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Purification and Characterization of a Plasminogen Activator Secreted by Cultured Human Pancreatic Carcinoma Cells†

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ABSTRACT: A plasminogen activator secreted by cultured human pancreatic carcinoma (Mia PaCa-2) cells has been purified to apparent homogeneity by procedures including Sepharose-L-arginine methyl ester affinity chromatography, Sephadex G-200 gel filtration, isoelectric focusing, and sodium dodecyl sulfate gel electrophoresis. The plasminogen activator shares many properties with urokinase including: molecular weight (55 000), isoelectric point (8.7), heat stability (60 °C, 30 min), pH stability (1.5-10), and its mode of activation of plasminogen. The intracellular enzyme is membrane bound and can be solubilized by detergent. Solubilized activator has a molecular weight similar to that of the secreted enzyme as determined by sodium dodecyl sulfate gel electrophoresis. The production of plasminogen activator by Mia PaCa-2 cells is totally inhibited by actinomycin D and cycloheximide.

Many publications appeared recently describing increased fibrinolytic activity associated with viral transformation. Unkeless et al. (1973) and Ossowski et al. (1973) described increased fibrinolytic activity in chick embryo fibroblast after transformation by Rous sarcoma virus and in hamster fibroblast after transformation by SV-40 virus. In subsequent studies, they provided evidence that their fibrinolytic system consisted of two proteins: a serum factor from the medium identified as plasminogen (Quigley et al., 1974), and a cell factor, a specific serine protease which functioned as a plasminogen activator (Unkeless et al., 1974). The activator secreted by SV-40 transformed hamster fibroblast has also been purified and characterized by Christman and Acs (1974). Recently, Chen and Buchanan (1975) reported on a plasminogen-independent fibrinolytic activity secreted by Rous sarcoma transformed chicken fibroblast cells.

In an earlier communication Yunis et al. (1973) reported the secretion of "fibrinolysin" by rat ovarian tumor cells in culture. In subsequent studies, we identified in conditioned serum-free medium, prepared from cultured rat breast carcinoma R2426 cells, two separable fibrinolytic activities, a direct fibrinolysin and a plasminogen activator (Wu et al., 1975; Schultz et al., 1975). The direct fibrinolysin is distinct from plasmin both biochemically and immunologically. The activator is similar to urokinase in molecular size, mode of activation of plasminogen, and other properties (Wu et al., 1976).

This report will describe the purification and characterization of a plasminogen activator from conditioned serum-free medium of cultured human pancreatic carcinoma cells.

Materials and Methods

Human plasminogen was prepared from fresh plasma by the procedure of Deutsch and Mertz (1970). Urokinase, Trasylol, and streptokinase were purchased from Calbiochem, La Jolla, Calif.; thrombin was from Upjohn, Kalamazoo, Mich.; human fibrinogen was from Cutter, Berkeley, Calif.; *p*-tosyl-*N*-arginine methyl ester, Sepharose 4B, arginine methyl ester, *N*-α-aceryl-L-lysine methyl ester, soybean trypsin inhibitor, Sephadex, and Triton X-100 were from Sigma Chemical Co., St. Louis, Mo.; cyanogen bromide was from Eastman, Rochester, N.Y.; Ampholine was from LKB, Rockville, Md.; acrylamide, bisacrylamide, and sodium dodecyl sulfate were from Bio-Rad, Richmond, Calif. Culture media, fetal calf serum, and horse serum were purchased from GIBCO, Grand Island, N.Y. Dulbecco's modified Eagle medium (DME)[1] was prepared from powder form and fortified with 10% fetal calf serum and 2.5% horse serum (DME-HSFC).

Cell Culture. Human pancreatic carcinoma cell line, Mia PaCa-2 (Yunis et al., 1977), was established according to previously described procedures (Wu et al., 1975; Yunis et al., to be published). Cells are grown in Dulbecco's modified Eagle's medium fortified with 10% fetal calf serum and 2.5% horse serum.

