Interactions Between Fibrin and the Plasminogen Activators Produced by Cultured Endothelial Cells

By David J. Loskutoff and Luciana Mussoni

Serum-free conditioned medium (CM) from cultured bovine aortic endothelial cells (BAEs) was fractionated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and analyzed for plasminogen activator (PA) activity by fibrin autography. Distinct size forms of PA with molecular weights (mol wt) of 100,000, 74,000, and 52,000 were readily identified by this approach. When fibrinogen and thrombin were added to CM containing these forms, approximately 55% of the total activity was found to associate with the resultant fibrin clot. The other 45% remained free in the supernatant. This relationship did not change at higher fibrin concentrations. Subsequent analysis revealed that only the larger PA forms (mol wt 74,000–100,000) were recovered in the clot. The 52,000 mol wt form did not bind to the polymerizing fibrin under these conditions. The fibrin-binding forms also bound to immobilized concanavalin-A and could be separated from those forms that did not interact with fibrin by concanavalin-A affinity chromatography. The PA activity of the separated forms was then compared by assessing their ability to cleave 125I-plasminogen. Although cleavage by the 52,000 mol wt form was apparent, little if any cleavage was initiated by the mixture containing the 74,000–100,000 forms. The addition of fibrin to this sample resulted in the generation of a potent PA activity. These results indicate that cultured BAEs produce multiple forms of PA that differ both in size and in behavior toward fibrin and concanavalin-A. These forms include molecules that functionally and immunologically resemble human urokinase, and others that resemble human tissue-type PA.

The experiments summarized here complement those data by demonstrating that the cellular t-PAs require fibrin to express PA activity. No interaction between the cellular u-PA and fibrin was apparent.

MATERIALS AND METHODS

Cell Culture

BAEs were isolated from the aortae of freshly killed cows and cultured as indicated. BAECs support the growth of these studies had been passaged 5–20 times. All cultures were grown to confluency in 60-mm dishes before use. To prepare cellular extracts, cultures were washed with cold phosphate-buffered saline (PBS) (0.14 M NaCl, 0.01 M sodium phosphate, pH 7.2), extracted with 1 ml of Triton X-100 (0.5% v/v in PBS; Sigma Chemical Co., St. Louis, MO), and removed with a rubber policeman. Conditioned medium (CM) was prepared by incubating washed monolayers in serum-free minimal essential medium (MEM) for 24 hr. This CM was collected and then centrifuged at 1,000 g to remove floating cells and cellular debris. All cellular samples were stored in 0.5% Triton X-100 at –30°C until used.

Polyacrylamide Gel Electrophoresis (PAGE)

Sodium dodecyl sulfate (SDS) polyacrylamide slab gels and buffers were prepared by using resolving gels of 9% acrylamide and stacking gels of 4% acrylamide. Samples were applied to the gels and subjected to electrophoresis at 40 V at room temperature. Molecular weight standards included human transferrin (76,000), human serum albumin (67,000), ovalbumin (43,000), chymotrypsinogen (25,700), and soybean trypsin inhibitor (16,700). Portions of the gel containing these standards were removed and stained for 30 min with 0.1% Coomassie blue and 50% trichloroacetic acid, and then destained in 10% acetic acid. The remaining portions of the gel were either processed for fibrin autography to localize PA activity or subjected to autoradiography to localize radiolabeled plasminogen.

Fibrin Autography

To prepare fibrin agar indicator gels, a 2% solution of agarose (LPB agarose, Miles Laboratories, Elkhart, IN) was mixed with prewarmed (47°C) PBS containing plasminogen (19.5 µg/ml) and then centrifuged at 1,000 g to remove floating cells and cellular debris. All cellular samples were stored in 0.5% Triton X-100 at –30°C until used.
thrombin (0.6 U/ml). Fibrinogen (10 mg/ml) in PBS (37°C) was added, the solution was mixed rapidly and poured on a glass slide. After electrophoresis, the SDS gels were soaked in 2.5% Triton X-100 for 1.5 hr to remove the SDS, patted dry with paper towel, and placed on the surface of the fibrin-agar indicator gel. The indicator gel was allowed to develop at 37°C in a moist chamber and then photographed. The dark areas of the indicator gel correspond to lytic zones initiated by the interaction of PA from the SDS gel and plasminogen in the indicator gel. Indicator gels without plasminogen contained no lytic zones.

