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Detection of an unusually stable fibrinolytic inhibitor produced by bovine endothelial cells

(vascular endothelium/plasminogen activators/regulation/reverse fibrin autography)

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ABSTRACT **Fibrin/agar films were prepared and used to detect plasminogen activators produced by cultured bovine aortic endothelial cells (fibrin autography). One preparation of fibrin underwent spontaneous lysis upon incubation at 37°C. This lysis was prevented by antibodies to tissue-type plasminogen activator but not by antibodies to urokinase. Conditioned medium from the confluent endothelial cells was fractionated by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$. The gels were analyzed on indicator films prepared with the spontaneously lysing fibrin (reverse fibrin autography). Unexpectedly, as the opaque fibrin film cleared, a distinct lysis-resistant zone appeared in the indicator gel at a region corresponding to M_r 55,000. Experiments were devised to determine whether the lysis-resistant zone in the indicator film reflected the presence of a cellular inhibitor in the polyacrylamide gel. The corresponding region was excised from a polyacrylamide gel, extracted with buffer, and tested directly for antifibrinolytic activity by the ^{125}I-labeled fibrin plate method. Urokinase-mediated fibrinolytic activity was inhibited by the gel extract in a dose-dependent manner indicating the presence of such an inhibitor. Inhibitor activity was detected in Triton X-100 extracts of washed monolayers and in conditioned medium, where it accumulated with time. The endothelial cell inhibitor not only survived exposure to $NaDodSO_4$ but also was active after incubation at pH 12 or treatment with 5% (vol/vol) 2-mercaptoethanol, 6 M urea, 4 M guanidine hydrochloride, or 1 M acetic acid. Considerable activity also remained after heating at 100°C for 30 min. These results indicate that cultured bovine aortic endothelial cells synthesize and secrete a previously undetected, unusually stable fibrinolytic inhibitor of M_r 55,000. Reverse fibrin autography offers a convenient approach for studying such molecules.**

The production of both urokinase-like and tissue-type plasminogen activators (PAs) by cultured endothelial cells (1) emphasizes the potential role of endothelium in the specific catabolism of locally deposited fibrin and in the general maintenance of blood vessel patency. Expression of these cellular activities should be regulated precisely to ensure that the normal hemostatic role of endothelium (2) is not compromised. Plasma contains a number of molecules that may function in this way by either promoting (3, 4) or inhibiting (5) endothelial cell-mediated fibrinolysis. In addition, cultured rabbit (6, 7) and human (8–11) endothelial cells were found to be associated with an antifibrinolytic activity, suggesting that endothelium itself also may produce molecules that modulate the fibrinolytic activity of the vessel wall. However, no such inhibitor activity has been detected in confluent bovine aortic endothelial cells (BAEs) (9, 12).

We have used cultured BAEs as a model to identify factors that influence the overall fibrinolytic activity of endothelium (13). Cellular fibrinolytic activity was found to change with the growth state of the cells (9, 12) and in response to the presence of fresh serum (14), thrombin (15), dexamethasone, and calcium ionophore (16). During the course of these studies, a technique was devised that unexpectedly revealed that these cells also synthesized and released an inhibitor of fibrinolysis. Here we present a preliminary characterization of this cellular inhibitor and discuss its potential contribution to the fibrinolytic activity of endothelium.