Preparation of Serum-Free Conditioned Medium. Serum-free conditioned medium from cultured pancreatic carcinoma was prepared as previously described (Wu et al., 1975). When cell growth had reached confluence, the medium was aspirated and the plates were rinsed three times with

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[1] Abbreviations used: DME, Dulbecco's modified Eagle medium; HSFC, 2.5% horse serum-10% fetal calf serum; SFCM, serum-free conditioned medium; Tame, *N*-*p*-tosyl-L-arginine methyl ester; Tris, tris(hydroxymethyl)aminomethane; Alme, *N*-α-acetyl-L-lysine methyl ester; EDTA, ethylenediaminetetraacetic acid.

TABLE I: Summary of Purification.

Purification	Total Protein (mg)[a]	Total Act. (unit)	Spec Act. (unit/mg)	Fold Purification	Recovery
1. Conditioned serum free medium	850	8000	9.4	1	100
2. Amicon	850	8000	9.4	1	100
3. Sepharose-arginine methyl ester column	38.2	6800	180	19	85
4. Sephadex G-200	0.630	5680	8 900	950	71
5. Isoelectric focusing	0.075	4960	66 000	7000	62

[a] Protein concentration was determined according to Lowry et al. (1951) from steps 1 to 3 and according to Waddell (1956) in steps 4 and 5, using bovine serum albumin as standard.

Hanks' balanced salt solution. Serum-free DME was then added to each plate and the incubation continued for an additional 72 h after which the serum-free conditioned medium (SFCM) was harvested and centrifuged to remove any cell debris.

Assay of Plasminogen Activator. The following assays were employed: (1) fibrin-agar plate assay described by Nilsson et al. (1972) was used in most experiments. The plasminogen activator activity from cultured cells was compared with standard urokinase (Calbiochem No. 400925) and expressed in plough units; (2) hydrolysis of *N*-*p*-tosyl-L-arginine methyl ester (Tame) according to the method of Hummel (1959) by following increase in absorbance at 247 nm using a Zeiss recording spectrophotometer. The incubation mixture contained 1 mM Tame-10 mM $CaCl_2$ in 0.04 M Tris-HCl, pH 8.1. The reaction was started by the addition of enzyme. (3) Hydrolysis of *N*-α-acetyl-L-lysine methyl ester (Alme) after the procedure of Sherry et al. (1964) with slight modification. Samples in volumes of 50 μL were incubated with 50 μL of 0.1 M Alme in 0.15 M NaCl-0.1 M potassium phosphate buffer (pH 7.6 at 37 °C). Methanol released during the hydrolysis was measured according to the procedure of Siegelman et al. (1962).

Sepharose-L-Arginine Methyl Ester Affinity Chromatography. Sepharose 4B was first activated by cyanogen bromide according to standard procedures (Cuatrecasas et al., 1968). It was then coupled with arginine methyl ester in 0.1 M $NaHCO_3$ (pH 8.9) overnight in the cold room. The gel was washed with 0.1 M sodium phosphate buffer (pH 7.4) containing 3 mM EDTA for at least 20 gel volumes before packing the column. For purification of plasminogen activator on affinity column, 1 L of SFCM prepared as described above was first concentrated to 50 mL by ultracentrifugation on Amicon using PM 10 membrane. The concentrated sample was then dialyzed overnight against 2 L of 0.1 M sodium phosphate buffer (pH 7.4) containing 3 mM EDTA. Triton X-100 was added to the dialyzed sample to the final concentration of 1% and the sample was applied to affinity column (bed volume 50 mL). The column was washed with the same phosphate buffer until the OD_{280} was less than 0.01 (10 column volumes) and then eluted with 0.5 M arginine in the same buffer. Fractions were collected and assayed for protein (OD_{280}) and fibrinolytic activity on fibrin-agar plate. The active fractions were pooled, concentrated by Amicon, and applied to a Sephadex G-200 column for further purification.

