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Urokinase-Type and Tissue-Type Plasminogen Activators Have Different Distributions in Cultured Bovine Capillary Endothelial Cells

David Moscatelli

Kaplan Cancer Center and Department of Cell Biology, New York University Medical Center, New York, New York 10016

The cell extracts and conditioned medium from cultured bovine capillary endothelial (BCE) cells were examined to determine the types of plasminogen activator (PA) present in each of these two fractions. The fractions were first analyzed by fibrin autography after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The cell extracts contained two species of PA of M_r 48,000 and 28,000. Multiple forms of PA were detected in the conditioned medium: variable amounts of the M_r 48,000 and 28,000 forms and a broad band of activity with M_r in the range of 67,000–93,000. The major fraction of the M_r 48,000 form was in the cell extract. Treatment of the cells with 12-0-tetradecanoyl phorbol-13-acetate or with a preparation containing angiogenic activity resulted in a proportionate increase in the levels of all forms. The M_r 48,000 form was demonstrated to be a urokinase-like PA, since it was immunoprecipitated with antibodies to urokinase. When conditioned medium or cell extracts from biosynthetically labelled BCE cells were incubated with antiserum to urokinase, the M_r 48,000 form was immunoprecipitated only from the cell extract. The M_r 67,000–93,000 forms were demonstrated to be tissue-type PAs, since they were immunoprecipitated with antibodies to tissue PA. When the same conditioned medium or cell extracts were incubated with antiserum to tissue-type PA, the M_r 67,000–93,000 forms were immunoprecipitated only from the conditioned medium. Therefore, BCE cells are able to produce both tissue-type PA, which is primarily secreted, and urokinase-type PA, which remains primarily cell associated.

Key words: tissue-type plasminogen activator, urokinase, capillary endothelial cells

Two immunologically and biochemically distinct forms of plasminogen activator (PA) have been identified, tissue-type PA (t-PA) and urokinase-type PA (u-PA). Tissue-type PA has been identified in the blood [1] and is thought to have a major role in thrombolysis because it has a high affinity for fibrin [2] and because its activity

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is strongly stimulated by interaction with fibrin [3,4]. Urokinase-type PA has also been identified in blood [5] as well as being associated with a variety of normal cell types [6–8]. Endothelial cells have been thought to be an important source of the t-PA present in the blood, since t-PA has been isolated from a perfusate of the vascular tree of cadavers [9], and t-PA activity has been identified in the wall of human vein [1]. Tissue-type PA has been shown to be produced by endothelial cells cultured from human umbilical vein [10] and bovine aorta [11]. Endothelial cells cultured from bovine aorta also produce u-PA [11]. However, the role of endothelial cells of the microvessels as a source of circulating PA is unknown, since perfusion of the vascular tree most likely recovers PA only from larger vessels, and production of PA by cultured endothelial cells has been investigated only in endothelial cells cultured from large vessels. Since the microvasculature accounts for a majority of the endothelial cells in the body, capillary endothelial cells are potentially a more important source of blood PA than large vessel endothelial cells. We have previously shown that cultured bovine capillary endothelial (BCE) cells produce PA at levels comparable to bovine aortic endothelial cells and that the levels of PA produced could be stimulated by the phorbol ester 12-0-tetradecanoyl phorbol-13-acetate (TPA) [12] as well as by preparations that stimulate angiogenesis [13]. In this report, we characterize the types of PA made by BCE cells under both unstimulated and stimulated conditions and their distribution in cell-associated and secreted fractions.

MATERIALS AND METHODS

Cells

Capillary endothelial cells were isolated by the procedure of Folkman et al [14] from the cortex of adrenal glands obtained from recently slaughtered yearling cattle. The endothelial cells were grown to confluence in medium conditioned by sarcoma 180 cells, as described previously [12]. When the cells reached confluence, the medium was changed to fresh α minimal essential medium containing 5% donor calf serum (Flow Laboratories, McLean, VA) with no conditioning factors. Two days later, the medium was changed again to fresh α minimal essential medium containing 5% donor calf serum. The cells were maintained for 2 more days in this medium before the start of the experiment. All experiments were performed with early passage cells.

