Cultured Bovine Endothelial Cells Produce Both Urokinase and Tissue-type Plasminogen Activators

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ABSTRACT  Cell extracts and conditioned media (CM) from cultured bovine aortic endothelial cells (BAEs) were fractionated by PAGE in the presence SDS, and plasminogen activator (PA) activity was localized by fibrin autography. Multiple molecular weight forms of PA were detected in both preparations. Cell-associated PAs had Mr of 48,000, 74,000, and 100,000 while secreted PAs showed Mr of 52,000, 74,000, and 100,000. A broad zone of activity (Mr 80,000–100,000) also was present in both cellular fractions. In addition, PAs of Mr 41,000 and 30,000 appeared upon prolonged incubation or repeated freezing and thawing of the samples, and probably represent degradation products of higher molecular weight forms. This complex lysis pattern was not observed when CM was subjected to isoelectric focusing. Instead, only two classes of activator were resolved, one at pH 8.5, the other at 7.6. Analysis of focused samples by SDS PAGE revealed that the activity at pH 8.5 resulted exclusively from the Mr 52,000 form; all other forms were recovered at pH 7.6. The activity of the Mr 52,000 form was neutralized by anti-urokinase IgG but was not affected by antitissue activator IgG indicating that it is a urokinaselike PA. The activities of the Mr 74,000-100,000 forms were not affected by anti-urokinase. They were blocked by antitissue activator suggesting that all the forms in this group were tissue-type PAs. The multiple forms of PA were differentially sensitive to inactivation by diisopropylfluorophosphate (DFP). Treatment of CM with 10 mM DFP for 2 h at 37°C only partially inhibited the 52,000-dalton form. However, it completely inactivated the 74,000-dalton PA. The activity of the Mr 100,000 form was not affected by this treatment, or by treatment with 40 mM DFP. Thus, cultured BAEs produce multiple, immunologically distinct forms of PA which differ in size, charge, and sensitivity to DFP. These forms include both urokinaselike and tissue-activator-like PAs. The possibility that one of these forms is a zymogen is discussed.
Preparation and Treatment of Cell Extracts and Conditioned Medium (CM)

To prepare cellular extracts, cultures were washed twice with cold phosphate buffered saline (PBS) (0.14 M NaCl, 0.01 M sodium phosphate, pH 7.2), extracted with 250 μl of Triton X-100 (0.5% vol/vol in PBS, Sigma Chemical Co., St. Louis, MO) and removed with a rubber policeman. The culture dish was washed once with 750 μl PBS, and the extract and wash were pooled. All samples were used immediately after preparation. Conditioned media was prepared by incubating washed monolayers in serum-free MEM for 24 h. This CM was collected, centrifuged at 1,000 g to remove cellular debris and stored in 0.5% Triton X-100 at −50°C until used.

PAGE

SDS polyacrylamide slab gels and buffers were prepared as described by Laemmli (16) with 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 (65,000), 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 1% Coomassie Blue and 50% trichloroacetic acid, and then destained in 10% acetic acid. Radioactivity measurements of 125I were performed in a Micromedic gamma spectrometer (Micromedic Systems, Horsham, PA). Plasminogen was detected by the development of lytic zones on fibrin-agar indicator gel mixture. A 9% SDS gel containing various PAs was placed on the antibody containing indicator gel and incubated at 37°C. Control fibrin agar plates containing normal rabbit IgG at the same concentrations were also tested.

Isoelectric Focusing and Two Dimensional Gel Electrophoresis

Isoelectric focusing of CM was performed in 5% polyacrylamide slab gels containing 6M urea (ultra pure, Schwarz-Mann, Orangeburg, NY). 0.5% Triton X-100, 10% glycerol, and 5% amphoteries (pH 3.5-10, 8-9.5, 9-11 in a ratio of 5:2:1, LKB Instruments, Inc., Rockville, MD). Conditioned media was dialyzed overnight in 0.5% Triton X-100, 6M urea to remove ions before application. The gels were electrofocused on an LKB multiphor system at 15 W for 3 h using electrode solutions of 1 M NaOH and 1 M H3PO4. After electrofocusing was completed, one lane was removed from the slab gel, sliced into 0.5-cm slices, and each slice was then soaked in 0.5 ml of 0.5% Triton X-100 for 30 min and the pH determined. The slices were allowed to continue soaking overnight and an aliquot assayed for PA activity on 125I-fibrin plates. Focused samples were also analyzed by SDS PAGE (2-dimensional gel electrophoresis). A duplicate lane was removed from the focusing gel and soaked for 60 min. in 0.05 M Tris-HCl, pH 8.4 containing 10% glycerol, 0.025% bromphenol blue, and 1% SDS. It was then placed on top of an SDS gel and subjected to fractionation by SDS PAGE as described above. PA activity was localized by fibrin autography.