Sephadex G-200 Gel Filtration. The concentrated sample from affinity column was applied to a Sephadex G-200 column (2.0 × 70 cm, bed volume 220 mL), equilibrated with 0.15 M NaCl-3 mM EDTA-0.1% NaN_3 in 0.1 M sodium phosphate buffer (pH 6.5). Again, fractions were collected and assayed for protein and fibrinolytic activity.

Isoelectric Focusing. This experiment was carried out on polyacrylamide gel according to standard procedures (Wrigley, 1971). The active concentrated pool from Sephadex was dialyzed against distilled water for 2 h, then against 5% sucrose overnight. It was then brought up to 10% sucrose and loaded on top of the gels (100-μL sample each) and protected with a layer of 1% ampholine in 5% sucrose (200 μL). Focusing was carried out in the cold for 2 h at 4 mA per gel and maximum of 500 V. The remainder of the protein samples that did not enter the gels were combined (pool 1). Gels were then sliced (2 mm/slice) and the pH and fibrinolytic activity of individual slices determined. The first slice from each gel was combined with the corresponding slices from others and homogenized in 0.1 M phosphate buffer (pH 7.4). The extract (pool 2) was combined with pool 1 and concentrated.

Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed by the method of Davis (1964). Sodium dodecyl sulfate gel electrophoresis was carried out by the procedure of Weber and Osborn (1969). Concentrated samples from isoelectric focusing were incubated with 0.1% sodium dodecyl sulfate at room temperature for 30 min and then electrophoresed in 10% acrylamide gel at 8 mA per gel for 7 h. Gels were sliced (2 mm/slice) and slices were assayed for fibrinolytic activity on fibrin-agar plates. Active slices were combined and extracted with 0.1 M phosphate buffer (pH 7.4) containing 0.1% sodium dodecyl sulfate. The gel extract was concentrated and part of the concentrate was rerun on sodium dodecyl sulfate gel to examine the homogeneity of the enzyme. The molecular weight was determined using the following standard proteins: (1) rabbit muscle phosphorylase (94 000), (2) bovine serum albumin (68 000), (3) γ-globulin heavy chain (50 000), and (4) γ-globulin light chain (23 500). (The numbers 1-4 correspond to those indicated in Figures 4 and 6.)

Results

Purification. The overall purification from five separate runs combined is summarized in Table I. The experimental results of each step were as follows.

(1) Sepharose-L-Arginine Methyl Ester Affinity Column. The binding of plasminogen activator from conditioned serum-free medium was poor if the medium was applied directly to the affinity column. The activity emerged immediately after the big protein peak, indicating that the enzyme was only partially retained. About 60% of the total activity was washed out of the column by 0.1 M phosphate buffer (pH 7.4) and the remaining 40% eluted by 0.5 M arginine. However, the addition of Triton X-100 (1%) greatly enhanced the binding of plasminogen activator with virtually all the activity (90%) becoming bound to the affinity column. A typical chromatography profile is shown in Figure 1. A 15-20-fold purification was consistently achieved. There was also considerable

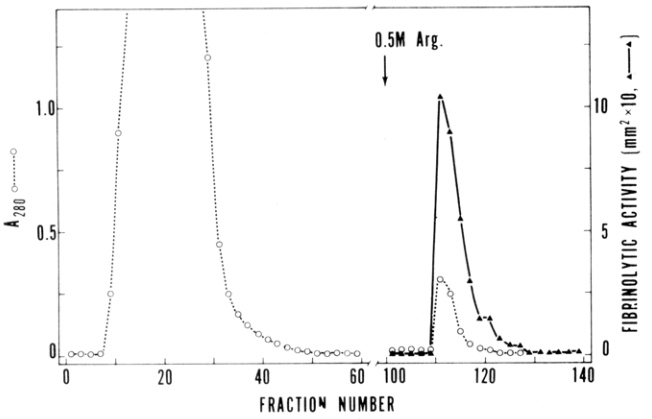

FIGURE 1: Sepharose–L-arginine methyl ester affinity chromatography. Conditioned serum-free medium (1 L) prepared from Mia PaCa-2 cell culture was concentrated and applied to the affinity column according to procedure described in Methods. Five milliliters per fraction was collected.

