Purification and Properties of a Plasminogen Activator from Cultured Rat Prostate Adenocarcinoma Cells†

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ABSTRACT: Zymographic analysis of the supernates from confluent cultures of a rat prostate adenocarcinoma cell line, PA-III, revealed the existence of two molecular forms of specific plasminogen activators, one of molecular weight of approximately 80 000 and another of approximate molecular weight of 45 000, in sodium dodecyl sulfate. The low molecular weight form has been purified 364-fold in 66% yield from the culture medium by a combination of gel filtration on Sephacryl S-200 and affinity chromatography on Sepharose 4B–benzamidine. The purified material possessed a specific activity of 192 000 urokinase CTA units mg^{-1}. This enzyme displayed activity toward human Glu_1-plasminogen, characterized by a K_m of 1.7 ± 0.2 μM and a V_{max} of 0.53 ± 0.1 pmol of plasmin min^{-1} $unit^{-1}$. A synthetic chromogenic substrate, H-D-Ile-Pro-Arg-*p*-nitroanilide (S-2288), was found for the activator. The enzyme possessed a K_m of 0.33 mM and a k_{cat} of 55 s^{-1} for S-2288. The activator was found to be a serine protease, inhibited by diisopropyl fluorophosphate (iPr_2PF). At a concentration of 1 mM iPr_2PF, and 30 nM enzyme, the half-time of this inhibition was 3.8 min. The 45 000 molecular weight enzyme was found to be inhibited by rabbit antibodies to human urokinase, thus characterizing the activator as a member of the urokinase class. The 80 000 molecular weight enzyme was not neutralized by anti-human urokinase but was neutralized by rabbit anti-human melanoma activator, likely allowing it to be classified as the tissue activator type.

Plasminogen is the plasma protein precursor of the fibrinolytic enzyme plasmin and occurs in the plasma of all mammalian species studied. Activation of plasminogen can be accomplished by proteins, termed plasminogen activators, which are found in many types of cells, various tissues, and body fluids. The molecular mechanism of activation of human plasminogen by all known activators involves cleavage of the Arg_{560}–Val_{561} peptide bond (Robbins et al., 1967), which results in conversion of the single-chain zymogen of 790 amino acid residues (Sottrup-Jensen et al., 1978) into a two-chain, disulfide-linked serine protease, plasmin (Robbins et al., 1967).

Plasminogen activators have been found in a variety of cells and cell supernates. Their presence in many cells which are migratory and/or invasive (Strickland et al., 1976; Beers et al., 1975; Unkeless et al., 1974) suggests that conversion of plasminogen to plasmin may be of importance in tissue remodeling and cell migration. Further, levels of plasminogen activators have been found to be significantly increased in cells which are malignant (Corasanti et al., 1980; Markus et al., 1980) and in culture supernates of neoplastic cells (Rifkin et al., 1974; Wilson et al., 1980). Although the plasminogen activators produced in these systems can possess a variety of molecular weights (Vetterlein et al., 1979; Harvey et al., 1982), they have been classified only into two groups. One type is neutralized by antibodies to human urokinase and does not interact with fibrin (Thorsen et al., 1972). These enzymes are called "urokinase-like" activators. Another type is not neutralized by anti-urokinase (Bernik & Kwaan, 1969) but is neutralized by antibodies prepared to certain tissue activators, such as pig heart activator (Cole & Bachmann, 1977), and does interact with fibrin (Thorsen et al., 1972). These activators are termed "tissue activator-like".

While elevated levels of plasminogen activators present in, and excreted by, transformed cells are well documented, the function of these proteins in development, propagation, and metastasis of neoplastic cells is not well understood. Thus, the characterization of activators produced in various tumor systems is of fundamental importance to achieving this goal. Of interest here is the utilization of animal model systems which resemble their counterpart diseases in man for this work. We have developed a rat prostate adenocarcinoma cell line (PA-III), derived from germ-free Lobund Wistar rats, which has served as a unique model for prostatic cancer (Pollard, 1977; Chang & Pollard, 1977; Pollard & Luckert, 1979). Since these cells have been shown to spread uniformly and predictably through the lymphatics only to lung (Pollard & Luckert, 1979; Pollard, 1980), this same cell line could be of significance in the study of metastasis. As an initial step in utilizing this cell line to study metastatic phenomena, and the possible involvement of plasminogen activators in such, we have examined the nature of the activators produced by these cells and have, in this report, biochemically characterized one such protein.