Assay of Fibrinolytic Activity

PA activity was assayed on 125I-fibrin coated multwell tissue culture dishes (24 wells, 16 mm; Costar Data Packaging, Cambridge, MA) as described (15). The standard cell-free assay contained in 1 ml: 4 μg human plasminogen, 0.1% gelatin, 0.1 M Tris-HCl, pH 8.1, and a source of PA. Fibrinolysis was not observed when plasminogen or PA was omitted from the reaction mixture, indicating that hydrolysis of the 125I-fibrin resulted from conversion of plasminogen into plasmin by PA.

Immunochemistry

Rabbit antiserum raised against human urokinase was a gift from Collaborative Research, Waltham, MA. To prepare IgG fractions, antiserum or nonimmune serum was combined with an equal volume of 0.15 M sodium chloride, and ammonium sulfate was added slowly to 45% saturation. This solution was stirred for 15 min and centrifuged at 20,000 g for 20 min. The pellet was resuspended in 0.15 M sodium chloride and the ammonium sulfate fractionation repeated two more times. The final precipitate was redissolved and dialyzed extensively against 0.07 M potassium phosphate, pH 7.2, and then chromatographed on a DEAE-cellulose column equilibrated in the same buffer. The unbound protein was collected, dialyzed against PBS and concentrated to 2 mg/ml of IgG. The rabbit antihuman tissue activator IgG (2 mg/ml) was isolated by adsorption onto Protein A-Sepharose columns as described (4) and was a generous gift from Dr. D. Collen, University of Leuven, Leuven, Belgium.

Miscellaneous

Protein was determined by the method of Bradford (18) using bovine serum albumin as a standard. Radioactivity measurements of 125I were performed in a Micromedic gamma spectrometer (Micromedic Systems, Horsham, PA). Plasminogen was prepared by affinity chromatography on lysine-Sepharose as described (15). Bovine fibrinogen fraction 1 (CalBiochem-Behring Corp., San Diego, CA) was purified free of plasminogen by ethanol precipitation in the presence of lysine (20). Human α-thrombin was a generous gift from Dr. Fenton (Albany, NY). Purified human urokinase (W.H.O. Standard) was supplied by Dr. A. Johnson, New York University. Human tissue plasminogen activator was purified from cultured melanoma cells and was a gift from Dr. D. Collen (21).
100,000 but its detection required a longer incubation time (not shown). Characteristically, lysis was also observed in the area between the 74,000- and 100,000-dalton PAs but it appeared as an unresolved smear rather than as distinct bands (lanes 4 and 5). In a few preparations, additional forms of PA with molecular weights of 51,000 (extract) and 55,000 (CM) were also detected, although conditions under which they were generated have not been established. No activity was observed in the absence of plasminogen. These complex lysis patterns were not altered when samples were prepared in the presence of soybean trypsin inhibitor (50 µg/ml) and analyzed in the presence of 8 M urea (not shown). However, prolonged incubation of cell extract or CM at 37°C, or repeated freezing and thawing of the samples generated PAs of Mr, 41,000 and 30,000. These forms are probably degradation products of higher molecular weight forms. The molecular weights of the various cell-associated and secreted PAs are summarized in Table I. Similar results were obtained when CM and extract from cloned BAEs was analyzed by these techniques (unpublished observation).

**Fractionation of Cellular PAs by Isoelectric Focusing**

The isoelectric points (pI) of the various forms of PA in CM were determined (Fig. 2). Dialyzed CM was electrophoresed as indicated. The gel was sliced into 5-mm sections and each slice was then tested for fibrinolytic activity on 125I-fibrin coated dishes. Two areas of activity were observed. The most active material accumulated between pH 8.4-8.6 with the peak at pH 8.5 (Fig. 2A). The less active material corresponded to a pI value of 7.4-7.8, with the peak of activity at pH 7.6. To relate these isoelectric forms to the molecular weight forms shown in Fig. 1, focused samples were subjected to additional fractionation by SDS PAGE (Fig. 2B). The PA form of pI 8.5 consisted of a single molecular species of Mr, 52,000. Unexpectedly, the smaller peak of activity (pI 7.5) contained all the higher molecular weight forms (Mr, 74,000-100,000). The pI of the various molecular weight forms of activator are summarized in Table I.