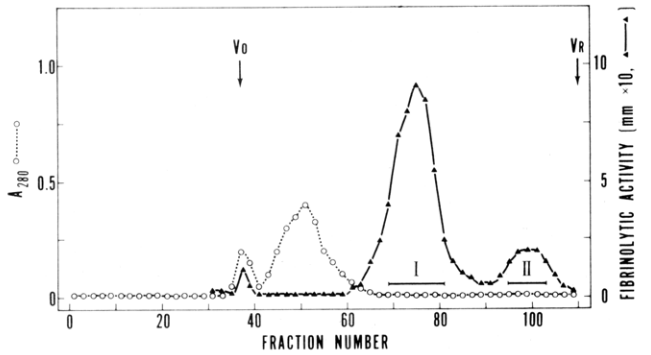

FIGURE 2: Sephadex G-200 gel filtration. Fractions containing activity from affinity column were pooled, concentrated to 2.0 mL (3.9 mg/mL, 1360 units), and filtered on Sephadex G-200. Two and one-half milliliters per fraction was collected. See Methods.

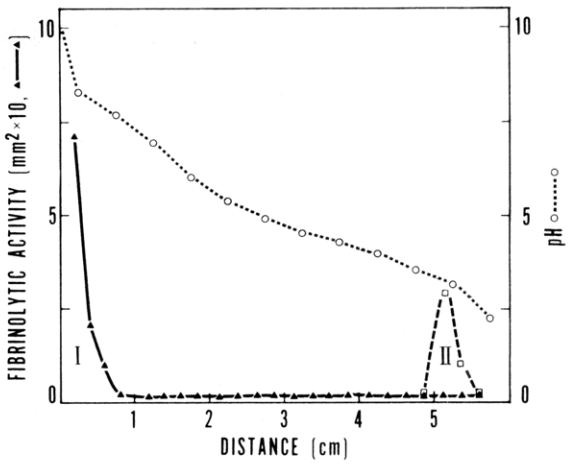

FIGURE 3: Isoelectric focusing. Activity peaks (I and II) from Sephadex G-200 were focused on polyacrylamide gel. Activity I (▲—▲); activity II (□—□). For detailed procedures, see Methods.

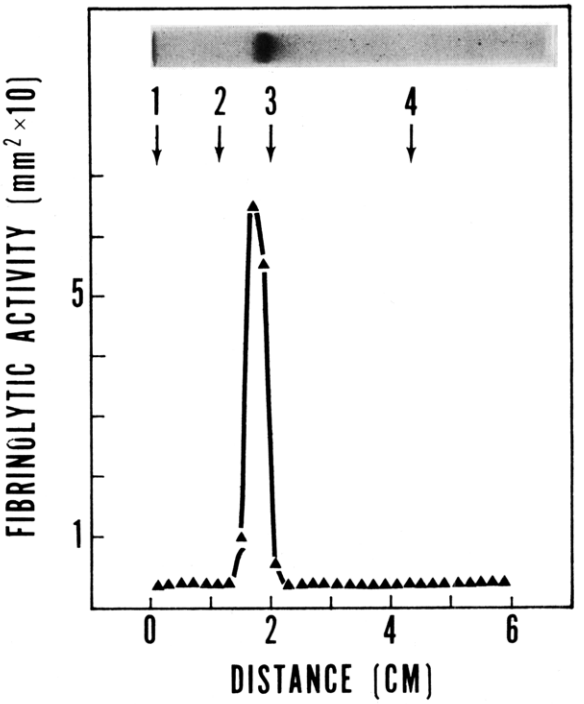

FIGURE 4: Homogeneity of Mia PaCa-2 plasminogen activator. Purified plasminogen activator from sodium dodecyl sulfate gel electrophoresis is rerun on duplicate sodium dodecyl sulfate gel (15 μg of protein each) along with standard proteins. For detailed procedures, see Methods.

nonspecific binding of proteins, the bulk of which was identified as albumin by analytical gel electrophoresis. However, most of this could subsequently be separated from plasminogen activator activity by Sephadex G-200 gel filtration.