**Assay of Fibrinolytic Activity**

The fibrinolytic activity initiated by various PAs was determined by the 121I-fibrin plate assay as described.2 The standard assay mixture (1 ml) included 4 µg human plasminogen, 0.1% gelatin, 0.25% Triton X-100, 0.1 M Tris-HCl, pH 8.1, and a source of PA. No fibrinolysis was observed when plasminogen or PA was omitted from the reaction mixture, indicating that hydrolysis of the 121I-fibrin resulted from conversion of plasminogen into plasmin by PA.

**Plasminogen Cleavage Assay**

Plasminogen purified by lysine-affinity chromatography9 was iodinated with insolubilized lactoperoxidase29 to a specific activity of 1–5 x 107 cpm/µg of protein and was employed as a direct substrate for PA as described.31 The samples were incubated for 60 min at 37°C, and then the reaction was stopped by boiling the mixture in 3% SDS in the presence of 5% β-mercaptoethanol.17 PA activity was indicated by cleavage of single-chain plasminogen into the characteristic plasmin “heavy” and “light” chains,2 as revealed by SDS-PAGE and autoradiography. Autoradiograms were prepared by placing dried gels (Hoeffer Scientific Instruments, San Francisco, CA) on Kodak X-omat film for various times to localize the radiolabeled protein. The final reaction mixture contained 2 x 106 cpm/ml 121I-plasminogen, 1,000 U/ml Trasylo (Sigma), and 100 µg/ml albumin, all in PBS. Various PAs were added as indicated. The influence of fibrin on the PA-mediated cleavage of 121I-plasminogen was also assessed. Fibrinogen (30 µl of a stock solution at 1 mg/ml) was added to the standard 121I-plasminogen cleavage reaction in the presence of either the concanavalin-A binding or nonbinding PAs (see below). Thrombin (1 U/ml) was added, and the mixtures were incubated for an additional 60 min. The samples were then extracted with sample buffer and analyzed by SDS-PAGE and autoradiography.

**Concanavalin-A Affinity Chromatography**

CM (75 ml) was passed over a 10-ml concanavalin-A-Sepharose (Pharmacia, Piscataway, NJ) column previously equilibrated with 1 M NaCl in 0.01 M phosphate buffer, pH 7.2. The column was washed with this “equilibration buffer” and then eluted with the same buffer to which 0.5 M α-methyl-D-mannoside (Sigma) was added. Protein fractionation was monitored by changes in OD280. The PA activity of each fraction was assessed by the 121I-fibrin plate assay. Additionally, aliquots of the pooled run-through fractions and the α-methyl-D-mannoside wash were analyzed by SDS-PAGE and fibrin autography and by the 121I-plasminogen cleavage assay.

**Preparation and Analysis of Fibrin Clots**

The ability of various PAs to bind to fibrin was assessed. One unit (100 µl) of either purified human urokinase or purified t-PA23 in PBS containing 0.01% Tween 80, or of CM (dialyzed versus PBS-Tween 80), was added to a mixture containing fibrinogen, 5 mM EDTA, and 0.01% Tween 80 in PBS. The final volume was 1 ml. The samples were clotted by the addition of 1 U of thrombin, incubated for 15 min at 37°C, and then centrifuged (5,000 g for 10 min) to separate the resultant clot and supernatant. Control clots and supernatants from mixtures lacking PA were prepared and added to the experimental supernatants and vice versa so that all samples contained both clot and supernatant. The samples were solubilized by gentle rocking in 0.5% SDS for 2 hr at 37°C. An aliquot of each sample was then assayed for PA activity by the 121I-fibrin plate assay and for PA forms by SDS-PAGE and fibrin autography.