MATERIALS AND METHODS

Cell Culture. BAEs were isolated from the aortae of cows and cultured in minimal essential medium containing 10% calf serum (Irvine Scientific, Santa Ana, CA) as indicated (15). In general, the cells employed for these studies had been passaged 5–20 times. The results were confirmed with a cell line we cloned from a single cell (1). All cultures were grown to confluency in 60-mm dishes or T75 flasks (Falcon) before use. To prepare cellular extracts, confluent cultures were washed three times with cold phosphate-buffered saline (P_i/NaCl; 0.14 M NaCl/0.01 M sodium phosphate, pH 7.2) and then extracted into 1 ml of P_i/NaCl containing 0.5% Triton X-100 (Sigma). Conditioned medium (CM) was prepared by incubating washed monolayers in serum-free minimal essential medium or growth medium for 24 hr. This CM was collected and then centrifuged at 1,000 × *g* to remove floating cells and cellular debris. Cell extracts and CM were stored in 0.5% Triton X-100 at −30°C until used. Samples (1 ml) of freshly prepared CM were treated for 30 min at 37°C under various conditions, dialyzed overnight against 0.1 M Tris, pH 8.1, containing 0.5% Triton X-100 and then stored at −30°C until analyzed. Treatments included incubation in the presence of 5% (vol/vol) 2-mercaptoethanol, 1 M acetic acid, 6 M urea, or 4 M guanidine hydrochloride, and at pH 12 after titration with 1 M NaOH. Samples also were heated to either 70°C or 100°C for 30 min, dialyzed, and analyzed.

Polyacrylamide Gel Electrophoresis. $NaDodSO_4$/polyacrylamide slab gels and buffers were prepared (17), using 10-cm resolving gels containing 9% acrylamide, and 2-cm stacking gels of 4% acrylamide. Samples were applied to the gels as indicated and subjected to electrophoresis for 16 hr at room temperature. Molecular weight (M_r) 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 0.1% Coomassie blue and 50% (wt/vol) trichloroacetic acid, and then destained in

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Abbreviations: PA, plasminogen activator; BAEs, bovine aortic endothelial cells; CM, conditioned medium; P_i/NaCl, phosphate-buffered saline.

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10% (wt/vol) acetic acid. The remaining portions of the gel were processed for reverse fibrin autography to localize PA and inhibitor activity. In one experiment, CM was fractionated as above but in tube gels (0.5 cm diameter). The gels were cut into 1-mm slices and assayed for inhibitor activity by the ^{125}I-labeled fibrin (^{125}I-fibrin) plate method as described below.

^{125}I-Fibrin Plate Assay of Inhibitor Activity. Gel slices were incubated for 16 hr in 0.5 ml of 0.1 M Tris, pH 8.1/0.5% Triton X-100 to elute the protein. In preliminary experiments (not shown), the inhibitor activity of each of these gel extracts was determined in mixing experiments by testing their capacity to depress the rate of urokinase-mediated fibrinolytic activity (6). The inhibitor assay was terminated when the untreated urokinase controls hydrolyzed 10–20% of the total radioactive material. All inhibitor activity localized to a single region of the gel, at M_r 50,000–60,000. The gel extracts containing inhibitor were pooled (12 gel slices, 6 ml of extract) and retested for inhibitor activity as shown in *Results*. The pooled gel extracts also were acidified by incubation for 60 min in 0.05 M glycine buffer, pH 2.7, containing 0.1% gelatin. The samples were neutralized by the addition of 0.1 vol of 1 M Tris, pH 8.1, before testing. In control experiments, 12 slices from a blank gel were extracted, pooled, and tested for inhibitor activity.

Reverse Fibrin Autography. To prepare fibrin agar indicator gels (1, 18), a 2% solution of agarose (LPG agarose, Miles Laboratories) was mixed with prewarmed (47°C) P_i/NaCl containing plasminogen (75 μg/ml) and thrombin (0.6 unit/ml). Fibrinogen (bovine, fraction I; Calbiochem–Behring, San Diego, CA; 10 mg/ml) in P_i/NaCl (37°C) was added, and the solution was mixed rapidly and poured onto a glass plate or glass slides. Final concentrations were 1% agarose, plasminogen at 25 μg/ml, thrombin at 0.18 unit/ml, and fibrinogen at 2 mg/ml. After electrophoresis, the polyacrylamide gels were soaked in 250 ml of 2.5% Triton X-100 (two changes, 45 min each) to remove the $NaDodSO_4$, patted dry with a 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 was photographed at various times. The dark areas of the indicator gel correspond to plasmin-mediated lytic zones initiated by the interaction of PA from the polyacrylamide gel with plasminogen in the indicator gel. Continued incubation of indicator gels prepared with this fibrin resulted in a general clearing (lysis) of the opaque fibrin film. The white areas that develop in these gels reflect areas relatively resistant to such lysis. Analysis of samples on lysing fibrin films is referred to as reverse fibrin autography. Where indicated, bovine plasminogen-free fibrinogen (Calbiochem–Behring) was used instead of fraction I fibrinogen. Films prepared with this fibrinogen did not lyse during a 48-hr incubation period (unpublished observation). Lysis of these indicator films was initiated by the addition of urokinase (0.05 unit/ml, final concentration) to the fibrinogen-agarose solution. In some experiments, the IgG fractions of rabbit antisera to purified human urokinase (20 μg/ml) or human tissue-type PA (10 μg/ml) were incorporated into the fibrin-agar indicator gels as described (1).

