 in the presence or absence of 5.0 mM EACA. Under the conditions of our studies, EACA does not affect the hydrolysis of S-2251 by plasmin in solution (the catalytic constant and Michaelis constant were 11.4±0.3 seconds⁻¹ and 190±13 μM in the absence of EACA; 12.4±0.2 seconds⁻¹ and 201±13 μM in the presence of EACA).

Fibrinogenolysis

C6 cells were incubated with plasmin for 2 hours at 4°C. The monolayers were washed and then incubated with fibrinogen (250 μl, 0.16 mg/ml) at 22°C. At various times, solutions were recovered, treated with the immediate plasmin inhibitor PNPG (0.1 mM), denatured in 1.0% SDS, and subjected to SDS-PAGE on 6% slabs. Fragments X, Y, and D were identified by comparison with molecular-weight standards (BioRad).

A more quantitative method to monitor fibrinogenolysis was as follows. Washed cultures of C6 cells with bound plasmin were incubated with 125I-fibrinogen (0.5 μCi diluted with 0.2 mg nonradioactively labeled fibrinogen in 250 μl). At various times, solutions were recovered from the wells and incubated immediately with premixed solutions (25 μl) of thrombin (2 National Institutes of Health [NIH] units)/Ca²⁺ (15 mM), reptilase-R (0.01 unit), and aprotinin (0.02 trypsin inhibitory unit) (supplied in Split-Prest kit). The fibrinogen was allowed to clot for 15 minutes at 37°C. The clots were then removed from each tube with wooden applicator sticks and rinsed twice by gentle stirring in EBSS and 10 mM HEPES, pH 7.4. Radioactivity in each clot and in the unclotted solutions was determined.

Fibrinogenolysis in the Presence of Antiplasmins

Fibrinogen and α₂AP or αM were added simultaneously to cultures of C6 cells with bound plasmin. Fibrinogenolysis was then analyzed by SDS-PAGE or by 125I-fibrinogen incorporation into clots as described above. Identical experiments were performed with HUVEC cultures.

Fibrinolysis by Cell-Associated Plasmin

Plasmin was incubated with C6 cells for 2 hours at 4°C. After washing the monolayers, 150 μl of 125I-fibrinogen (2 mg/ml) was added immediately, followed by addition of thrombin (2 NIH units). A visible clot formed within 5 minutes at 22°C. EBSS and 10 mM HEPES, pH 7.4 (150 μl), was slowly added on top of each formed clot. Incubation was then continued for up to 2 hours at 22°C. At various times, additional buffer (200 μl) was added to each well, and the fibrin clots were carefully removed with wooden applicator sticks. The radioactivity present in the wells after removing the clots was determined.

Fibrinolysis in the Presence of α₂AP

Fibrinolysis experiments were performed in the presence of α₂AP (0.01-1.0 μM). The α₂AP was added either with the 125I-fibrinogen before forming the clot or in the 150 μl added gently above the clots 5 minutes after the thrombin was added.

Crosslinking of α₂AP Into Fibrin Clots

The fibrin clots that formed above C6 cell cultures were analyzed by SDS-PAGE under reducing conditions to study α- and γ-chain crosslinking. The fibrin clots were solubilized in 2.0 M urea, 1.0% SDS, and 3 mg/ml dithiothreitol for 12 hours at 22°C. SDS-PAGE was performed on 8.0% slabs. In some experiments, 125I-α₂AP was included with the fibrinogen before addition of thrombin. Covalent binding of α₂AP to the clot, which is a function of factor XIIIa activity, was judged by the appearance of a band with an apparent M₆ of 120,000 by autoradiography. To quantify the amount of 125I-α₂AP in this band, gels were sliced into 3-mm sections, and each section was counted in a gamma counter.