(2) Sepharose G-200 Gel Filtration. As shown in Figure 2, the bulk of proteins eluted from Sepharose have high molecular weights and are almost completely separable from the plasminogen activator. A very small amount of activity appeared on the void volume and probably represents aggregated enzyme as indicated from our previous studies. The major activity peak (PI) comprised about 90% of the total activity but had no measurable absorbance at 280 nm. The lower molecular weight peak (PII) which comprised about 10% of the total activity in this preparation was probably a degradation product of PI as observed with urokinase (Lesuk et al., 1967) and with plasminogen activator from rat breast carcinoma R2426 (Wu et al., 1976). The active fractions from PI were pooled and concentrated by Amicon. An additional 50-fold purification was achieved by this step.

(3) Isoelectric Focusing. The partially purified plasminogen activator form Sephadex G-200 was subjected to isoelectric focusing for further purification. A typical profile is shown in Figure 3. The activity peak I from Sephadex G-200 was focused on the first slice of the gel and some of it remained in the top of the gel, indicating a PI value greater than 8.0. The PI value was reexamined using narrow pH range gels (8–10) and was determined to be 8.7. The second peak (II) was focused at the very end of the gel, indicating a PI value of approximately 3. The high isoelectric point for the plasminogen activator was an advantage allowing an additional eight-fold purification from this step.

(4) Sodium Dodecyl Sulfate Gel Electrophoresis. The Mia PaCa-2 plasminogen activator from step 3 (isoelectric focusing) was further purified by sodium dodecyl sulfate gel electrophoresis as described under Methods. The apparent homogeneity of the purified enzyme is shown in Figure 4. A single active protein band was obtained.

Characterization of Mia PaCa-2 Plasminogen Activator. (1) Molecular Weight. The molecular weight of activator was estimated from the gel filtration profile of Figure 2 and sodium dodecyl sulfate gel electrophoresis of Figure 4 to be 55 000. The low molecular weight activator shown in Figure 2 has an

TABLE II: Thermostability.[a]

Temp (°C)	Time of Incubation (min)	% Activity Mia PaCa-2 Activator	% Activity R2426 Activator	% Activity Urokinase
Room temp	30	100	100	100
50	30	100	100	100
60	30	100	100	100
70	30	19	20	21
80	5	5	4	6
90	5	0	0	0

[a] Samples were incubated at the indicated temperatures and time intervals and then assayed on fibrin–agar plates.

TABLE III: pH Stability.[a]

pH	Percent Activity Mia PaCa-2 Activator	Percent Activity R2426 Activator	Percent Activity Urokinase
1.5–10	100	100	100
11	15	10	10
12	0	0	0

[a] Samples were adjusted to indicate pH in citrate–phosphate–borate buffer and kept in the cold for 24 h and then assayed on fibrin–agar plates after neutralizing to pH 7.5.

estimated molecular weight of 30 000. For comparison the plasminogen activator was run simultaneously with urokinase on 10% sodium dodecyl sulfate gel. The activity profiles showed both urokinase and the plasminogen activator to have identical mobility, similar to what we have observed in our studies on plasminogen activator from R2426 rat breast carcinoma cells (Wu et al., 1976).

(2) Thermostability. The thermostability of the plasminogen activator is summarized in Table II. Samples were heated at temperatures from 40 to 70 °C for 30 min and 80 to 100 °C for 5 min. After heating, samples were chilled in ice and assayed on fibrin–agar plate. The activity remaining was expressed as percentage of control samples kept at room temperature. The results indicated that plasminogen activator of Mia PaCa-2 is stable at 60 °C for 30 min. However, almost all activity was lost at 80 °C for 5 min. This thermostability is very similar to plasminogen activator from rat breast carcinoma R2426 and to urokinase.