Materials and Methods

Proteins. Human Glu_1-plasminogen (Glu_1-Pg), variant 2, was purified by affinity chromatography (Deutsch & Mertz, 1970), as modified by Brockway & Castellino (1972).

Human fibrinogen was purified by the procedure described by Morris et al. (1981).

Low molecular weight kidney urokinase was generously provided by Dr. William H. Holleman of Abbott Laboratories. High molecular weight urokinase, as well as rabbit antibodies against low and high molecular weight urokinases, was provided by Dr. Grant H. Barlow of the Michael Reese Research Foundation. The urokinase activity reference standard was purchased from Calbiochem.

Cell Culture and Plasminogen Activator Harvest. The rat prostate adenocarcinoma cell line PA-III was a cloned epithelial cell line which was originally derived from the prostate adenocarcinoma of an aged germ-free Lobund Wistar rat (Pollard, 1973; Chang & Pollard, 1977). The PA-III cells have been adapted to grow in a serum-free, chemically defined medium (Chan, 1981). The cells were propagated in the MB–DF medium plus penicillin (100 units/mL) and strep-

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tomycin (100 μg/mL) in a 2.5% CO_2 environment at 37 °C.

Confluent monolayers of PA-III cells growing in Corning Flasks (150-cm^2 growing area) were washed 3 times with Hank's balanced salt solution and incubated with 15 mL of MB–DF medium without any growth factors, except 5α-dihydrotestosterone (100 ng/mL). After 72 h, the spent medium was harvested and centrifuged at 9000*g* for 15 min at 4 °C. The supernate was employed for purification of plasminogen activator.

Purification of the Low Molecular Weight Plasminogen Activator. The culture supernatant was placed in dialysis bags (M_r cutoff <20 000) around which was packed solid poly(ethylene glycol) (M_r 20 000), at 4 °C. After concentration of the medium to 50–100-fold was effected, the contents of the bags were removed and layered on a Sephacryl S-200 column (1.5 cm × 90 cm), equilibrated and eluted at 8 mL/h with a buffer consisting of 0.05 M tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl)/0.5 M NaCl/0.01% Triton X-100, pH 7.4, at 4 °C. An aliquot (0.025 mL) of each fraction (1.5 mL) was removed and assayed for plasminogen activator activity.

Fractions containing such activity were pooled and directly applied to a column of Sepharose 4B–benzamidine (1.0 cm × 3.0 cm) equilibrated with the above buffer at 4 °C. After the column was washed with 3–4 bed volumes of this buffer, the activator was eluted with a solution consisting of 0.05 M Tris-HCl/0.5 M NaCl/2.0 M guanidine hydrochloride (Gdn·HCl)/0.01% Triton X-100, pH 7.4. Fractions containing the desired activator activity were pooled, dialyzed against a buffer consisting of 0.025 M Tris-HCl/0.1 M NaCl, pH 7.4, and stored at 4 °C.

Assay for Plasminogen Activator. Here, two assays were employed, one for screening column eluates and another used to quantitate levels of plasminogen activator.

In the former assay, 0.05-mL aliquots of Glu_1-Pg (1 mg/mL in 50 mM Tris-HCl/0.1 M lysine, pH 7.4) were added to separate test tubes, followed by 0.025 mL of the column eluate. The resulting solution was allowed to incubate for 1 h at 37 °C. A total of 0.1 mL of a 1 mM solution of substrate, H-D-Val-Leu-Lys-*p*-nitroanilide (S-2251), was added and the reaction allowed to proceed for 5 min at 37 °C. After this point, 0.15 mL of a solution of 50% acetic acid was added to inactivate the enzyme. The absorbance of *p*-nitroanilide released was determined at 405 nm and used as a monitor of the plasmin produced (the incubation conditions should be adjusted such that amounts not greater than 30% of the substrate are hydrolyzed).