Miscellaneous. Protein was determined by the method of Bradford (19) with bovine serum albumin used as a standard. ^{125}I measurements were performed in a Micromedic gamma spectrometer. Plasminogen was prepared by affinity chromatography on lysine-Sepharose as described (20). Human α-thrombin was a generous gift from J. Fenton (Albany, NY). Purified human urokinase containing both M_r 54,000 and M_r 33,000 forms (World Health Organization standard) was supplied by A. Johnson (New York University). Human tissue PA was purified from cultured human melanoma cells (21) and was a generous gift from D. Collen (University of Leuven, Leuven, Belgium). The IgG fractions of rabbit antisera to human urokinase and human tissue-type PA were prepared as described (1).

RESULTS

Analysis of CM by Reverse Fibrin Autography. We previously showed that cultured rabbit (6, 7) and human (8, 9) endothelial cells contained an inhibitor of fibrinolysis. However, no inhibitor activity was detected in samples prepared from confluent BAEs (9, 12). We have modified the fibrin autography technique used for PA detection (1, 18) and can now detect inhibitor activity in these cells as well. This approach is based on the observations shown in Fig. 1. In these experiments, one lot of bovine fraction I fibrinogen was found to spontaneously lyse upon continued incubation at 37°C. The unincubated control fibrin film (lane 1) completely cleared when incubated for 8 hr at 37°C (lane 2). This clearing of the opaque fibrin film was not affected by the presence of the IgG fraction of antisera to urokinase (lane 3) but was blocked by the IgG fraction of antisera to tissue-type PA (lane 4). Thus, lysis of the fibrin film seemed to result from the presence of contaminating PA in the fibrin preparation.

When CM from confluent BAEs was fractionated by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ and analyzed on these lysing fibrin films (reverse fibrin autography), one area of the indicator gel now seemed to resist lysis (Fig. 2). This region was not initially apparent (lane 1). At these early times (2 hr of incubation) only the rather characteristic (1) PA profile of these cells was revealed in the indicator film as lysis zones at M_r 52,000, 74,000, and 100,000. With continued incubation, however (4 hr; lane 2), the lytic zones became more distinct, and the fibrin film itself began to lyse (note general darkening of the background compared to the earlier time). At this time, the lysis-resistant zone became apparent in the region of the indicator gel immediately above the low molecular weight PA form. When the fibrin film had completely lysed (8 hr; lane 3), this lysis-resistant zone was revealed as a distinct, sharply defined band of M_r approximately 55,000.

Demonstration of Inhibitor Activity in BAE CM. Mixing experiments were devised to confirm that the lysis-resistant zones in the indicator gel (Fig. 2) reflected the presence of a fibrinolytic inhibitor in the $NaDodSO_4$ gel. Those portions of a $NaDodSO_4$ tube gel containing the presumed inhibitor were identified as described in *Materials and Methods*, pooled, and