Plasminogen Activator Assays

Plasminogen activator was assayed by the fibrin-plate procedure as described previously [12]. Purified human urokinase (Leo Pharmaceuticals, Copenhagen, Denmark) was used as a standard. Results were expressed in Ploug units.

Polyacrylamide Gel Electrophoresis (PAGE)

SDS polyacrylamide slab gels and buffers were prepared as described by Laemmli [15], with resolving gels of 8% acrylamide and stacking gels of 3% acrylamide. Samples were applied to the gels and subjected to electrophoresis at 25 V at room temperature.

Fibrin Autography

Fibrin-agar indicator gels were prepared by a modification of the method of Granelli-Piperno and Reich [16]. A 2.5% solution of agar (Difco Laboratories,

Detroit) was boiled, cooled to 45°C, and mixed with warmed phosphate buffered saline (PBS) containing plasminogen (125 μg/ml) and thrombin (0.3 U/ml) in a 45°C water bath. Fibrinogen (10 mg/ml in PBS) was added, the solution was mixed rapidly and poured into a slab gel mold, and the agar solution was allowed to solidify at room temperature. Final concentrations were 1.3% agar, 32.3 μg/ml plasminogen, 0.077 U/ml thrombin, and 2.25 mg/ml fibrinogen.

Samples containing plasminogen activator activity were run on an SDS-8% polyacrylamide gel under nonreducing conditions. The gel was soaked in 2.5% Triton X-100 for 1 hr to remove the SDS. Then the polyacrylamide gel was laid on top of a freshly made fibrin-agar gel and incubated at 37°C in a humidified atmosphere. Periodically, the fibrin-agar gel was examined for lysis zones. When lysis zones were observed, the gel was fixed and stained with 0.1% amido black in 70% methanol and 10% acetic acid. Upon destaining, fibrin-containing areas were stained blue, and lysis zones were clear.

Immunoprecipitation

For biosynthetic labelling of plasminogen activators, confluent cultures were preincubated for 2 hr in serum-free Eagle's minimal essential medium with 1/10 the standard concentrations of amino acids. Then the cultures were incubated for 5 hr in fresh serum-free Eagle's medium with 1/10 amino acids containing 25 μCi/ml (250 mCi/mmole) [^{35}S]cystine (New England Nuclear, Boston, MA). At the end of this period, the conditioned medium was collected, and the cells were washed twice with cold PBS, scraped from the plate with a rubber policeman, and extracted with 0.5% Triton X-100 in 0.1 M sodium phosphate (pH 8.1).

Before immunoprecipitation, samples of both the conditioned medium and the cell extract were preabsorbed with normal rabbit serum to reduce nonspecific binding. This was achieved by incubating 15 μl of normal rabbit serum with the samples in 1 ml of radioimmunoprecipitation assay (RIPA) buffer (0.15 M NaCl, 50 mM Tris (pH 7.4), 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM ethylenediamine tetraacetic acid [EDTA], 2 mM phenylmethylsulfonylfluoride) for 2 hr at room temperature. Then 20 μl packed protein A-Sepharose beads (Sigma, St. Louis, MO) were added, the samples were agitated at 4°C for 45 min, and the protein A-Sepharose beads were collected by centrifugation in an Eppendorf centrifuge for 30 sec. The supernatants were used for the specific immunoprecipitation, which was performed by a modification of the procedure of Stanley et al [17]. Fifteen microliters of rabbit antiserum to human t-PA were added to the supernatants, and the samples were incubated at 4°C overnight. Then 20 μl of protein A-Sepharose were added, the samples were agitated at 4°C for 45 min, and the beads were collected by centrifugation. The supernatants were used for a second immunoprecipitation with rabbit antiserum to human urokinase. The pellets from both immunoprecipitations were washed three times with 1 ml of RIPA buffer, three times with 0.5 ml of RIPA buffer and two times with 1 ml of water. The washed beads were resuspended in a buffer containing 5% (v/v) 2-mercaptoethanol, heated in a boiling water bath for 3 minutes, and loaded on an SDS-8% polyacrylamide gel. The polyacrylamide gels were processed for fluorography by the method of Bonner and Laskey [18], and the dried PPO-impregnated gels were exposed to Kodak XAR-5 film for 2 wk at −70°C.