Results

Plasmin Binding to C6 Cells

The binding of DIP-plasmin to C6 cells has been studied previously. The Kₐ and Bₐₐ were 0.9 μM and 2.6×10⁶ sites per cell, respectively. In this investigation, incubation of C6 cells with 0.05 μM 125I-plasmin for 2 hours at 4°C yielded 1.0±0.2 pmol of specifically bound radioligand per well (7.5×10⁵ molecules per cell). After incubation with 0.1 μM 125I-plasmin, specific binding was 1.7±0.3 pmol/well (1.3×10⁶ molecules per cell). Specific binding represented greater than 75% of total binding, as determined with 10 mM EACA or a 50-fold molar excess of plasminogen.

Plasmin Dissociation From C6 Cells

The apparent dissociation of 125I-plasmin from C6 cell binding sites into buffered EBSS at 22°C was relatively slow (Figure 1). BSA at concentrations up to 10 mg/ml did not affect the rate of 125I-plasmin dissociation. Therefore, plasmin dissociation experiments were performed in a standard manner with EHB buffer, which contains BSA. When DIP-plasmin (0.3 μM) was included in the medium to prevent 125I-plasmin reassociation, the radioligand accumulated in solution somewhat more rapidly (30% dissociation in 15 minutes). A further increase in apparent plasmin dissociation was not observed when the concentration of DIP-plasmin was increased to 1.0 μM.

A small increase in apparent 125I-plasmin dissociation rate was observed when fibrinogen (0.16 mg/ml) was included in solution; 31% of the plasmin was recovered in solution after incubation for 15 minutes. Comparable data were obtained with 0.8 mg/ml fibrinogen (35% dissociation in 15 minutes; complete study not shown). The increase in apparent 125I-plasmin dissociation rate caused by fibrinogen probably reflects the ability of fibrinogen to bind dissociated plasmin and prevent reassociation. A slight increase in apparent 125I-plasmin dissociation was also observed with 1.08 mM S-2251 (compared with EHB buffer). When DIP-plasmin was present, S-2251 did not further increase the apparent rate of plasmin dissociation (data not shown).

While it is well documented that EACA and other lysine analogues inhibit specific binding of plasminogen to cell surfaces, the effect of EACA on plasmin dissociation rate has not been fully characterized. The apparent rate of 125I-plasmin dissociation was markedly increased by EACA (Figure 1); more than 50% of the plasmin was recovered in solution by 2.5 minutes. The dissociation curve in the presence of EACA was not monophasic; approximately 30% of the
FIGURE 1. Line plots showing dissociation of 

\[^{125}\mathrm{I}\text{-plasmin}\] from C6 cell receptors. Panel A: Cells were incubated with 0.1 \(\mu\)M 

\[^{125}\text{I}\text{-plasmin}\] for 2 hours at 4°C. After washing, medium supplemented with the following was added: fresh medium alone (○), 0.3 \(\mu\)M diisopropyl phosphoryl (DIP)–plasmin (○), 1.08 mM S-2251 (○), 0.16 mg/ml fibrinogen (△), 5.0 mM e-amino-n-caproic acid (EACA) (★), or 0.3 \(\mu\)M DIP-plasmin and 5.0 mM EACA (×). At various times, the media and the cells were separated and the radioactivity in each was determined. Plasmin bound to cells at each time was expressed as a percentage of that bound immediately after washing. Dissociation of nonspecifically bound plasmin was determined after incubating 

\[^{125}\text{I}\text{-plasmin}\] with cells in the presence of 5.0 mM EACA. Binding at each time was expressed as a percentage of total binding observed in the absence of EACA immediately after washing (—). Panel B shows the dissociation curves recalculated to represent only specifically bound plasmin (specifically bound plasmin at time=“x” divided by specifically bound plasmin at time=0) (mean±SEM, n=4).

plasmin remained bound and relatively stable after 5 minutes. DIP-plasmin did not further accelerate the rate of plasmin dissociation in the presence of EACA. C6 cell cultures with only nonspecifically bound 

\[^{125}\text{I}\text{-plasmin}\] were prepared by incubating plasmin (0.1 \(\mu\)M) with the cells in the presence of EACA. The nonspecifically bound plasmin dissociated slowly (Figure 1). Dissociation was not promoted by DIP-plasmin, S-2251, or 5.0 mM EACA. The curves in panel A of Figure 1 were corrected to represent only specifically bound plasmin (panel B). This correction demonstrated that greater than 90% of the specifically bound plasmin was dissociated within 15 minutes in EACA.