**Miscellaneous**

Protein was determined by the method of Bradford,24 with bovine serum albumin used as a standard. Measurements of 121I were performed in a Micromedica gamma spectrometer (Micromedica Systems, Inc., Horsham, PA). Plasminogen was prepared by affinity chromatography on lysine-Sepharose as described.18 Bovine fibrinogen, fraction I (CalBiochem, La Jolla, CA), was purified free of plasminogen by ethanol precipitation in the presence of lysine.35 Human α-thrombin was a generous gift from Dr. J. Fenton (Albany, NY). Purified human urokinase (WHO Standard) was supplied by Dr. A. Johnson, New York University, New York, NY. Human t-PA was purified from cultured melanoma cells27 and was a gift from Dr. D. Collen, University of Leuven, Belgium.

**RESULTS**

**Interactions Between Cellular PAs and Fibrin**

BAEs produce two immunologically distinct classes of PA14, those similar to human urokinase and those that resemble human t-PA. To develop both a functional and immunochemical distinction between these molecules, their interaction with fibrin was examined. Fibrinogen was mixed with CM from cultured BAEs and the mixtures clotted with thrombin. The clots were removed by centrifugation, extracted with SDS, and then tested for PA activity by the 121I-fibrin plate assay. The interaction of urokinase and t-PA with fibrin was also evaluated under similar conditions. As expected24 urokinase showed no association with the fibrin, while more than 98% of the recovered t-PA activity was found in this fraction (Table 1). When CM was analyzed, significant activity was found both in association with the clot and free in the supernatant. The amount of cellular PA activity recovered in the clot increased as the fibrinogen concentration was increased, reaching 50%-60% of the total at approximately 250 µg/ml (Fig. 1). Increasing the fibrinogen concentration above this value did not raise the relative amount of activity recovered in this fraction. Less than 3% of the urokinase activity was detected in the clot, even at the highest (2 mg/ml) fibrinogen concentration.

The SDS extracts of the clots and supernatants listed in Table 1 were fractionated by SDS-PAGE and analyzed by the fibrin autograph technique to evaluate the possibility that only specific molecular weight (mol wt) forms of BAE PA bound to the clot. The controls for this approach are shown in Fig. 2. In agreement
with the results of Table 1, none of the urokinase activity (mol wt 55,000 or 33,000) was found in the clot, all remaining free in the supernatant. In contrast, t-PA (mol wt 70,000) was detected only in the clot.

Again, cellular PA activity was detected in both fractions (Fig. 3). Two classes of cellular PA were in fact revealed by these experiments. The first, which bound to the fibrin (Fig. 3, lanes 2 and 3), included only the larger molecular weight forms (74,000–100,000). These forms were selectively removed from the supernatant as the fibrinogen concentration was increased (Fig. 3, lanes 2, 3, and 4). The second class, which did not appear to associate with the fibrin (Fig. 3, lane 4), included only the lower molecular weight forms (from 48,000 to 52,000).

Fractionation of Cellular PAs by Affinity Chromatography on Concanavalin-A-Sepharose

To determine whether any of the PA forms actually required fibrin as a cofactor for expression of their activity, we developed fibrin-independent methods for the separation and detection of the two classes of PA. Concanavalin-A affinity chromatography was used previously in the purification of t-PA from human uterine tissue26 and from human melanoma cells.23 In addition, we have observed that urokinase does not bind to concanavalin-A (unpublished observation), suggesting that affinity chromatography on concanavalin-A-Sepharose might also prove useful for the separation of the two classes of cellular PA. When CM was passed over a concanavalin-A-Sepharose column, the majority of the protein and PA activity was found to come through in the effluent (Fig. 4). However, peaks of both protein (about 10% of the input) and PA activity eluted with α-methyl-D-mannoside. Analysis of these fractions by SDS-PAGE and fibrin autography (Fig. 5) revealed that the run-through contained the low molecular weight forms (Fig. 5, lane 2) and essentially none of the higher molecular weight forms. These forms were subsequently recovered in the α-methyl-D-mannoside wash (Fig. 5, lane 3). In order to