Reagents

Thrombin was obtained as topical thrombin (bovine origin) from Parke-Davis (Detroit, MI). Plasminogen was prepared from human plasma by the method of Deutsch and Mertz [19]. Fibrinogen was purified from commercial bovine fibrinogen (Miles Laboratories, Elkhart, IN) by the method of Laki [20]. Human urokinase was obtained from Leo Pharmaceutical Products (Copenhagen, Denmark). Rabbit antiserum to human urokinase was kindly provided by Dr. L. Ossowski, The Rockefeller University. Rabbit antiserum to human tissue-type plasminogen activator was kindly provided by D. Weil, New York University Medical Center. D. Weil also supplied conditioned medium and cell extracts of cultured RPMI 7272 human melanoma cells that were used as a source of control human t-PA in these experiments.

RESULTS

BCE cells were incubated in serum-free medium with or without 10^{-7} M TPA. After 24 hr, the conditioned medium was collected, the cells were extracted, and the media and cell extracts were assayed for PA activity by their ability to stimulate plasminogen-dependent degradation of ^{125}I-fibrin (fibrin-plate assay). As reported previously [12], TPA treatment stimulated both cell-associated PA activity (40-fold) and PA activity in the conditioned medium (eight-fold) (Table I). More than eight times as much PA activity was present in the cell-associated fraction of the TPA-treated cells as was present in the conditioned medium from these TPA-treated cells (Table I). Both the extent of stimulation and the relative amounts of PA in cell-associated and secreted fractions varied from experiment to experiment, but TPA treatment always stimulated both cell-associated and secreted PAs.

The molecular size of the PAs in the cell-associated and conditioned medium fractions of both the unstimulated and TPA-stimulated BCE cells from the above experiment were determined by fibrin autography of samples run on an SDS-polyacrylamide gel. Fibrin autography of cell extracts from unstimulated BCE cells showed a single zone of lysis corresponding to an M_r of approximately 48,000 (Fig. 1, lane 1). This activity comigrated with the PA from MDBK cells, a bovine u-PA (data not shown). Fibrin autography of an equal amount of protein from a cell extract of BCE cells that had been treated with TPA for 24 hr showed the presence of much more fibrinolytic activity at this same molecular weight (Fig. 1, lane 2). In addition, activity appeared at a position corresponding to an M_r of 28,000. Both the M_r 48,000

TABLE I. Effect of TPA on PA Activity in Cell-Associated and Secreted Fractions of BCE Cells*

	PA mU/culture	
	Cell-associated	Secreted
Control	100	60
TPA-treated	3,963	480

*Confluent cultures of BCE cells on 35-mm dishes were changed to fresh α minimum essential medium (MEM) or fresh α MEM containing 10^{-7} M TPA. After a 24-hr incubation at 37°C, the conditioned media were collected, and the cells were extracted with detergent. Aliquots of the conditioned media and the cell extracts were assayed for PA activity by the fibrin-plate assay.

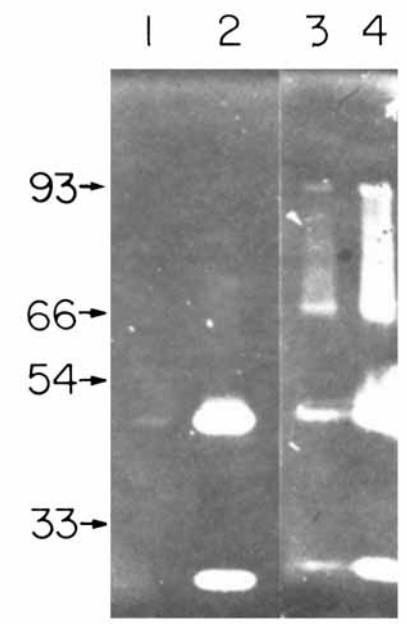

Fig. 1. Fibrin autography of plasminogen activators from BCE cells. Confluent cultures of BCE cells on 35-mm dishes were incubated in α-Minimal Essential Medium containing no additions or 10^{-7} M TPA for 24 hr. Then the conditioned medium was collected, and the cells were extracted. The conditioned medium (1.5 ml/culture) was dialyzed against 1/10 PBS, was lyophilized, and was reconstituted in 1/10 of its original volume. One-sixth of the cell extract (30 μg of cell protein) and two-thirds of the conditioned medium from the untreated or TPA-treated BCE cells were run on SDS-PAGE and analyzed by fibrin autography. Lane 1) cell extract from untreated cells, lane 2) cell extract from TPA-treated cells, lane 3) conditioned medium from untreated cells, lane 4) conditioned medium from TPA-treated cells.