Plasmin Amidase Activity

To compare the amidase activities of specifically and nonspecifically bound plasmin, C6 cells were incubated with plasmin in the presence or absence of EACA. After washing the cultures to remove unbound plasmin, S-2251 hydrolysis was examined (Figure 2). Within the S-2251 concentration range studied (0.09–0.54 mM), amidase activity was a property of specifically bound plasmin only.

We demonstrated that 5.0 mM EACA does not affect the hydrolysis of S-2251 by plasmin in solution (see “Methods”). This allowed us to exploit the ability of EACA to rapidly dissociate specifically bound plasmin to compare the amidase activities of identical amounts of free and cell-associated plasmin. C6 cells with bound plasmin were incubated with S-2251 or with S-2251 and 5.0 mM EACA at 22°C (Figure 3). When plasmin dissociation was promoted with EACA, the amidase activity was slightly increased or unchanged compared with wells that did not receive EACA. These studies indicate that the amidase activities of free and C6 cell–associated plasmin are similar. It should be noted that when the S-2251 concentration was low, substrate consumption may have affected the apparent amidase activity after the first 3–5 minutes.

We have previously demonstrated that the amidase activity of C6 cell–associated plasmin is at least partially resistant to inhibition by \(\alpha_2\)AP.\(^{24}\) In the present investigation, we confirmed these results. C6 cells were incubated with 0.1 \(\mu\)M plasmin, washed, and then incubated with S-2251 and \(\alpha_2\)AP simultaneously for 30 minutes. Activities were compared with those observed when only S-2251 (no \(\alpha_2\)AP) was added. Table 1 shows that cell-associated plasmin retained significant activity in the presence of concentrations of \(\alpha_2\)AP that completely inactivated a comparable amount of plasmin in solution. When incubations with S-2251 and \(\alpha_2\)AP were conducted for less than 30 minutes, the calculated percentages of retained activity were higher (data not shown). This result was expected, since plasmin slowly but
progressively dissociates from cellular binding sites and is then rapidly inhibited by α2-AP. In addition, α2-AP may slow inhibit plasmin at the cell surface.

**Fibrinogenolysis by Cell-Associated Plasmin**

Plasmin (0.05 or 0.1 μM) was incubated with C6 cells. After washing the cultures to remove unbound plasmin, lysis of 125I-fibrinogen was studied. As determined by the SPli-Prest method, fibrinogen was digested, and the extent of fibrinogenolysis depended on the initial level of cell-associated plasmin and the time of incubation (Figure 4). Similar results were obtained when SDS-PAGE was used to analyze fibrinogen digestion. The lysis of fibrinogen in these experiments reflected the activity of cell-associated plasmin and/or plasmin that dissociated into solution.

C6 cells that had been incubated with 0.05 μM plasmin digested 24±3% of the 125I-fibrinogen within 30 minutes. When 125I-fibrinogen was incubated with a comparable amount of plasmin in solution (4.0 nM) for 30 minutes, 83±5% digestion was observed. Therefore, fibrinogenolysis was more rapid when the plasmin was in solution compared with plasmin that was initially cell associated.

To determine whether plasmin expresses fibrinogenolytic activity while bound to C6 cells and thus protected from rapid antiplasmins, 125I-fibrinogen digestion was studied in the presence of α2-AP. Fibrinogeno-

**Figure 2.** Line plots showing S-2251 hydrolysis by plasmin bound to C6 cells in a specific or nonspecific manner. Plasmin (0.1 μM) was incubated with C6 cells for 2 hours at 4°C without (panel A) or with (panel B) 5.0 mM e-amino-n-caproic acid. Monolayers were washed, and fresh medium containing S-2251 was added. The initial concentrations of S-2251 were 0.54 mM (○), 0.36 mM (●), 0.18 mM (△), and 0.09 mM (■). Absorbance at 405 nm was determined for up to 60 minutes (mean±SEM, n=4). pNA, p-nitroanilide.