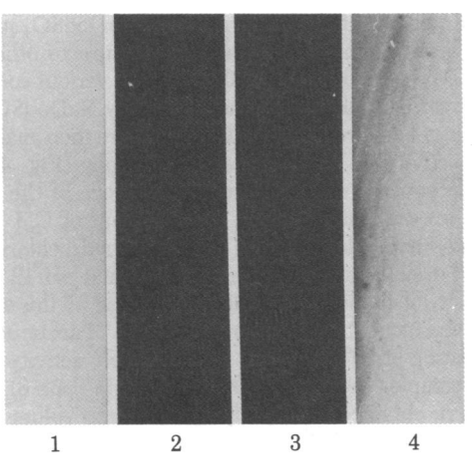

FIG. 1. Spontaneous lysis of indicator films. Fibrin indicator gels were prepared on slides and photographed after 8-hr incubation at 37°C in the absence (lane 2) or presence of the IgG fraction of antisera to either urokinase (lane 3) or tissue-type PA (lane 4). The unincubated control slide (lane 1) was photographed immediately after it was prepared.

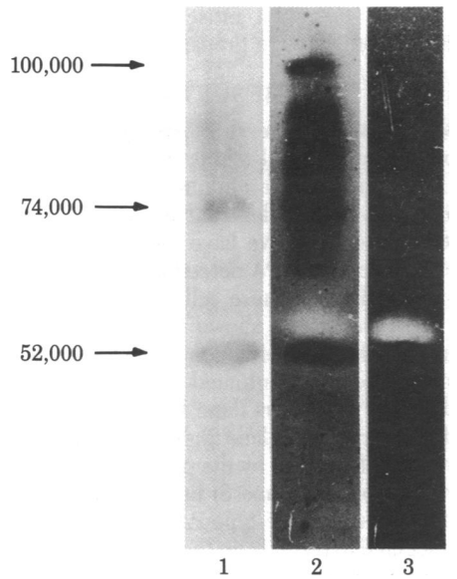

FIG. 2. Analysis of CM by reverse fibrin autography. CM (100 μl) was fractionated by polyacrylamide slab gel electrophoresis in the presence of $NaDodSO_4$ and then analyzed by reverse fibrin autography. The fibrin film was photographed after 2-hr (lane 1), 4-hr (lane 2), and 8-hr (lane 3) incubation at 37°C.

then tested directly for antifibrinolytic activity by the ^{125}I-fibrin plate method. A lysis-resistant zone was formed when the eluted material was reanalyzed by $NaDodSO_4$ slab gel electrophoresis and reverse fibrin autography (not shown). It was in the same position of the indicator gel as the material in CM. Urokinase-mediated fibrinolytic activity was inhibited by the eluant in a dose-dependent manner (Fig. 3), with less than 10 μl of gel extract resulting in 50% inhibition. The extracted material also inhibited the fibrinolytic activity initiated by tissue-type PA (data not shown). Unlike the inhibitor activities associated with rabbit endothelial cells (6) and plasma (5, 22), the BAE inhibitor was resistant to inactivation by acidification. In fact, acid treatment seemed to enhance inhibitor activity rather than destroy it. The reasons for this stimulation of activity are not apparent. Little inhibitor activity was detected in the eluant from control gel slices prepared in parallel from a blank gel.

Stability of Inhibitor. The unusual apparent stability of the BAE inhibitor to both acid treatment and $NaDodSO_4$ suggested that this agent might also withstand exposure to other denaturants. CM was incubated for 30 min under various conditions, dialyzed overnight, and then fractionated by $NaDodSO_4$/polyacrylamide gel electrophoresis. The gels were then analyzed for inhibitor activity by reverse fibrin autography (Fig. 4*A*). The untreated control is shown in lane 1. The size of the lysis-resistant zones was unaffected by pretreatment of CM with 5% 2-mercaptoethanol (lane 2), 4 M guanidine hydrochloride (lane 5), or 6 M urea (lane 6), or upon incubation at pH 12 (lane 3). The majority of the inhibitor activity revealed by this approach also survived incubation in the presence of 1 M acetic acid (lane 4) and heating at 70°C (lane 7). Considerable activity was detected in samples heated at 100°C for 30 min (lane 8). Similar results were obtained when only one-fifth the volume of CM was analyzed (Fig. 4*B*).