and 28,000 forms are PAs, since no lysis was observed if plasminogen was omitted from the assay (data not shown). Although the M_r 28,000 form appears to be a major form in Figure 1, this result is an artifact that is due to the length of time the gel was incubated in order to detect the PA from unstimulated cells. The band appeared much later than the band of M_r 48,000 and developed after the band of M_r 48,000 had reached a maximum zone of lysis. The presence of this band was variable and often did not appear at all when incubations were done for shorter periods. Thus, the cell extracts from both untreated and TPA-stimulated cultures contained a major species of PA of M_r 48,000, and cell extracts from TPA-treated cultures also contained a minor species of M_r 28,000.

The 24-hr conditioned medium from unstimulated BCE cells showed the presence of distinct bands of lysis corresponding to M_r of 67,000 and 93,000, with a more diffuse area of lysis extending between these bands (Fig. 1, lane 3). In the 24-hr conditioned medium from TPA-stimulated BCE cells, a greater amount of fibrinolytic activity could be detected in this region (Fig. 1, lane 4). These zones could be shown to be due to the presence of PAs, since no lysis was observed in the absence of plasminogen (data not shown). In addition, bands corresponding to M_r 48,000 and 28,000 could also be detected in the 24-hr conditioned medium from both stimulated

and unstimulated cultures. However, the presence of the M_r 48,000 and 28,000 forms in the conditioned medium was variable from experiment to experiment. When the M_r 48,000 and 28,000 forms were present in the conditioned medium, they represented only a minor fraction of the total amount of these two forms. For example, in the experiment of Figure 1, since the M_r 48,000 and 28,000 species accounted for all of the activity in the cell-associated fraction and since this fraction contained 8.3 times as much PA activity as was present in the 24-hr conditioned medium (Table I), the amount of M_r 48,000 and 28,000 species present in the cell-associated fraction must be at least eight times greater than the amount in the 24-hr conditioned medium. Thus, BCE cells can be shown to produce several size classes of PA: M_r 48,000 and 28,000 species, which are predominantly cell associated, and several species in the range M_r 67,000 to 93,000, which are secreted. Stimulation with TPA increases the production of both the M_r 48,000 species and the higher molecular weight species.

A better estimation of the relative amounts of the various forms of PA in the different fractions can be obtained by analyzing equal amounts of activity from untreated and stimulated cultures so that incubations can be performed for a shorter period. Therefore, equal amounts of activity from extracts of unstimulated and TPA-stimulated BCE cells were analyzed after SDS-PAGE by fibrin autography. Comparison of lanes 4 and 5 in Figure 2 show that only the M_r 48,000 species and a minor

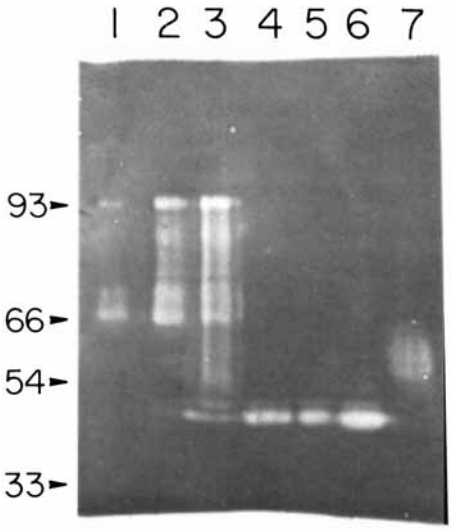

Fig. 2. Comparison of PAs from stimulated and unstimulated BCE cells. Confluent cultures of BCE cells were incubated in α-Minimal Essential Medium containing no additions, 10^{-7} M TPA, or 300 μg/ml hepatoma sonicate for 24 hr. Then the conditioned medium was collected, and the cells were extracted. Cell extracts and conditioned media were assayed for PA by the fibrin plate assay, and the amount of activity was compared to a commercial urokinase standard. Approximately 200 milli-Ploug units of PA activity from each of the cell extracts and from each of the conditioned media were run on SDS-PAGE and were analyzed by fibrin autography. Lane 1) conditioned medium from untreated cells, lane 2) conditioned medium from TPA-treated cells, lane 3) conditioned medium from hepatoma sonicate-treated cells, lane 4) cell extract from untreated cells, lane 5) cell extract from TPA-treated cells, lane 6) cell extract from hepatoma sonicate-treated cells, lane 7) hepatoma sonicate at the concentration added to BCE cells, showing endogenous PA.