Another assay was employed to determine specific activities of the activator. In this case, the following solutions were added to cuvettes at 37 °C; 0.4 mL of a 1 mM solution of S-2251; 0.31 mL of a buffer consisting of 0.05 M Tris-HCl/0.1 M NaCl, pH 7.4; 0.04 mL of a 20 μM solution of Glu_1-Pg. The activation of Glu_1-Pg was initiated by addition of 0.05 mL of plasminogen activator, or standard urokinase. The rate of plasmin generation was continuously monitored by measurement of the increase in absorbance at 405 nm, due to hydrolysis of S-2251 by the resulting plasmin formed, in a Cary 219 recording spectrophotometer. The curve obtained was replotted according to $\Delta A_{405}/t$ vs. t. The slope of this final graph was determined and plotted against the activity of urokinase in order to generate a standard activity curve, which was then employed to quantitate plasminogen activity in terms of urokinase units.

Fibrin Autography. The location of plasminogen activators on sodium dodecyl sulfate slab gels, by their ability to lyse fibrin/agar indicator gels, was evaluated by the procedure of Levin & Loskutoff (1982).

Steady-State Kinetics. K_m and V_{max} values for the purified activator toward the synthetic substrate D-Ile-Pro-Arg-*p*-nitroanilide (S-2288) were determined in a buffer of 0.05 M Tris-HCl/0.1 M NaCl, pH 7.4, at 37 °C. Substrate levels were varied between 0.1 and 3.0 mM. For the evaluation of hydrolytic rates, a Cary 219 recording spectrophotometer was employed at 37 °C.

K_m and V_{max} values for the purified activator (and urokinase) toward human Glu_1-Pg were evaluated essentially as described by Strickland et al. (1982), in the same buffer as used above. Plasminogen concentrations were varied between 0.25 and 4.0 μM. Glu_1-Pg was incubated with 6.7 units of the activator for 2 min at 37 °C. An aliquot (0.15 mL) was then removed, and the amount of plasmin generated was measured by initial rate analysis toward S-2251. This latter substrate was present at a concentration of 0.5 mM. The plasmin concentration was determined by comparison of the rate obtained to a standard curve, generated by measuring the initial hydrolysis rates of different plasmin concentrations at 0.5 mM concentrations of S-2251. In all cases, less than 3% of the plasminogen was converted into plasmin, thereby ensuring that the activation was terminated at a point at which a linear rate of plasmin production was occurring.

Comparative Binding of the Adenocarcinoma Cell Activator and Urokinase to Fibrin. A total of 0.2 mL of a solution of human fibrinogen (1 mg/mL) in 0.01 M phosphate/0.15 M NaCl, pH 7.4 (PBS), was mixed with 0.1 mL of a PBS solution of the activator or urokinase (14–15 units) and 0.1 mL of a PBS solution of human serum albumin (Miles). The fibrinogen was clotted by addition of 0.001 mL of bovine thrombin (0.25 unit/mL), and the mixture was allowed to incubate at 37 °C for 30 min. After this time, the sample was subjected to low-speed centrifugation and the supernatant removed. The clot was washed twice with 0.3 mL of a buffer consisting of 0.05 M Tris-HCl/0.5 M NaCl/0.01% Triton X-100, pH 7.4. After each wash, the samples were subjected to centrifugation and all washes combined with the original clot supernate. The clots were then extracted overnight at 4 °C with a solution of 0.05 M Tris-HCl/1.0 M KSCN/0.01% Triton X-100, pH 7.4 (Rijken & Collen, 1981).

The amount of activator or urokinase in the total clot supernate and washes, as well as in the clot extract, was determined by the assay described above.

Analytical Procedures. Isoelectric focusing was performed at 4 °C by using an LKB 110-mL column and LKB ampholines (pH range 3–10), as described by Sodetz & Castellino (1972).