Table 1. Binding of Various PAs to Fibrin

<table>
<thead>
<tr>
<th>PA Source*</th>
<th>PA Activity (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supematant</td>
</tr>
<tr>
<td>Human urokinase, purified</td>
<td>99.5</td>
</tr>
<tr>
<td>Human melanoma t-PA, purified</td>
<td>1.5</td>
</tr>
<tr>
<td>BAE conditioned medium</td>
<td>55</td>
</tr>
</tbody>
</table>

*One unit of PA activity from each PA source was added to the reaction mixture containing 1 mg/ml of fibrinogen (see Materials and Methods); the samples were clotted with thrombin and then incubated for 15 min at 37°C. The clot and supernatant were separated by centrifugation, dissolved in SDS, and then assayed for PA activity.

†Determined by the 125I-fibrin plate method and expressed as the percentage of the total activity recovered.

Fig. 1. Fractionation of cellular fibrinolytic activity by fibrin binding. CM or urokinase was mixed with increasing amounts of fibrinogen; the mixtures were clotted with thrombin, and the clots and supernatants recovered by centrifugation. The clots were washed with PBS, extracted with SDS, and then assayed for fibrinolytic activity. (• • •) CM, (□ □ □) urokinase. The fibrinolytic activity of the supernatants from the CM (○) or urokinase (□) samples was also determined at the highest fibrinogen concentration.

Fig. 2. Interactions among urokinase, melanoma t-PA, and fibrin. Purified human urokinase (lanes 1–3) or melanoma t-PA (lanes 4–6) were mixed with 1 mg/ml fibrinogen; the samples were clotted with thrombin, and the resultant clots and supernatants recovered by centrifugation. The samples were extracted with SDS and subjected to SDS-PAGE. The presence of PA activity in the clots (lanes 2, 5) and supernatants (lanes 3, 6) was detected by fibrin autography and compared to that in the unfraccionated controls (lanes 1, 4). The indicator gels were allowed to develop for 3 hr and then photographed.
reveal the presence of low levels of cross-contamination in these fractions, the fibrin films were allowed to develop for 1 hr longer than those shown in Figs. 2 and 3. This prolonged development time resulted in the formation of a large lysis zone, which extended from mol wt 74,000 to 100,000 (lanes 1 and 3). In spite of this prolonged assay, no cross-contamination of the forms could be demonstrated.

Cleavage of $^{125}$I-Plasminogen by Cellular PAs in the Presence or Absence of Fibrin

The ability of each of the two concanavalin-A-separated PA classes to cleave plasminogen, their natural substrate, was assessed (Fig. 6). Cleavage was monitored as described, employing $^{125}$I-plasminogen to increase the sensitivity of the assay. The iodinated product consisted of a single polypeptide of mol wt 89,000, even after incubation for 1 hr at 37°C (Fig. 6, lane 1). This plasminogen was cleaved into its characteristic plasmin heavy (mol wt 65,000) and light (mol wt 25,000) chains when incubated with the fraction that did not bind to concanavalin-A (Fig. 6, lane 2). Little or no cleavage was observed when the concanavalin-A-binding material was similarly tested (Fig. 6, lane 4). However, the addition of fibrin to this latter sample generated PA activity (Fig. 6, lane 5). The addition of fibrin to samples containing the concanavalin-A-nonbinding fraction did not stimulate its inherent PA activity (Fig. 6, compare lane 2 and lane 3). No cleavage was observed when fibrin was incubated with plasminogen in the absence of the cellular source of PA (unpublished observation).

