

An Inhibitor of Plasminogen Activator in Rabbit Endothelial Cells*

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The antifibrinolytic activity of cytosols obtained from cultured rabbit endothelial cells was studied to determine whether it resulted from the presence of an antiplasmin or an antiactivator. These cytosol preparations inhibited the fibrinolytic activity initiated by some plasminogen activators (e.g. urokinase, rabbit endothelial cell activator), but not others (e.g. activators associated with bovine endothelial cells and Rous sarcoma virus-infected chick embryo fibroblasts), suggesting that inhibition occurred at the level of plasmin formation, not plasmin activity. The fibrinolytic activity of plasmin itself was unaffected by concentrations of cytosol that completely blocked urokinase-mediated fibrinolysis, consistent with this conclusion. In addition, the ability of urokinase to cleave ^{125}I -plasminogen into its characteristic activation fragments was inhibited by cytosol in a dose-dependent manner. When urokinase was analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, two peaks of activity were detected, corresponding to $M_r = 55,000$ and $32,000$. Urokinase preincubated with cytosol and analyzed in a similar manner demonstrated no activity in any portion of the gel, suggesting that its ability to function as a plasminogen activator was irreversibly lost following its interaction with cytosol. These results indicate that the antifibrinolytic activity of rabbit endothelial cells results from the presence of a molecule or molecules with antiactivator activity. The cellular location and unusual degree of specificity distinguish the endothelial cell inhibitor(s) from antifibrinolytic agents observed in other cells and in plasma and platelets.

The endothelium is thought to play a pivotal role in maintaining vascular homeostasis, not only because of its strategic location as an interface between blood and tissue, but also because of its potential to inhibit platelet aggregation (1-3), to promote thrombosis and hemostasis (4), and to function in the catabolism of locally deposited fibrin (5). However, this fibrinolytic activity might also compromise hemostasis by initiating premature lysis of fibrin at sites of injury. Proteolytic

activity in the vicinity of a thrombus is thought to be regulated by inhibitors found in plasma and platelets.

We recently demonstrated that cultured rabbit endothelial cells had the potential to initiate fibrinolysis since they contained a membrane-associated plasminogen activator (6); however, the presence of this molecule was obscured initially because the cells also contained an inhibitor(s) of fibrinolysis within the cytosol. The availability of this inhibitor activity may introduce yet another mechanism for regulating local fibrinolytic activity and the fibrinolytic process. The present study demonstrates that this endothelial cell inhibitor(s) specifically and irreversibly inactivates endothelial plasminogen activator and urokinase, although it is apparently without effect on plasmin. This unique specificity distinguishes the endothelial cell inhibitor(s) from other fibrinolytic inhibitors detected in plasma, platelets, and tissues.

EXPERIMENTAL PROCEDURES

Cell Culture—Endothelial cells were isolated from intact rabbit vena cava or bovine aorta as described (7). Rabbit kidney cells were obtained from minced, trypsin-dispersed whole kidneys, and chick embryo fibroblasts infected with the Schmidt-Rupin strain of Rous sarcoma virus were a kind gift from Dr. B. Sefton, The Salk Institute, La Jolla, CA. All cells were grown in Falcon disposable plasticware (Falcon Plastics, Oxnard, CA) in modified Eagle's minimal essential medium supplemented with 10% fetal bovine serum, $100\text{ }\mu\text{g/ml}$ of streptomycin, and 100 units/ml of penicillin (Grand Island Biological Co., Grand Island, NY). Cultures were maintained at 37°C in a 95% air/5% CO_2 atmosphere, and were routinely subcultured once a week (1:5) after trypsinization of confluent monolayers. Cell number was determined by counting trypsinized cells in a hemocytometer. All cells used in these studies had been subcultured 3-10 times.

Conditioned Medium—Confluent cultures were washed with phosphate-buffered saline (7) to remove serum and then incubated for 16 h in serum-free Eagle's minimal essential medium. The culture fluid conditioned by the cells was removed, centrifuged at $600 \times g$ for 10 min at 22°C to remove detached cells and cellular debris, and stored at -30°C until assayed.