Protein concentrations at the various stages of activator purification were determined by using the Bio-Rad protein assay kit, with bovine serum albumin (Miles) as a standard.

Results

It has previously been shown, qualitatively, that cell culture supernates from the rat prostate adenocarcinoma cell line PA-III produced and secreted plasminogen activator (Pollard & Chan, 1981). However, the types of activator produced were not characterized. Prior to our undertaking such an investigation, we required a reproducible, sensitive, and readily quantifiable assay for detection of plasminogen activator. Such an assay has been developed, based upon activation of human Glu_1-plasminogen by test samples and determination of the rate of plasmin production by the chromogenic substrate S-2251 as described under Materials and Methods. Since the substrate is present throughout the activation, the spectro-

Table I: Purification of a Plasminogen Activator from the Culture Supernates of the Rat Prostate Adenocarcinoma Cell Line PA-III

material	volume (mL)	total protein (μg)	activity (urokinase units)	sp act. (units/mg)	yield (%)	x-fold purification
culture supernate	300	5460	2886	528	100	1
Sephacryl S-200	13.4	549	2036	3707	71	7
Sepharose–benzamidine	7.4	10	1920	192000	66	364

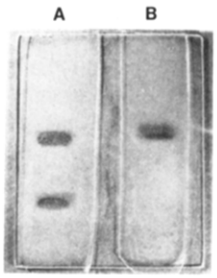

FIGURE 1: Fibrin autographs of the activators present in the 72-h supernate from culture rat prostate adenocarcinoma cell line PA-III after separation by sodium dodecyl sulfate gel electrophoresis. (A) Culture supernate. (B) As in (A) except that rabbit anti low molecular weight urokinase was added to the fibrin plate.

photometer trace will be a parabola, which can be fitted to equations similar to those described for rectilinear motion with constant acceleration (Kosow, 1975). Plots of $\Delta A_{405}/t$ vs. t, at different levels of activator (in this case, urokinase), yield a series of straight lines, with the slope of each proportional to the initial rate of activation. A replot of the slopes obtained, vs. the concentration of urokinase, yields a straight line, which can be employed as a standard curve for measurement of activator concentrations in terms of urokinase units. The advantages of this assay are that the initial rates of plasmin generation are being measured, resulting in a linear dependence of urokinase or plasminogen activator concentration, and that the assay is readily performed and sufficiently sensitive, since as little as 0.25 urokinase unit/mL can be easily measured.

The total levels of activator secreted from the cell line PA-III as a function of time after confluent monolayers have been established, employing the assay above, have been determined. After an initial lag period, activator activity is produced in the supernate and increases up to at least a 3-day period. For the purpose of this study, we have harvested the medium at 72 h as a balance between sufficient production of activator and possible degradation of such by other secreted proteases. At this point, we have made no attempts to maximize activator production through media manipulations, since our object was to chemically define the activator(s) secreted.

After separation of the proteins present in the culture supernatants, fibrin autographic analysis of the activators secreted into the culture medium (Figure 1A) showed only two lysis bands, revealing the existence of two activator types. No lysis zones were present when plasminogen was omitted from the fibrin plate, demonstrating that the lytic properties of the activators were dependent on the presence of plasminogen.

Subsequent to concentration of the culture supernate, the material was chromatographed on Sephacryl S-200. The elution profile is presented in Figure 2. Clearly, activator activity, as monitored by the assay described under Materials and Methods, is well separated from the bulk protein. All high molecular weight activator is present in the bulk protein of Figure 2, well resolved from the lower molecular weight activator. The activity of the high molecular weight activator is not observed in Figure 2, due to the nature of the assay shown here. This activator is virtually nondetectable in the absence of fibrin, which greatly stimulates its activity. Fibrin