**Immunological Characterization of Cellular PAs**

Urokinase and tissue activator were fractionated by SDS PAGE and then analyzed by fibrin autography. The fibrin indicator gels contained either anti-urokinase IgG or antitissue activator IgG. Nonimmune IgG was added to parallel fibrin indicator gels as controls. The presence of antitissue activator IgG blocked the development of tissue activator initiated lytic zones (Fig. 3) but did not affect the activity of purified urokinase. Conversely, the presence of antiurokinase IgG neutralized urokinase activity but was not effective against tissue activator. The activities of the multiple cellular PA forms also showed differential sensitivity to these IgG fractions (Fig. 4). Anti-urokinase completely neutralized the activity of the Mr, 52,000 form in CM (lane 2) but had no effect on the activity of the other forms (Mr, 74,000–100,000). In contrast, the presence of antitissue activator IgG (lane 3) blocked the activities of the high molecular weight forms but had little detectable effect on the activity of the lower molecular weight forms. These results were confirmed with CM prepared from cloned BAEs (unpublished observation). Fibrin-agarose plates containing nonimmune IgG did not affect the activity of any of the PA forms.

**Sensitivity of Cellular PAs to Inactivation by DFP**

The relative sensitivity of the various PA forms to inactivation by DFP was assessed. CM was treated with DFP for...
molecule and was not simply due to hydrolysis and inactivation with 1 mM DFP for 2 h (the sample shown in lane A). The inactivated by this treatment (lane A). The activity was completely analyzed for urokinase activity. The urokinase was completely secreted forms (not shown).

The 74,000 form was almost completely inactivated by 10 mM DFP (lane A). The activity of this form was observed after 2 h of exposure even though cellular forms behaved as a single class of biochemically similar molecules. This resistance suggests that the larger forms may be tissue-activator-like PAs, direct immunochemical and functional evidence for this conclusion is lacking. The relationship of the various forms to each other is not at all clear from these studies. Our studies with cultured endothelial cells resolve many of these uncertainties. Various times, fractionated by SDS PAGE, and residual PA activity was then localized by fibrin autography (Fig. 5). Although the 74,000 form was almost completely inactivated by treatment with 1 mM DFP (lanes A2 and B2), the 52,000-dalton form remained relatively active even after incubation with 10 mM DFP (lanes A3 and B3). The 100,000-dalton form was apparently refractory to DFP treatment since no change in the activity of this form was observed after 2 h of exposure to 0 mM DFP (lane B3). Treatment of CM with 40 mM DFP did not decrease the activity of this molecule (not shown). To insure that residual PA activity represented a DFP-insensitive molecule and was not simply due to hydrolysis and inactivation of the DFP during the incubation period, 0.5 U of urokinase was added to the CM sample that previously had been treated with 1 mM DFP for 2 h (the sample shown in lane A2). The mixture was incubated for an additional 30 min and then analyzed for urokinase activity. The urokinase was completely inactivated by this treatment (lane C2) even though cellular PA activity was still detected at Mr 52,000. The DFP sensitivity of cell associated PAs was identical to their corresponding secreted forms (not shown).

**DISCUSSION**

A variety of normal and transformed cells in culture have been shown to produce multiple, molecular weight forms of PA (22-25). These PAs were found to range in size from Mr 36,000 to Mr 100,000 and larger. The lower molecular weight PAs from several of these cell lines (Mr ~50,000) were neutralized by antitissue activator-like PAs, but the activity of the larger molecular weight forms was apparently resistant to such treatment. Although this resistance suggests that the larger forms may be tissue-activator-like PAs, direct immunochemical and functional evidence for this conclusion is lacking. The relationship of the various forms to each other is not at all clear from these studies. Our studies with cultured endothelial cells resolve many of these uncertainties.