Accumulation of Inhibitor Activity in CM. Inhibitor activity was detected in both cell extracts and CM (Fig. 5). The molecular weight of the cell-associated inhibitor (lane 1) was similar to that in CM (lanes 3–5). This material was released from the cells into the medium, where it was first detected at 6 hr (lane 3) and appeared to accumulate with time (lanes 4 and 5).

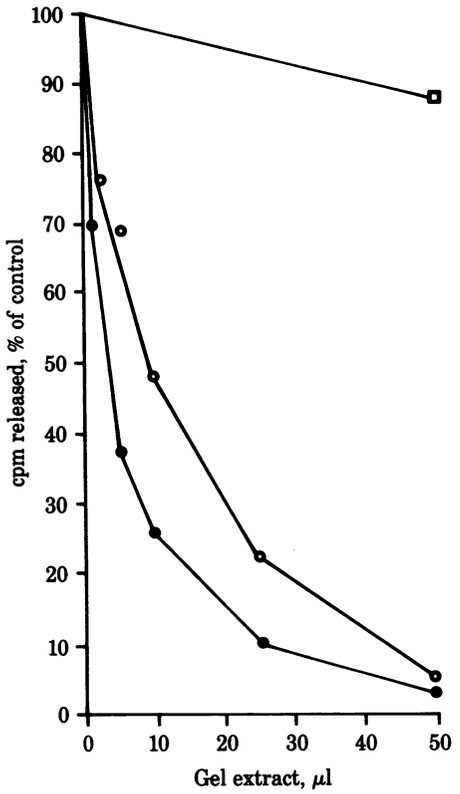

FIG. 3. Inhibitor activity of gel extracts. CM (300 μl) was fractionated by electrophoresis through a $NaDodSO_4$/polyacrylamide tube gel. The gel was sliced and each slice was eluted into 0.1 M Tris, pH 8.1, containing 0.5% Triton X-100. The inhibitor activity of each slice was determined by the ^{125}I-fibrin plate method with urokinase at 0.0025 unit/ml. The assay was terminated after 2 hr. The active fractions were pooled (12 fractions, 6 ml), and retested for inhibitor activity before (○) or after (●) acidification to pH 2.7. The inhibitor activity of extracts prepared from a blank gel was also determined (□).

The amount present in the medium after 12 hr (lane 4) exceeded that detected in the cell extract (lane 1). No inhibitor activity was evident in the growth medium itself (lane 2). The amount of inhibitor in the extracts remained constant over the entire incubation period (not shown). These results were sim-

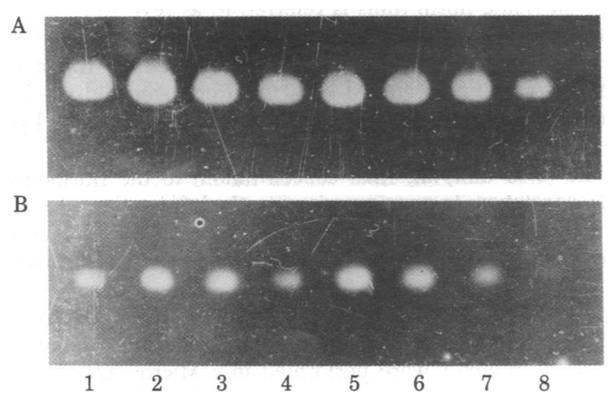

FIG. 4. Effect of various treatments on inhibitor activity of CM. CM was treated for 30 min as indicated, dialyzed, fractionated by polyacrylamide slab gel electrophoresis in the presence of $NaDodSO_4$, and tested for residual inhibitor activity by reverse fibrin autography. Both 25 μl (*A*) and 5 μl (*B*) of CM were analyzed. Lane 1, untreated control; lane 2, 5% 2-mercaptoethanol; lane 3, pH 12; lane 4, 1 M acetic acid; lane 5, 4 M guanidine·HCl; lane 6, 6 M urea; lane 7, 70°C; lane 8, 100°C. The gels were photographed after 8-hr incubation.