(3) pH Stability. For this experiment citrate–phosphate–borate buffer (pH 3 to 10) was used. However, at lower and higher pH range, the pH was adjusted by adding either HCl or NaOH. Samples at the indicated pH were kept in the cold room for 24 h. They were dialyzed against 0.05 M Tris-HCl buffer (pH 7.5) before assay. As shown in Table III, plasminogen activator from cultured human pancreatic carcinoma cells was stable at the pH range from 1.5 to 10 similar to urokinase and plasminogen activator from rat breast carcinoma R2426.

(4) Activation of Human Plasminogen. The mode of activation of human plasminogen by urokinase has been studied extensively (Summaria et al., 1975; Walther et al., 1974). Human plasminogen from plasma is a single peptide protein with a molecular weight of 87 000. Upon activation by urokinase, it is converted into two peptides with molecular weights of 55 000 and 25 000, respectively. These two peptides are cross-linked by a disulfite bond. The Mia PaCa-2 activator activates human plasminogen similar to urokinase as shown in Figure 5. The sodium dodecyl sulfate gel patterns resulting from the activation of plasminogen by both Mia PaCa-2 activator and urokinase are identical, indicating a similar or identical mode of activation.

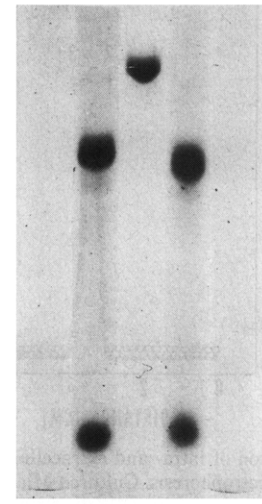

FIGURE 5: Activation of human plasminogen. Human plasminogen (500 μg) was incubated with either Mia PaCa-2 plasminogen activator or urokinase (200 units) in 25% glycerol and Trasylol (2000 units) for 18 h at 25 °C. They were heated in the presence of mercaptoethanol (5%) and sodium dodecyl sulfate (0.1%). From left to right: (1) Mia PaCa-2 plasminogen activator; (2) activator and plasminogen; (3) human plasminogen; (4) plasminogen + urokinase; (5) urokinase.

(5) Esterase Activity on Synthetic Substrate. The Mia PaCa-2 activator also showed esterase activity toward some synthetic substrate such as *N*-*p*-tosyl-L-arginine methyl ester (Tame) and *N*-α-acetyl-L-lysine methyl ester (Alme). Although the activator will specifically cleave the arginyl–valine bond in plasminogen during activation, the hydrolytic activity with Tame is only about $\frac{1}{20}$ of that with Alme. The pH optimum for the esterase activity was about 7.5. It is inhibited by benzamidine and diisopropyl fluorophosphate but not by trasylol or soybean trypsin inhibitor. These properties are also similar to those of urokinase, although detailed kinetic studies were not done.

Comparison of Intra- and Extracellular Enzymes. We have found that the intracellular enzyme is membrane bound. When cultured cells were harvested, homogenized, and centrifuged at 40 000*g* (Sorvall) for 30 min, virtually all the activator remained in the pellet. The activator could be solubilized from the pellet by either sodium dodecyl sulfate or Triton X-100 detergent. The intracellular and the secreted enzyme were compared on sodium dodecyl sulfate gel electrophoresis illustrated in Figure 6. Activator activity, as assayed on fibrin–agar plates from both enzyme samples showed identical mobility on the sodium dodecyl sulfate gel, indicating identical molecular weight. The intracellular enzyme in transformed chicken fibroblast was reported to be located in the lysosomal fractions as studied by differential centrifugation fractionation (Unkeless et al., 1974). The intra- and extracellular enzymes also have identical molecular weight.