Analysis of cell extracts and conditioned media from cultured BAEs by SDS PAGE revealed the presence of at least three distinct molecules with PA activity, and a number of others not clearly resolved by this approach (Fig. 1). That these activities result from the presence of only two classes of activator can be argued from the following observations. First, the complex molecular weight pattern observed by SDS PAGE reduced to a rather simple, two component pattern upon isoelectric focusing (Fig. 2A). Symmetrical peaks of PA activity were detected at pH 8.5 and at pH 7.6. Subsequent fractionation of the focused samples by SDS PAGE (Fig. 2B) revealed that the activity recovered at pH 8.5 contained only one molecular species of activator, that of Mr 52,000. This form was not detected at the lower isoelectric point. The size and charge properties of the Mr 52,000 form are similar to those reported for urokinase (26). All of the higher molecular weight forms (Mr 74,000-100,000) migrated to one position in the gel, at pH 7.5. The higher molecular weight, lower pl of these other forms is consistent with the properties of tissue-activator-like PAs (21, 27). Second, and in agreement with these conclusions, are the antibody neutralization experiments. Only the activity of the Mr 52,000 form was blocked by antitissue activator (Fig. 3); it was not affected by antitissue activator (Fig. 4). The activity of all the high molecular weight forms (Mr 74,000-100,000) was neutralized by antitissue activator (Fig. 4) but was not affected by antiurokinase (Fig. 3). Third, the high molecular weight forms behaved as a single class of biochemically similar mole-
cules with respect to their interaction with fibrin (Loskutoff and Mussoni, unpublished observations). All of these forms not only bound to fibrin, but also appeared to require fibrin for their activity. This fibrin cofactor requirement is the primary functional distinction between tissue-type and urinary PAs (6). The M, 52,000 form resembled urokinase in that it showed no association with fibrin.

Thus, it is clear from the above considerations that the M, 52,000 is a urokinaslike PA, while the M, 74,000--100,000 forms behave in all respects like tissue-type PAs. Whether these two classes of PA are actually products of two separate genes (22) remains to be determined. Similarly, the exact relationship among the multiple forms of tissue-type PA (M, 74,000--100,000) remains unclear. Although it is possible that they are products of multiple genes, the fact that they demonstrate similar pls, are sensitive to the same antisera, and interact similarly with fibrin, suggests a common origin from a single gene. The DFP-inactivation studies (Fig. 5) would seem to suggest a precursor-product relationship. That is, the relative insensitivity to DFP of the M, 100,000 form is consistent with the behavior of a proenzyme. Like other zymogen activation reactions (28), activation in this case would be expected to be a proteolytic process resulting in the formation of a lower molecular weight, DFP-sensitive molecule. The M, 74,000 form has these properties. The relative insensitivity of the unresolved activity between M, 80,000--100,000 suggests that this material may represent inactive degradation products of the larger form. The ability to detect PA activity at all in DFP-resistant material seems at first glance, to be inconsistent with this model. However, this activity may arise after treatment with DFP, perhaps during SDS PAGE, and may only reflect a small percentage of the potential activity of the molecule. Conclusive support for these speculations awaits purification of the relevant molecules and specific activity determinations.

It is intriguing that cells of the vascular wall should be associated with both tissue-type (fibrin-dependent) and urokinaslike (fibrin-independent) PA activity. It may be 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 urokinase is a relatively ineffective thrombolytic agent when compared to tissue-type PAs (8), suggesting that it may be important for processes other than fibrinolysis (29). Rijken et al (4) detected only tissue-type 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. It is also possible that the presence of both types of activator reflects a heterogeneity of cells in our cultures. If so, it is unlikely that such heterogeneity is due to the presence of a subpopulation of nonendothelial vascular cells (i.e., smooth muscle cells or fibroblasts) since the morphology of cultures maintained at confluenve for 2 wk and fed every 3 d, did not change from the “cobblestone” appearance of the original cultures. If the other cells were present, the cultures would quickly convert to a multilayered population of elongated cells under these conditions (30). Laug (14) recently observed multiple molecular weight forms of PA in a cloned isolate of BAEs. We also have confirmed our results with six separate clonal isolates, indicating directly that BAEs produce both types of PA. Because BAEs may be phenotypically unstable (31), it is possible that the cultures consisted of two subpopulations of endothelial cells with one producing only the urokinaslike molecule and the other the tissue-type PAs. Satisfactory resolution of this possibility will depend upon development of techniques to detect both activities in single cells. In any case, it is clear that endothelium has the potential to produce both classes of activator. How expression of each is regulated, and the relative importance of each in vivo fibrinolysis will await further studies.

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