**Figure 3.** Line plots showing the amidase activities of free and C6 cell-associated plasmin. Plasminogen was activated in the presence of C6 cells for 1 hour at 22°C. Monolayers were washed, and fresh medium containing S-2251 was added. Initial concentrations of S-2251 were 0.54 mM (○), 0.36 mM (●), 0.18 mM (△), and 0.09 mM (■). In panel B, 5.0 mM e-amino-n-caproic acid (EACA) was added simultaneously with the S-2251. In panel A, no EACA was added. Absorbance at 405 nm was determined for up to 30 minutes (mean±SEM, n=4). pNA, p-nitroanilide.
Table 1. Plasmin Amidase Activity in the Presence of α2-Antiplasmin

<table>
<thead>
<tr>
<th>Concentration (nM)</th>
<th>Cell-associated plasmin</th>
<th>Plasmin in solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100±8</td>
<td>100±4</td>
</tr>
<tr>
<td>10</td>
<td>35±1</td>
<td>3±2</td>
</tr>
<tr>
<td>100</td>
<td>22±2</td>
<td>2±1</td>
</tr>
</tbody>
</table>

Plasmin that was bound to C6 cells or a comparable amount of plasmin in solution was incubated with 0.54 mM S-2251 and the specified concentrations of α2-antiplasmin (α2AP) for 30 minutes. Absorbance at 405 nm was then determined. Activity in the presence of α2AP is expressed as a percentage of that observed in the absence of α2AP.

Lysis by cell-associated plasmin was completely inhibited by 0.1 μM α2AP (Table 2). Fibrinogen digestion was inhibited 86% by 10 nM α2AP; equivalent inhibition was observed when fibrinogen and 10 nM α2AP were incubated with plasmin in solution. These results were confirmed by SDS-PAGE.

α2M also inhibited fibrinogenolysis whether the plasmin was initially free in solution or cell associated (Table 2). The slight difference in the ability of 10 nM α2M to inhibit the plasmin that was initially bound to cells compared with free plasmin was not statistically significant. These experiments strongly suggest that the digestion of fibrinogen caused by C6 cell–associated plasmin is primarily a function of the plasmin that gradually dissociates from cellular binding sites. Plasmin that is bound to the cell membrane and is thus protected from α2AP or α2M does not digest significant levels of fibrinogen. When fibrinogen digestion experiments were performed with HUVEC cultures instead of C6 cells, equivalent results were obtained; fibrinogenolysis mediated by HUVEC-associated plasmin was completely inhibited by solution-phase proteinase inhibitors, indicating the requirement for plasmin dissociation (data not shown).

Fibrinolysis by Cell-Associated Plasmin

Fibrinogen above C6 cell monolayers clotted when thrombin was added, forming uniform translucent layers. From measurements of visible light diffraction, fibrin polymerization was complete within 5 minutes. A small gap (<0.05 mm) typically formed between the fibrin clot and the walls of the tissue-culture well as determined by examination with a ×15 dissecting microscope. Analysis of the fibrin clots by SDS-PAGE showed almost complete loss of the γ-chain monomer band and a prominent y-chain dimer band within 5 minutes. Some α-chain polymers were also evident (data not shown). Therefore, the fibrin in solution above C6 cells polymerized and was crosslinked by factor XIIa (an expected component of the fibrinogen preparation).