band with slightly lower mobility were present in cell extracts from unstimulated and TPA-stimulated cells, and these were present in about the same proportions in both extracts. Because so little PA is secreted by unstimulated cells, it was difficult to obtain enough PA activity from the 24-hr conditioned medium of unstimulated BCE cells to compare to 24-hr conditioned medium from TPA-stimulated cells on the basis of equal amounts of activity. However, when roughly equal amounts of activity from the conditioned medium of unstimulated cells and from the conditioned medium of TPA-treated cells were compared, it appeared that the majority of the PA secreted by both unstimulated and treated cultures had M_r 67,000–93,000 (Fig. 2, lanes 1 and 2). Very little M_r 48,000 form was detected. All of the species present in the conditioned medium of TPA-treated BCE cells were also present in the conditioned medium of unstimulated cells and were present in about the same proportions. Therefore, it appears that when BCE cells are stimulated by TPA, there is a quantitative but not a qualitative change in the species of PA produced.

We have previously shown that certain preparations that are angiogenic *in vivo* also stimulate PA production in BCE cells [13]. Cell extracts and 24-hr conditioned medium from BCE cells stimulated with one of these preparations, a sonicate of cultured human hepatoma cells, were also analyzed by fibrin autography after SDS-PAGE. Lanes 3 and 6 in Figure 2 show that the same species of PA present in TPA-stimulated BCE cells were present in both the conditioned medium and in the cell layer of hepatoma sonicate-stimulated BCE cells. In the conditioned medium, an additional faint zone of lysis appeared between the M_r 48,000 species and the M_r 67,000 species. This zone can be attributed to a human u-PA that is present in the hepatoma cell sonicate (Fig. 2, lane 7) that is added to the medium. Thus, stimulation of PA production with either TPA or hepatoma sonicate causes the induction of the same species of PAs.

For preliminary characterization of the PAs produced by BCE cells, conditioned medium from TPA-treated cultures was passed over a column of concanavalin A-Sepharose. The M_r 48,000 and 28,000 forms passed over the column without binding (data not shown). The 68,000–93,000 forms bound to concanavalin A-Sepharose and could be eluted with 0.6 M α-methy mannoside (data not shown). Since human t-PA will bind to concanavalin A [21] while human u-PA does not, these results suggested that the 68,000–93,000 forms were t-PAs and the M_r 48,000 and 28,000 forms were u-PAs.

The types of PA produced by BCE cells were further characterized immunologically. TPA-stimulated BCE cell cultures were labelled for 5 hours with [^{35}S]cystine. Then the conditioned media were collected, the cells were extracted, and samples of each were immunoprecipitated with preimmune serum, with antiserum raised against human t-PA, or with antiserum raised against human urokinase. Autoradiograms of SDS-PAGE gels of the immunoprecipitate obtained with antiserum raised against t-PA showed the presence of a diffuse band with M_r in the range 67,000–74,000 and a fainter band with an M_r of 93,000 in the sample from the conditioned medium (Fig. 3, lane 1, I). These bands were not present in the immunoprecipitate obtained with preimmune serum (Fig. 3, lane 1, PI). No radiolabelled proteins were specifically immunoprecipitated from cell extracts (Fig. 3, lane 2). No immunoprecipitate was obtained when the antiserum against human t-PA was incubated with labelled PA from MDBK cells, demonstrating that the antiserum does not cross-react with bovine u-PA (data not shown). Thus, the high molecular weight PAs that are predominantly