Cell Extracts and Subcellular Fractions—To prepare cell homogenates for differential centrifugation, the cultures were washed three times with cold phosphate-buffered saline, and the cells were removed from the surface of the culture dish with a rubber policeman. The cells were washed again by low speed centrifugation ($800 \times g$ for 5 min), resuspended in a hypotonic homogenization medium (0.25 M sucrose, 0.1 mM EDTA, 0.01 M Tris-HCl pH 8.1) at 4°C and homogenized with a tight-fitting Teflon pestle tissue grinder until 90-95% of the cells were disrupted. The nuclei were removed by centrifugation at $600 \times g$ for 10 min, and the supernatant was further fractionated to yield a cytosol (the supernatant resulting from centrifugation at $100,000 \times g$ for 60 min) and a particulate fraction (the pellet from this step). Whole cell extracts were prepared by scraping the washed cell monolayer into 0.5% Triton X-100 (Sigma).

Fibrinolytic Activity— ^{125}I -Labeled fibrin-coated multiwell culture dishes (24 wells/dish, 16 mm diameter, Costar) were prepared as described (6) and employed to measure the fibrinolytic activity of cell extracts and conditioned medium prepared from confluent REVC,¹ bovine aortic endothelial, kidney, and RSV-CEF cells. Each well contained $20\text{ }\mu\text{g}$ of fibrinogen and 100,000 cpm. Fibrinolytic activity was determined by measuring the release of radioactivity from the surface of the dish into the assay buffer. The standard assay buffer contained in 1 ml: $4\text{ }\mu\text{g}$ of human plasminogen, 0.1% gelatin, and 0.1 M Tris-HCl, pH 8.1, supplemented with either $10\text{ }\mu\text{g}$ of extract, 10-50

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¹ The abbreviations used are: REVC, rabbit endothelial vena cava; RSV-CEF, Rous sarcoma virus-infected chick embryo fibroblasts; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

μ l of conditioned medium, or 0.025 International Units of urokinase (kindly provided by Dr. A. Johnson, New York University). Background fibrinolysis was less than 2% of the total counts per min/h of assay time and was subtracted from each sample. The results are an average of duplicate determinations and were subject to less than a 15% coefficient of variation. Fibrinolytic activity was not observed in the absence of plasminogen. The fibrinolytic activity of samples subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was assessed in a similar manner. Following electrophoresis, the gel was soaked in 2% Triton X-100 (8) for 1 h at 22 °C and then cut into 1-mm fractions. Each slice was added to assay buffer lacking a plasminogen activator source. The position of plasminogen activator in the gel was indicated by the release of radioactivity. Again, fibrinolysis required the presence of plasminogen.

Inhibitor Activity—Inhibitor activity was determined in three ways. In the first method, inhibitor activity was quantitated in mixing experiments in which the capacity of endothelial cell cytosol to depress the rate of fibrinolysis initiated by various plasminogen activators in the standard assay was determined. One unit of inhibitor activity was defined as the amount of cytosol required for a 50% reduction. The assay was terminated when the untreated controls hydrolyzed 15–25% of the total radioactivity. In the second approach, the effects of the endothelial cell cytosol inhibitor on fibrinolysis initiated by plasmin or urokinase were compared. Plasmin and urokinase were diluted to approximately equivalent fibrinolytic activities. The enzymes were then incubated for 5 min at 37 °C in the presence or absence of 10 μ g of cytosol protein. The mixtures were transferred to 125 I-labeled fibrin-coated wells, plasminogen was added to the urokinase sample, and the release of radioactivity from the surface of the dish into the buffer was monitored. Finally, plasminogen was iodinated with insolubilized lactoperoxidase (9) to a specific activity of $1-5 \times 10^6$ cpm/ μ g of protein and employed as a direct assay for plasminogen activator as described (10). The assay system was modified to contain 0.5% Triton X-100 in 0.1 M phosphate buffer, pH 7.2, supplemented with 100 μ g of albumin/ml, 1000 units of trypsin/ml (Calbiochem), 10 μ g of soybean trypsin inhibitor/ml (Miles Laboratories), 2×10^6 cpm of 125 I-plasminogen/ml, and 10 units of urokinase/ml. The samples were incubated for 30 min at 37 °C, and the reaction was stopped by boiling the sample in sample buffer (11). In this assay system, antiactivator activity was indicated by the ability of cytosol to block urokinase-mediated cleavage of single chain plasminogen into the characteristic plasmin "heavy" and "light" chains (12) as revealed by SDS-PAGE in the presence of β -mercaptoethanol, and by autoradiography.