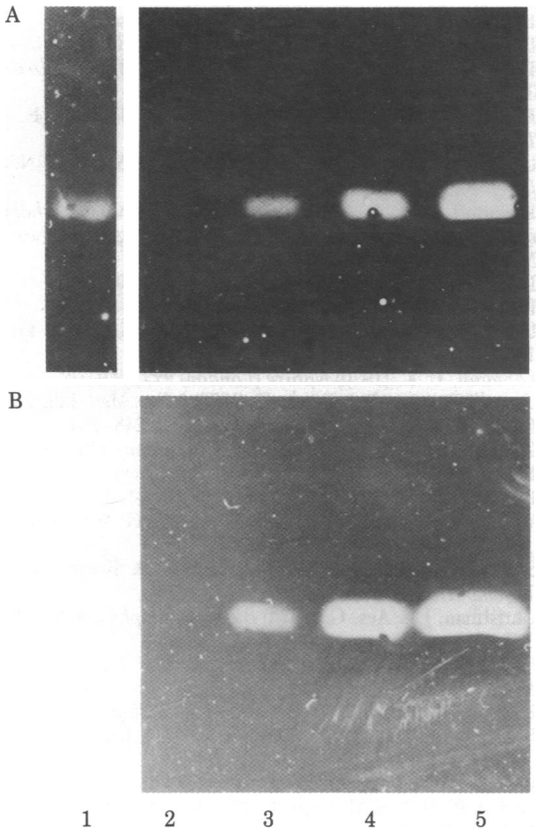

FIG. 5. Accumulation of inhibitor in CM. Confluent cultures were washed and then incubated in minimal essential medium for various times in the absence (*A*) or presence (*B*) of 10% serum. An aliquot of the resulting CM (75 μl, or 1/20th of the total) and extracts (25 μg of protein, or 1/20th of the total) was fractionated by $NaDodSO_4$/polyacrylamide slab gel electrophoresis and tested for inhibitor activity by reverse fibrin autography. The fibrin/agar gels were prepared as usual except that plasminogen-free fibrinogen containing urokinase was used instead of fraction I fibrinogen. Lane 1, cell extract; lane 2, growth medium alone; lane 3, CM after 6 hr; lane 4, CM after 12 hr; lane 5, CM after 18 hr. The gels were photographed after 2-hr incubation.

ilar whether the CM was prepared in the absence (Fig. 5*A*) or presence (Fig. 5*B*) of serum. $NaDodSO_4$-resistant inhibitor activity of the same molecular weight was detected in cell extracts and CM from an isolate of BAEs (1) cloned from a single cell (not shown).

DISCUSSION

Fibrin autography has proven to be an especially convenient tool for the detection and preliminary characterization of PAs in complex biological mixtures (1, 18, 23, 24). The success of this approach is based upon the ability to detect these molecules after their fractionation by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ (25). The presence of PAs in regions of the gel is revealed by the formation of clear lytic zones in corresponding areas of opaque fibrin indicator films. The results presented here indicate that a similar approach can be taken to detect molecules also resistant to $NaDodSO_4$ but with antifibrinolytic activity. In this case, however, the indicator film itself is lysed by lytic agents present in the fibrin preparation. Inhibitor activity is then revealed as an opaque, lysis-resistant band in the otherwise clear indicator film. We have termed this approach reverse fibrin autography. In the preliminary studies summarized here, lysis apparently was initiated by contaminating tissue-type PA in the fibrin (Fig. 1). Although the fibrinogen lot used for those studies was not available for retesting and other lots were found to vary considerably in their lytic properties (unpublished observation), we were able to reconstruct this detection system by mixing known amounts of purified urokinase (Fig. 5) or tissue-type PA (not shown) with fibrin. In general, fibrin films containing urokinase at 0.05 unit/ml or tissue-type PA at 0.1 unit/ml lyse within 2 hr and in so doing reveal the presence of inhibitor activity.