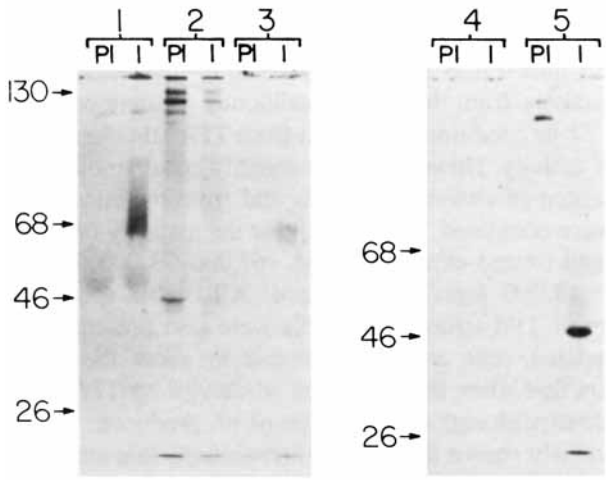

Fig. 3. Immunoprecipitation of secreted and cell-associated BCE PAs. Confluent cultures of BCE cells were incubated in α-Minimal Essential Medium containing 5% calf serum and 10^{-7} M TPA. After 15 hr, the cells were labelled for 5 hr with [^{35}S]cystine, as described in Methods. Then the conditioned media were collected, and the cells were extracted. Aliquots containing 100,000 cpm. of TCA-precipitable [^{35}S]cystine from both conditioned medium (lanes 1 and 4) and cell extract (lanes 2 and 5) were incubated either with preimmune serum (PI) or with antiserum (I) against human t-PA (lanes 1 and 2), and immune complexes were precipitated with protein A-Sepharose. The supernatants were then incubated either with preimmune serum or with antiserum against human urokinase (lanes 4 and 5), and immune complexes were precipitated with protein A-Sepharose. As a control, labelled cell extracts from human melanoma cells were incubated either with nonimmune serum or antiserum against human t-PA (lane 3). The immunoprecipitates were run on SDS-PAGE under reducing conditions, and the gels were analyzed by fluorography.

secreted are t-PAs. Autoradiograms of SDS-PAGE gels of the immunoprecipitate obtained with antiserum raised against human urokinase showed the presence of a single band with an M_r of 48,000 that comigrated with the PA immunoprecipitated from labelled MDBK cells. The BCE cell immunoprecipitate was obtained only with the cell extract (Fig. 3, lane 5), not with conditioned medium (lane 4). Thus, the M_r 48,000 PA that is predominately cell associated is a u-PA.

It has been demonstrated that u-PAs are synthesized as inactive single chain prourokinases [26]. Prourokinase can be converted to active urokinase by proteolytic cleavage at an internal site, producing a molecule with two chains of about equal size that are held together by disulfide bonds [26]. The above experiments demonstrated that the BCE M_r 48,000 u-PA contained a single protein chain, since it showed the same size on the nonreducing polyacrylamide gels used to assay activity (Figs. 1, 2) and on the reducing polyacrylamide gels used to analyze the immunoprecipites (Fig. 3). These results suggested that this molecule was a prourokinase that could be detected because it was activated by small amounts of proteolytic enzymes present in the assays. To test whether the cell-associated M_r 48,000 u-PA was a prourokinase, cell extracts were incubated with the serine protease inhibitor phenylmethylsulfonylfluoride (PMSF), which inhibits active urokinase but does not react with prourokinase [27]. The PMSF was removed by dialysis, and the extracts were assayed for PA activity with the fibrin-plate assay. With a protocol that completely inactivated

commercial human urokinase, there was no effect on the cell-associated PA activity from either stimulated or untreated cultures (Table II). These results suggest that the M_r 48,000 cell-associated u-PA produced by BCE cells is a prourokinase.

DISCUSSION

We have demonstrated that extracts of BCE cells contain two species of PA, a M_r 48,000 form and a M_r 28,000 form. The M_r 48,000 form, which is the predominant species, has been identified as u-PA by its ability to cross-react with antibodies to human urokinase. The fact that this u-PA contains a single peptide chain, coupled with the fact that the PA activity in BCE cell extracts is not affected by PMSF treatment, suggests that this molecule is a prourokinase. The M_r 28,000 form seems to be present in much lower amounts and is detected only after long exposure of fibrin-autography gels. This form may represent the bovine equivalent of the human M_r 33,000 single chain form of urokinase, which is a degradation product of the higher molecular weight form [28].