SDS-PAGE—SDS-PAGE was performed in slab gels according to Laemmli (11). The upper stacking gel consisted of 4% acrylamide and the lower separating gel contained 10% acrylamide. The samples were brought to 2% SDS and applied to the gel. When β -mercaptoethanol (5%) was employed, the samples were boiled for 3 min immediately prior to electrophoresis. Gels to be assayed were sliced and treated as indicated above, while gels to be used for autoradiography were fixed and stained in 50% trichloroacetic acid, 0.1% Coomassie brilliant blue R-250 (Bio-Rad). After destaining in 10% glacial acetic acid, these gels were dried with a slab gel drier (Hoeffer Scientific Instruments) and placed on Kodak X-omat film to localize the radiolabeled protein. Apparent molecular weights were estimated from the relative migration of internal standards including human plasminogen (89,000), human serum albumin (65,000), ovalbumin (43,000), chymotrypsinogen (25,700), and bromphenol blue.

Miscellaneous—Plasminogen was isolated from human serum by affinity chromatography on lysine Sepharose (13). Protein was determined by the method of Lowry *et al.* (14). Radioactivity measurements were performed in a Packard γ -spectrophotometer. All chemicals and reagents were of the highest grade available.

RESULTS

Specificity of Inhibitor—We showed previously that the cytosol fractions from cultured REVC cells inhibited the fibrinolytic activity initiated both by human urokinase and by the membrane-associated plasminogen activator isolated from the REVC cells themselves (6). Based on these observations, we prepared a variety of samples to determine whether the inhibitor(s) also was capable of inhibiting fibrinolysis generated by other plasminogen activator sources. The plasminogen activator source was preincubated for 5 min with increasing concentrations of cytosol and then the mixture was added to

125 I-fibrin plates in the presence of human plasminogen. The fibrinolytic activity generated by human urokinase and by cultured rabbit kidney and endothelial cells was inhibited by the cytosol (Table I). In contrast, neither the fibrinolytic activity initiated by plasminogen activator extracted from bovine aortic endothelial cells, nor that released by RSV-CEF cells was inhibited, even in the presence of relatively high concentrations of cytosol.

The extent of inhibition varied considerably, in spite of the fact that the fibrinolytic activity of all mixtures resulted from the formation and activity of the same species of plasmin (*i.e.* human). These results suggested that the inhibitor activity attenuated plasminogen activator rather than plasmin *per se*. Therefore, the capacity of the endothelial cell cytosol to inhibit plasmin itself was tested. Cytosols were preincubated with the active enzyme, the mixture was added to 125 I-fibrin plates, and the release of radioactivity was monitored and compared to that observed for the enzyme in the absence of cytosol. The fibrinolytic activity of plasmin was not affected by the presence of cytosol (Fig. 1B). However, when urokinase was preincubated with the cytosol and then added to the plate in the presence of plasminogen, the rate of fibrinolysis was depressed relative to the untreated control (Fig. 1A).

Inhibition of Plasminogen Activation by Cytosol—Plas-

TABLE I
Specific activity of the cytosol inhibitor determined against different sources of plasminogen activator

Source of plasminogen activator	Specific activity inhibitor units/mg cytosol	Control %
Urokinase	1000	100
Kidney cells (conditioned medium)	1000	100
Kidney cells (extract)	333	33
REVC cells (extract)	200	20
RSV-CEF (conditioned medium)	<10	<1
Bovine aortic endothelial cells (extract)	<10	<1

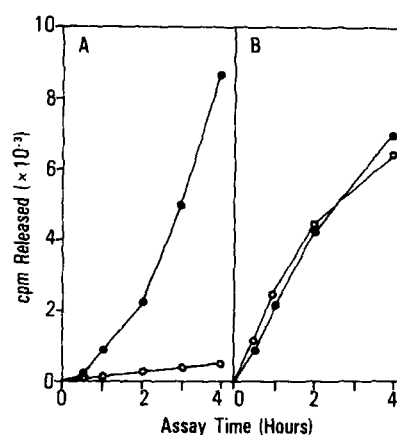


FIG. 1. Effect of cytosol on urokinase and plasmin-mediated fibrinolysis. Urokinase (A) or plasmin (B) were incubated with cytosol for 5 min at 37 °C and then added to 125 I-fibrin-coated wells. Plasminogen was added to the urokinase sample at this time. The fibrinolytic activity of each of these mixtures (○—○) was followed and compared to that of the control enzymes in the absence of cytosol (●—●).