This approach was used to demonstrate the existence of a previously undetected cellular fibrinolytic inhibitor (Fig. 2). This inhibitor was associated with cultured BAEs, had an apparent M_r of 55,000, and appeared to retain activity after treatment with reducing agents, denaturants, or extremes in pH (Figs. 3 and 4). This unusual apparent stability makes this inhibitor unique among fibrinolytic components identified in serum and plasma (5, 22, 26), most of which are readily inactivated upon exposure to mildly acidic (pH 3) conditions. However, an acid-stable inhibitor of fibrinolysis was previously detected in human endothelial cells (8, 27). The BAE inhibitor blocked the ability of human urokinase and human tissue-type PA to cleave ^{125}I-labeled plasminogen (unpublished observation), suggesting not only that it is an antiactivator but also that it may recognize both types of PA (13).

That the inhibitor was actually synthesized by the cells can be inferred from the data shown in Fig. 5. Although inhibitor activity was present in both cell extracts and CM, none could be detected in the growth medium itself. In addition, the amount of inhibitor in CM increased with time, and by 12 hr exceeded that present initially in the cell. This accumulation of inhibitor activity in CM continued for at least 18 hr and occurred in the presence or absence of serum. Thus, the serum did not influence the accumulation of inhibitor activity in the medium. It seems unlikely from these results that a serum inhibitor is internalized by the cells and subsequently released back to the medium (28). We have also purified the inhibitor from BAEs grown in the presence of [^3H]leucine (unpublished results). The final product was electrophoretically homogeneous, biologically active, and radiolabeled, indicating that the inhibitor was a protein synthesized by the cells. An inhibitor activity with similar properties (i.e., molecular weight, stability) was detected in and subsequently purified from cloned BAEs, indicating that the inhibitor originated from endothelial cells and not from a minor population of contaminating cells (data not shown). Another cellular protease inhibitor of similar molecular weight, protease nexin, was recently described (29). The relationship of protease nexin to the BAE inhibitor is unknown at present, although protease nexin is apparently acid labile (J. Baker, personal communication).

The results presented here complement previous studies on cultured BAEs and indicate that these cells have evolved a relatively complex fibrinolytic system. They not only produce the fibrinolytic inhibitor but also synthesize and secrete multiple molecular forms of PA (30) that immunochemically (1) and functionally (13, 31) include both urokinase and tissue-type PA, two molecules detected in blood and essential to fibrinolytic phenomena *in vivo* (26). The overall fibrinolytic potential of these cells can be stimulated by agents such as phorbol myristate acetate (9) and suppressed by serum (14), thrombin (15), dexamethasone, and calcium ionophore A23187 (16). In some cases, this altered fibrinolytic activity can be correlated directly to changes in the activity of only one species of PA, the M_r 52,000 urokinase-like molecule (16). It has not yet been determined whether this altered activity reflects increased production of inhibitor or decreased production of PA (6, 32–34). In any case, it is clear that a thorough characterization of the inhibitor, its specificity and mechanism of action, and its relationship to the

various PAs produced by the cells is required to understand endothelial cell-mediated fibrinolysis.

It is clear from these and other studies (32–35) that cells may contain both fibrinolytic activators and inhibitors. This may be especially true of cultured endothelial cells because all endothelial cells tested, including those isolated from humans (8–11, 36), rabbits (6, 7), and now cows have been shown to contain both activities. Obviously, then, special consideration should be given in designing any experimental approach to characterize and quantitate the fibrinolytic components of such cells. If inhibitor and activator are not first separated or selectively inactivated (6, 35) they may neutralize each other. Resultant measurements would then reflect only that activity present at the higher concentration. The data presented here suggest that the fractionation of samples by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ and subsequent analysis of the gels by reverse fibrin autography offers a convenient approach to identify and quantify $NaDodSO_4$-resistant fibrinolytic activators and inhibitors present not only in endothelial cells but also in plasma and other complex biological mixtures.

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