When C6 cells were not preincubated with plasmin, the fibrin clots were stable for up to 2 hours (after which monitoring was discontinued). Cells that were preincubated with 0.05 μM plasmin degraded 16.3±0.6% of the fibrin clot within 1 hour (determined by the radioactivity released from the clot); 39±6% of the clot was digested within 2 hours. Initially, the ability of α2AP to inhibit fibrin clot digestion by cell-associated plasmin was studied by adding α2AP after the fibrin clot had formed (Figure 5). When 0.01 μM α2AP was added, the amount of α2AP exceeded the total level of available plasmin by about a factor of three; however, significant inhibition of fibrinolysis was not observed. Fibrin clot lysis was partially inhibited by 0.1 or 1.0 μM α2AP; however, inhibition was still incomplete. The incomplete effectiveness of α2AP in this system is best explained by the idea that the fibrin clot acts as a physical barrier that separates plasmin from solution-phase α2AP. This barrier permits the partial digestion of the clot either by cell-associated plasmin or by plasmin that is transferred from the cell surface to the clot in an inhibitor-free microenvironment.

When 125I-α2AP (0.1 μM) was added to the cell culture wells with the fibrinogen before thrombin was added, 17.2% of the α2AP was covalently incorporated into the clot as determined by SDS-PAGE, autoradiography, and gel slicing. After incubation with 10 nM 125I-α2AP, 25.1% incorporation was demonstrated. The addition of α2AP before fibrin clot formation greatly increased the effectiveness of α2AP as an inhibitor of clot lysis. Only 7.2% of maximum lysis was achieved in the presence of 1.0 μM α2AP; 10.4% of maximum lysis was achieved with 0.1 μM α2AP. Based on a Student’s paired t test, these percentages were not statistically different from complete inhibition of fibrinolysis (no plasmin present) at the p=0.05 level.

Discussion

Cell-associated plasmin(ogen) and uPAR-associated high-molecular-weight u-PA interact to strongly promote plasminogen activation.13,17,20 Cell-associated plasmin effectively converts scu-PA into the more active two-chain form.13,17 Both forms of u-PA (scu-PA and two-chain u-PA) activate plasminogen with increased catalytic efficiency at the cell surface.50,31 In addition, some cell types promote the activation of plasminogen by tissue-type plasminogen activator.19,29 As a result of these reactions, substantial levels of cell-associated plasmin may be generated. Apart from scu-PA, physiologically significant substrates for cell-associated plasmin remain to be defined.

The studies presented here and elsewhere15 demonstrate that plasmin dissociates from cellular binding...
sites relatively slowly. Therefore, it is possible that plasmin, while bound to cell membranes, cleaves physio-
logically significant substrates other than scu-PA. In
this investigation, we have demonstrated that plasmin
that is bound to C6 cells retains substantial catalytic
efficiency against a small chromogenic substrate. Nev-
evertheless, the activity of C6 cell- or HUVEC-associated
plasmin toward fibrinogen in a “dissociation-con-
trolled” experimental system (α2AP or α2M present) is
essentially undetectable.

The choice of C6 cells as a model system for plasmin-
ogen receptors is based on extensive comparisons with
other cell types, including endothelial cells and hepato-
cytes. In previous studies of plasmin binding
affinity, capacity, dissociation rate constant, and
reactivity of bound plasmin with protease inhibitors,
C6 cells have behaved equivalently to these other cell
types. The greatly decreased activity of plasmin toward
fibrinogen when the former is bound to C6 cells or
HUVECs represents another equivalence that is dem-
onstrated here.

Every large solution-phase plasmin substrate studied
thus far reacts minimally with plasmin that is bound to
cell surfaces. The inhibition of plasmin by α2AP, which
mechanistically is initiated as an enzyme-substrate in-
teraction, occurs at a rate that is decreased dramatically
when the plasmin is cell associated. This decrease
might reflect occupancy of the plasmin kringle domains
and/or steric constraints imposed by the plasma membrane. The complete or
nearly complete inability of α2M to react with cell-
associated plasmin probably represents entirely steric
constraints. Fibrinogen digestion by cell-associated
plasmin is probably restricted by steric constraints
and/or occupancy of the kringle domains by cell surface
macromolecules. Therefore, when plasmin is generated
on the surfaces of cells that are exposed to blood, the
fibrinogen within the plasma will not be compromised so
long as α2AP or α2M is present.