DISCUSSION

The fibrinolytic potential of cells can apparently be accounted for entirely by the presence of PAs that are either sensitive or resistant to antibodies against urokinase. Moreover, some of these cells may actually contain both activities. We recently demonstrated that cloned BAEs not only produced both classes of activator, but also that the anti-urokinase-resistant material was in fact immunologically similar to human mela-
The primary functional distinction between the two PA classes resides in the observation that while t-PAs seem to require a cofactor for their activity, no such requirement can be demonstrated for u-PAs. The most studied cofactor is fibrin itself, although recent observations suggest that other molecules may have cofactor activity as well. Approximately 55% of the total PA activity in BAE CM was found to bind to polymerizing fibrin, the remainder showing no such association (Fig. 1). For some reason, the relative amount of fibrin-binding activity in CM varied over a rather large range (10%–70% of the total recovered activity) when different cultures were compared (unpublished observation). These relationships did not change, even in the presence of high concentrations of fibrin (Fig. 1), suggesting both that fibrin was not limiting in this system and that the observed fractionation of activity reflected fractionation of specific PA forms. Analysis of the clots and supernatants by SDS-PAGE and fibrin autography substantiated these conclusions. Only the higher molecular weight forms (74,000–100,000) bound to the clot (Fig. 3) and thus resembled human t-PA in this respect (Fig. 2). Some heterogeneity in the fibrin-binding properties of this material is suggested, however, since under conditions in which essentially all of the mol wt 74,000 form was recovered in the clot, considerable mol wt 100,000 PA was still detected in the supernatant (Fig. 3, lanes 3 and 4). The lower mol wt form (52,000) resembled urokinase and remained free in the supernatant, even at the highest fibrin concentration.

The fibrin-binding, t-PA-like forms were separated from the u-PA-like forms by concanavalin-A affinity chromatography (Figs. 4 and 5), and the effect of fibrin on their respective activities was determined (Fig. 6). Again, the mol wt 52,000 form resembled urokinase in that it cleaved plasminogen equally well in the presence (Fig. 6, lane 3) or absence (Fig. 6, lane 2) of fibrin. No cleavage of plasminogen was observed in the sample containing the high molecular weight fibrin-binding forms unless fibrin was also present (Fig. 6, lanes 4 and 5). The fibrin dependency of this activity again supports the immunochemical data that the latter forms are t-PAs only, not u-PAs. The addition of fibrinogen, fibrin, or thrombin to the 125I-plasminogen in the absence of a PA source did not initiate cleavage of plasminogen (unpublished observation).

Taken collectively, the immunochemical, biochemical, and functional data provided here argue rather persuasively that cultured bovine endothelial cells produce both types of PA. Cultured human umbilical vein endothelial cells may also produce both classes of PA. It is intriguing that cells of the vascular wall should be associated with both fibrin-dependent and fibrin-independent PA activities. These observations may imply that expression of both activities is critical for the role of the endothelium in maintaining vessel patency. On the other hand, recent observations indicate that u-PAs are relatively ineffective thrombolytic agents compared to t-PAs, suggesting that they are important for processes other than fibrinolysis. Rijken et al. detected only t-PA activity in sections of the vascular wall. This finding differs from the results presented here and may reflect species or tissue specificity or changes that occur in cultured cells. Nevertheless, the endothelium clearly has the potential to produce both classes of activator. The regulation of each, and the definition of their relative importance for in vivo fibrinolysis, warrant further research.
ENDOTHELIAL CELL PLASMINOGEN ACTIVATORS

Fig. 6. Autoradiogram showing cleavage of $^{125}$I-plasminogen by various PA forms. $^{125}$I-plasminogen (lane 1) was incubated with 30 µl of the concanavalin-A run-through (lanes 2, 3) or eluant (lanes 4, 5), fractionated by SDS-PAGE, and analyzed by autoradiography. The effect of fibrin on cleavage by these samples was assessed (lanes 3, 5).

ACKNOWLEDGMENT

The authors wish to thank Meredith Alexander and Elizabeth Simpson for preparing the manuscript and Karen Roegner for excellent technical assistance. We would also like to acknowledge Dr. Desire Collen not only for providing tissue activator and its antiserum, but also for revealing many of his observations to us prior to their publication.

REFERENCES

Interactions between fibrin and the plasminogen activators produced by cultured endothelial cells

DJ Loskutoff and L Mussoni