Synthesis of Plasminogen Activator. In order to investigate whether the plasminogen activator in conditioned serum-free DME medium is preformed or is synthesized and secreted into the medium during the incubation, the following experiment was done. Cells were grown until confluence and rinsed with

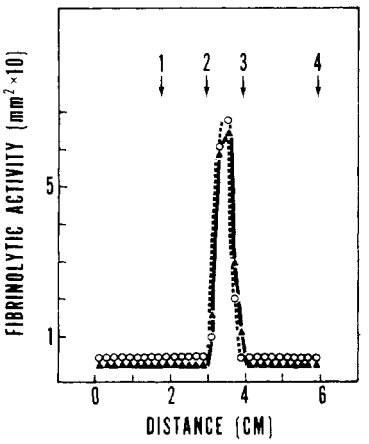

FIGURE 6: Comparison of intra- and extracellular enzyme on sodium dodecyl sulfate gel electrophoresis. Cultured Mia PaCa-2 cells in 0.1 M phosphate buffer (pH 7.0) were homogenized with a Virtis homogenizer at maximum speed for three interrupted 1-min periods and centrifuged at 40 000*g* for 30 min. The pellet was extracted with 0.1% sodium dodecyl sulfate in 0.1 M phosphate buffer, pH 7.0. The plasminogen activator from cell extract (200 μg, 1.5 units) and conditioned medium (200 μg, 2 units) was compared on sodium dodecyl sulfate gel electrophoresis. The activity profile of intracellular enzyme (O—O) superimposed on that of extracellular enzyme (▲—▲).

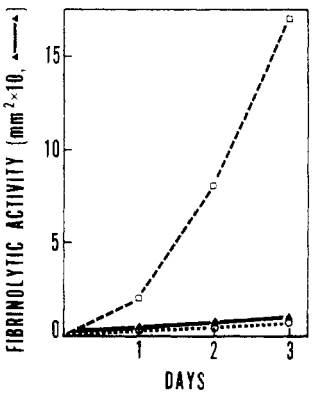

FIGURE 7: The effect of actinomycin D and cycloheximide on the production of plasminogen activator. When Mia PaCa-2 cells were grown to confluence, the medium was aspirated, each plate was rinsed with Hanks' balanced salt solution three times, and serum-free DME medium and drugs were added. Aliquot of the medium was withdrawn at time intervals and assayed for activator activity. Control (□); actinomycin D (0.1 μg/mL) (▲); cycloheximide (0.1 μg/mL) (O).

Hank's balanced salt solution three times, and serum-free DME containing drugs at the indicated concentration were added to the culture plates. Aliquots were withdrawn at time intervals and assayed for activator activity. As can be seen from Figure 7, actinomycin D and cycloheximide at the concentration of 0.1 μg/mL completely inhibited the appearance of activator activity in the medium. Cells from each plate were collected and homogenized in phosphate-buffered saline using a Virtis homogenizer. Activator activity was measured after solubilization by the addition of Triton X-100 (to 1%). The intracellular enzyme level thus determined was the same in drug treated cells and in controls, indicating no accumulation of intracellular enzyme in the drug-treated cells. These results suggest that the activator is synthesized and secreted into the surrounding medium during incubation.

Discussion

We have demonstrated in the present study that human pancreatic carcinoma cells in continuous culture secrete an activator of plasminogen which could be purified to homogeneity. The procedures used could be applied for the purification of activator both from serum-free conditioned medium or from whole medium containing fetal calf and horse sera. An important observation in this regard was the enhancement of binding of activator to the arginine methyl ester substituted Sepharose gel by the addition of Triton X-100. A similar observation has been reported by Unkeless et al. (1974) in their purification of plasminogen activator from transformed chicken embryo fibroblasts. These authors used the ionic detergent sodium dodecyl sulfate for the same purpose. Although the activator from Mia PaCa-2 is stable in 0.1% sodium dodecyl sulfate, the trace amount of sodium dodecyl sulfate bound to the enzyme was difficult to remove and thus hindered further purification.

The low molecular weight activator that we have observed in this study has also been found in conditioned medium from rat breast carcinoma R2426 cells and in urokinase preparations. Lesuk et al. (1967) reported an activator or molecular weight 34 000 in their urokinase preparations which had a specific activity twice that of urokinase. They provided evidence that this low molecular weight activator was a product of the proteolytic degradation or urokinase by uropepsin present in the urine. Mochan et al. (1975) also reported two forms of plasminogen activator from transformed adult kidney cells, a major one with an isoelectric point (PI) of 8.3 and another with a PI of 4.7. The two were immunologically identical as shown by immunodiffusion. The major and minor forms of activator from Mia PaCa-2 had isoelectric points of 8.7 and 3, respectively, the latter probably representing a degradation product of the former, but further studies are needed to confirm this conclusion.