The ability of EACA to promote plasmin dissociation
from cellular binding sites supports the hypothesis that
this interaction is mediated by multiple plasmin kringle
domains, as proposed by Miles et al. Each cell-
associated plasmin molecule may be viewed as having
multiple kringles reversibly engaging and disengaging

---

**Table 2. Inhibition of Fibrinogenolysis Mediated by Free and Cell-Associated Plasmin**

<table>
<thead>
<tr>
<th>Concentration (nM)</th>
<th>Cells</th>
<th>Solution</th>
<th>Cells</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100±11</td>
<td>100±5</td>
<td>100±11</td>
<td>100±5</td>
</tr>
<tr>
<td>10</td>
<td>14±5</td>
<td>14±5</td>
<td>69±14</td>
<td>58±4</td>
</tr>
<tr>
<td>500</td>
<td>2±1</td>
<td>1±1</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>125</td>
<td>α2-AP</td>
<td>α2-M</td>
<td>α2-M</td>
<td>α2-M</td>
</tr>
</tbody>
</table>

125I-Fibrinogen was added to cells with bound plasmin (cells) or to a comparable quantity of plasmin in solution
(solution). α2-Antiplasmin (α2AP) or α2-Macroj;oglobulin (α2M) was added simultaneously and incubated for 30 minutes
at 22°C. Fibrinogen digestion was then determined and is expressed as a percentage of maximum fibrinogenolysis.
Values are mean±SEM, n=4.
membrane binding sites. For plasmin dissociation to occur, all of the kringle domains must be disengaged simultaneously. We hypothesize that EACA promotes plasmin dissociation by binding to individual kringle domains as these domains disengage from membrane sites. This interaction then prevents the individual kringle from rebinding to the cell surface.

Under normal conditions, endothelial cells and other cells that are exposed to blood express a variety of macromolecules that inhibit thrombosis. For this reason, blood clot formation is usually limited to denuded blood vessel walls. Whether plasmin that is formed on intact cell surfaces adjacent to areas of thrombosis can digest and thereby restrict the extension of the clot is an important question that is partially addressed in the present study. The lysis of fibrin clots formed above cells in culture occurred only when α2AP was excluded during fibrin polymerization. These results suggest that α2AP, which is incorporated into fibrin, represents a major impediment to clot digestion whether the plasmin is derived from solution or the cell surface. Based on the ability of the fibrin clot to form a barrier between cell-associated plasmin and proteinase inhibitors in solution, we can speculate that fibrinolysis will be promoted from the cellular side once the fibrin-associated α2AP is overcome. The major limitation in determining the importance of cell surface–derived plasmin in the lysis of fibrin clots is the inability to predict how much cell-associated plasmin is generated under various conditions. If sufficient plasmin can be generated to overcome clot-associated α2AP, then lysis can occur from the cell surface, perhaps until communication with the vascular compartment is reestablished.

The exclusion of large soluble proteins from interaction with cell-associated plasmin is not complete, since plasmin-specific anticalytic antibodies bind receptor-bound plasmin. It is possible, however, that the primary substrates of cell-associated plasmin are not related to hemostasis. Other potential candidate substrates include cytokines, extracellular matrix proteins, cellular receptors, and the zymogen forms of other enzymes.

References

7. Wiman B, Liinen HR, Collen D: On the specific interaction between the lysine-binding sites in plasmin and complementary sites in α2-antiplasmin and in fibrinogen. Biochim Biophys Acta 1979;579:142–154
Fibrinogenolytic and fibrinolytic activity of cell-associated plasmin.
J E Humphries, J Vasudevan and S L Gonias

doi: 10.1161/01.ATV.13.1.48
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1993 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/13/1/48

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/