Analysis of conditioned medium shows the presence of several species of PA. A broad band of activity of M_r 67,000–93,000 has been identified as t-PA by its ability to cross-react with antibodies to human t-PA. In medium collected after a 5-hr labelling period, the M_r 67,000–93,000 t-PAs are the only species detected. However, when medium conditioned by cells for 24 hr is assayed with long exposures on fibrin autography gels, M_r 48,000 and 28,000 species are detected in addition. This suggests that eventually some of the cell-associated u-PA is released by the cells into the medium.

Thus, these results demonstrate that BCE cells are able to synthesize both t-PA and u-PA and that their fates differ. We had previously reported that we could detect only a u-PA in both cell extracts and conditioned medium from BCE cells [12]. The reason for the inability to detect t-PA in that study may be related to the assay

TABLE II. Treatment of BCE Cell-Associated PA With PMSF*

	PMSF treatment	Counts/min released from ^{125}I-fibrin wells
Cell extract		
Untreated cultures	−	783
	+	718
TPA-treated cultures	−	3,478
	+	3,553
Urokinase	−	1,865
	+	11

*Confluent cultures of BCE cells were given fresh α-MEM containing 5% calf serum with or without the addition of 10^{-7} M TPA. After a 24-hr incubation in this medium, the cells were washed with PBS and extracted with detergent. The extracts were then incubated with or without 2 mM PMSF for 60 min at 37°C. Commercial urokinase was diluted into the extraction buffer and was also incubated with or without PMSF under the same conditions. All of the samples were then dialyzed against PBS overnight at 4°C. The PA activity in 1 μg of each sample of cell extract was determined by the fibrin-plate assay.

procedures employed. The fibrin plate assay, which was used in that study, is much less sensitive to t-PA than the fibrin autography assay and would underestimate the amount of t-PA present. In addition, we have found that, although u-PA is predominately cell associated, there is some variation in the amount of u-PA released into the medium. Perhaps, in the previous study, the amount of u-PA released into the medium was greater than the amount of t-PA. This, in combination with the less sensitive assay for t-PA, would make it appear that no t-PA was synthesized.

Levin and Loskutoff [11] have shown that bovine aortic endothelial cells also produce both types of PA. The molecular weights estimated for the PAs synthesized by BCE cells are in very good agreement with the molecular weights of the PAs produced by the aortic endothelial cells. Since there seems to be little difference in the type of PA produced by capillary endothelial cells and aortic endothelial cells, the circulating t-PA may be derived from both large vessel and microvessel endothelial cells. However, since the surface area of the endothelial lining per unit volume of blood is 1,000 times greater in capillaries than in large vessels [22], capillary endothelial cells may be the major source of circulating t-PA.

The broad range of molecular weights obtained for BCE cell t-PA is similar to the broad range of molecular weights obtained for t-PA from bovine aortic endothelial cells by Levin and Loskutoff [11]. Human umbilical vein endothelial cells also produce a high molecular weight form of t-PA that has been shown to be due to complexes formed between the t-PA and an inhibitor of PA that is also produced by the cells [10]. Bovine aortic endothelial cells produce a similar inhibitor [23], suggesting that the high molecular weight forms of t-PA in these cells are also due to complexes with inhibitors. It is likely that the high molecular weight forms of t-PA produced by BCE cells are also enzyme-inhibitor complexes.

Since t-PA has been proposed to have the major role in clot lysis, it seems reasonable that the t-PA synthesized by endothelial cells would be secreted into the medium in culture and into the circulation *in vivo*. The fact that BCE cell u-PA is predominantly cell associated may indicate that BCE cells have membrane receptors for u-PA similar to the receptors demonstrated recently in 3T3 cells [24] and monocytes [25]. Endothelial cell u-PA may be important in tissue remodelling. In capillary endothelial cells, it may be important in angiogenesis. Angiogenesis is thought to require the elaboration of proteolytic enzymes locally, at the tip of the growing blood vessels, in order to digest barriers to the expansion of the new blood vessels. We have shown here that a preparation containing angiogenic activity, hepatoma sonicate, stimulates the production of u-PA by BCE cells. Perhaps, the fact that the u-PA is predominately cell associated may restrict it to only acting locally, where it is needed.

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