The thermostability and pH stability of the Mia PaCa-2 activator are similar to those of urokinase and the activator from R2426 cells. We have frequently observed an increase in activator activity if the pH is lowered to 3 prior to purification. This is probably due to acid inactivation of an inhibitor. Currently we routinely pretreat the conditioned medium at pH 3 for 2 h at room temperature prior to purification.

The activation of human plasminogen by Mia PaCa-2 activator yields two peptide subunits identical in electrophoretic mobility with those resulting from activation by urokinase. This suggests that Mia PaCa-2 activator and urokinase activate plasminogen by similar mechanisms with probably the same arginyl-valine bond cleavage (Summaria et al., 1967). Also, as with urokinase, the hydrolytic activity of Mia PaCa-2 activator or *N*-*p*-tosyl-L-arginine methyl ester is much lower than on *N*-α-acetyl-L-lysine methyl ester.

It would appear from our studies that Mia PaCa-2 activator is membrane bound but can be solubilized by detergents such as Triton X-100 and sodium dodecyl sulfate. The solubilized enzyme has a molecular weight identical with that of the secreted activator as determined by sodium dodecyl sulfate gel electrophoresis. Continued synthesis of activator appears to be required for the secretion of measurable activity into the surrounding medium; both actinomycin D and cycloheximide completely inhibited the appearance of activator activity in the medium. Of interest is the effect of cytosine arabinoside, an inhibitor of DNA synthesis, which actually caused a several-fold increase in activity in the medium. The mechanism of this effect is at present uncertain.

Acknowledgments

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Analysis and Computer Simulation of Aerobic Oxidation of Reduced Nicotinamide Adenine Dinucleotide Catalyzed by Horseradish Peroxidase†

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ABSTRACT: Under suitable experimental conditions the aerobic oxidation of NADH catalyzed by horseradish peroxidase occurred in four characteristic phases: initial burst, induction phase, steady state, and termination. A trace amount of H_2O_2 present in the NADH solution brought about initial burst in the formation of oxyperoxidase. About 2 mol of oxyperoxidase was formed per mol of H_2O_2. When a considerable amount of the ferric enzyme still remained, the initial burst was followed by an induction phase. In this phase the rate of oxyperoxidase formation from the ferric enzyme increased with the decrease of the ferric enzyme and an approximately exponential increase of oxyperoxidase was observed. A rapid oxidation of NADH suddenly began at the end of the induction phase and the oxidation continued at a relatively constant rate. In the steady state, oxygen was consumed and H_2O_2 accumulated. A drastic terminating reaction suddenly set in when the oxygen concentration decreased under a certain level. During the reaction, H_2O_2 disappeared accompanying an accelerated oxidation of NADH and the enzyme returned to the ferric form after a transient increase of peroxidase compound II. Time courses of NADH oxidation, O_2 consumption, H_2O_2 accumulation, and formation of enzyme intermediates could be simulated with an electronic computer using 11 elementary reactions and 9 rate equations. The results were also discussed in relation to the mechanism for oscillatory responses of the reaction that appeared in an open system with a continuous supply of oxygen.

The aerobic oxidation of NADH or NADPH catalyzed by plant peroxidases has been investigated mostly in reaction systems containing activators such as Mn^{2+} and certain phenols (Akazawa and Conn, 1958; Williams-Ashman et al., 1959; Klebanoff, 1960; Kalyanaraman et al., 1975). The reaction between horseradish peroxidase and NADH without activators has been studied by Yokota and Yamazaki (1965a). The nature of the reaction is found to be very peculiar. In the presence of a large amount of peroxidase, the oxidation of NADH sets in after a distinct lag period and the reaction comes to a rapid termination on account of O_2 consumption. During the ter-

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