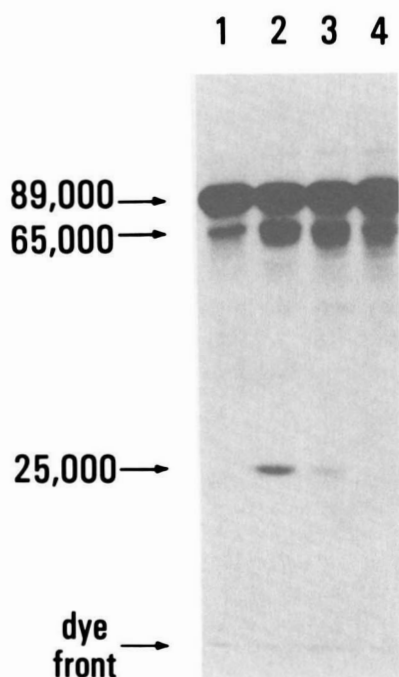


FIG. 2. Inhibition of plasminogen activation by cytosol. ^{125}I -Plasminogen (lane 1) was mixed with 10 units/ml of urokinase for 2 h at 37°C (lane 2) in the presence of 5 (lane 3) or 20 (lane 4) $\mu\text{g}/\text{ml}$ of cytosol. The samples were boiled in the presence of SDS and β -mercaptoethanol and analyzed by electrophoresis on 10% SDS slab gels. The gel was dried and subjected to autoradiography.

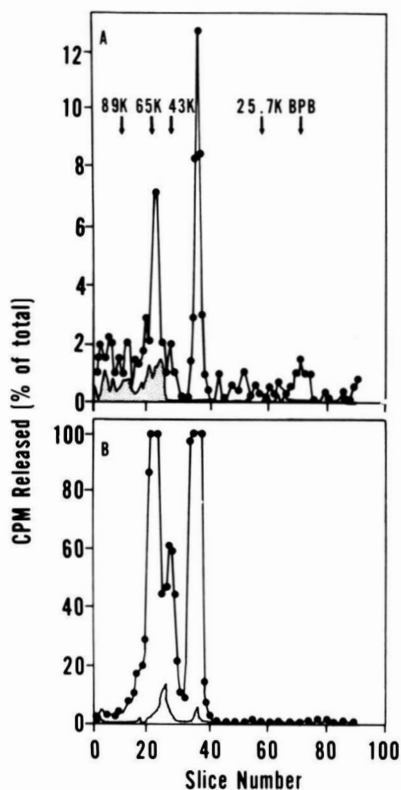


FIG. 3. Effect of cytosol inhibitor on the fibrinolytic activity of urokinase. Urokinase (●—●) and urokinase incubated with endothelial cell cytosol (stipple) were subjected to SDS-PAGE. The gels were cut and each slice was tested for plasminogen activator activity in the standard fibrin plate assay. Fibrinolysis was not observed in the absence of plasminogen. A, counts per min released after 4 h; B, counts per min released after 24 h. BPB, bromphenol blue.

minogen was iodinated and employed as a direct substrate for plasminogen activator. Although there was some variation from preparation to preparation, in all cases more than 75% of the total radioactivity was recovered in the plasminogen (Fig. 2, lane 1). Another 5 to 10% of the radioactivity was associated with a single polypeptide of $M_r = 65,000$ – $70,000$. The remaining 15 to 20% of the radioactivity was found as a rather homogeneous background throughout the gel. Incubation of the ^{125}I -plasminogen with urokinase resulted in conversion of the single chain disulfide-linked molecule into its characteristic "heavy" ($M_r = 65,000$) and "light" ($M_r = 25,000$) plasmin fragments (Fig. 2, lane 2). Although not shown, cleavage of plasminogen by urokinase was linear both with time (for at least 6 h) and concentration (from 0.1–100 units/ml of urokinase). When urokinase was added to plasminogen in the presence of 5 (lane 3) or 20 (lane 4) $\mu\text{g}/\text{ml}$ of cytosol, formation of the plasmin heavy and light chains was reduced in a dose-dependent manner.

The experiments illustrated in Fig. 3 indicate that the enzymatic activity of urokinase was irreversibly lost following exposure to cytosol. In these experiments, we took advantage of the observation that plasminogen activator activity can be recovered from polyacrylamide gels after electrophoresis in the presence of SDS (15). Urokinase and urokinase preincubated with endothelial cell cytosol were subjected to SDS-PAGE. After electrophoresis, the gels were sliced and each slice was added to ^{125}I -fibrin-coated Linbro wells and tested for its ability to activate plasminogen. Plasmin activity was observed in the untreated sample within 4 h, and was restricted to two regions of the gel (corresponding to M_r of 55,000 and 33,000) (Fig. 3A). These values are in agreement with the known M_r of urokinase (16). However, only minimal plasmin activity was observed in the gel containing the sample exposed to cytosol. When these fractions were assayed for an additional 20 h (Fig. 3B), a time sufficient to cause a number of the untreated control fractions to hydrolyze 100% of the fibrin, activity was still minimal.

DISCUSSION

A number of protease inhibitors have been detected in plasma (17) and the vessel wall (18). Those influencing fibrinolysis appear to act primarily by limiting the activity of plasmin. Platelets may also contribute to the inhibitor activity of the thrombus since they are known to contain antiplasmin activity (19, 20). Inhibitors of plasminogen activator may also be present in plasma (17) and platelets (20), but because of their extreme lability and the absence of direct, plasmin-independent assays for plasminogen activator, have been poorly characterized.

The results of this study indicate that rabbit endothelial cells also contain antifibrinolytic activity which may result from the presence of one or more inhibitors. This activity seems to differ in many important respects from inhibitors produced by other cells (21) or found in plasma. First of all, it is localized to the cytosol of a cell which does not circulate but rather is a component of the vessel wall itself. Unlike plasma inhibitors which may be retained in or circulate through the thrombus, and platelet-derived inhibitors which may be released into the thrombus during platelet aggregation and activation, the endothelial cell cytosol inhibitor(s) may be introduced into the forming thrombus as a direct consequence of local, endothelial cell damage. Localization of the inhibitor(s) to the cytoplasm of the cell would facilitate its efficient release at the earliest times after vascular injury. It is unclear whether this molecule(s) also may be released from these cells in response to specific physiologic stimuli, although preliminary studies indicate that an inhibitor activity is se-

creted into the medium conditioned by REVC cells.²

Unlike most fibrinolytic inhibitors, the endothelial cell inhibitor(s) appears to exert its effect on fibrinolysis at the level of plasmin formation, not plasmin activity. Evidence in support of this conclusion is based upon a number of direct and indirect observations. Thus, while the plasminogen-dependent fibrinolytic activity of a variety of rabbit plasminogen activators and of human urokinase was inhibited by the endothelial cell inhibitor (Table I), the fibrinolytic activity of a number of other plasminogen activator sources was relatively insensitive to the presence of this molecule. Since human plasminogen was employed in all of our experiments, the different sensitivities appear to reflect specificity at the level of plasminogen activator, not plasminogen or plasmin. This conclusion is consistent with the results shown in Fig. 1 where one can see that plasmin activity is unaffected by the cytosol under conditions in which urokinase-mediated fibrinolysis is completely blocked. In preliminary experiments,² we observed that the endothelial cell inhibitor blocked the activity of human urokinase but not human tissue activator (22), suggesting that the different specific activities listed in Table I may reflect the presence of either urokinase-like or tissue activator-like plasminogen activators. The results shown in Figs. 2 and 3 indicate that urokinase exposed to endothelial cell cytosol can no longer function as an activator of plasminogen. Thus, the ability of urokinase to cleave ¹²⁵I-plasminogen (10, 12) was inhibited by cytosol in a dose-dependent manner (Fig. 2); moreover, the ability of urokinase to convert plasminogen into plasmin was irreversibly lost (Fig. 3). In this latter case, our inability to detect urokinase activity either in the position of the enzyme or in the position of an enzyme-inhibitor complex after SDS-PAGE differs from the observations of Granelli-Piperno and Reich (8) who showed that protease-antiprotease complexes are often dissociated upon SDS-PAGE with subsequent regeneration of protease activity.

An inhibitor with similar antiactivator but not antiplasmin activity recently was detected in placenta (23). In addition, it has been reported that cultured human endothelial cells (24, 25), as well as dexamethasone-induced hepatoma cells (26), and uterine flushings from progesterone-treated pigs (27) may also contain an antiactivator. The use of the ¹²⁵I-plasminogen cleavage assay for plasminogen activator should facilitate the identification and characterization of other molecules with such activity.

² David J. Loskutoff, unpublished observation.

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