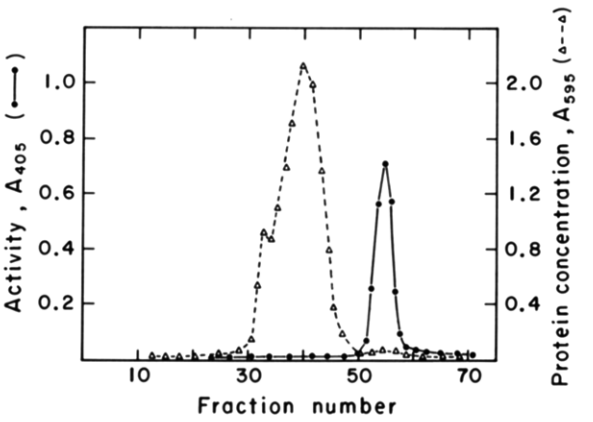

FIGURE 2: Chromatography of the culture medium on a Sephacryl S-200 column. Concentrated supernatant was layered on the column (1.5 × 90 cm) and eluted at a flow rate of 8 mL/h. The column was equilibrated with 50 mM Tris-HCl/500 mM NaCl/0.01% (v/v) Triton X-100, pH 7.4. Aliquots from each fraction were assayed for plasminogen activator activity and for protein content by using the procedures described under Materials and Methods.

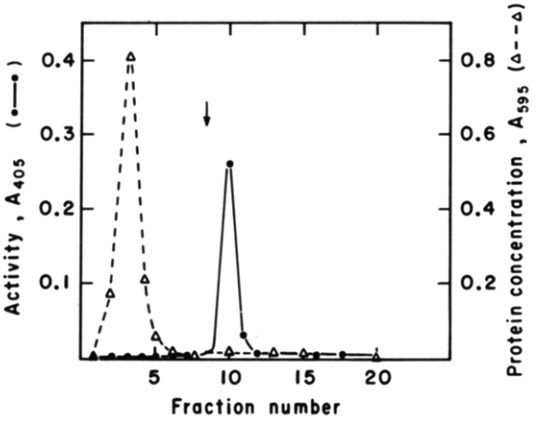

FIGURE 3: Further purification of the plasminogen activator on a Sepharose–benzamidine column. The pool from the Sephacryl S-200 column was applied directly to a Sepharose–benzamidine column (1.0 × 3.0 cm) equilibrated with 50 mM Tris/500 mM NaCl/0.01% Triton X-100, pH 7.4. The column was eluted at a flow rate of 60 mL/h, and 1-mL fractions were collected. Plasminogen activator was eluted with 50 mM Tris-HCl/500 mM NaCl/2.0 M Gdn·HCl/0.01% Triton X-200, pH 7.4. Aliquots from each fraction were assayed for plasminogen activator activity and for protein content by using the procedures described under Materials and Methods.

is not present in the assays of Figure 2. Identification of the fractions containing high molecular weight activator was based upon sodium dodecyl sulfate gel electrophoresis of each of the fractions, followed by fibrin autography. The high molecular weight activator was not significantly contaminated by the low molecular weight activator, and the opposite is also the case. Further purification of the lower molecular weight activator was achieved by adsorption to a column of Sepharose 4B–benzamidine, as shown in Figure 3. Again, significant purification is readily observable. A summary of the purification steps and the purification achieved is presented in Table I. A 364-fold purification, in 66% yield, resulted from these steps. The specific activity of the final product was approximately

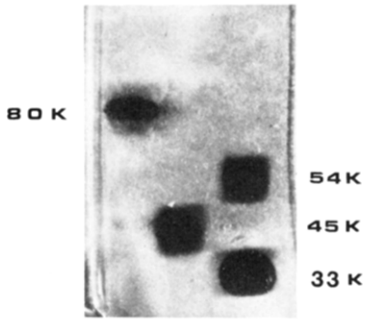

FIGURE 4: Fibrin autographs of various activators after separation by nonreduced sodium dodecyl sulfate gel electrophoresis. Tissue activator (left lane), purified rat prostate adenocarcinoma cell activator (middle lane), and a mixture of low and high molecular weight urokinases (right lane) were subjected to electrophoresis on nonreduced sodium dodecyl sulfate slab gels. The activator activity was localized, as the dark zones, by overlay on a fibrin/agar slab. The molecular weights of the zones were determined by calibrating a duplicate-stained sodium dodecyl sulfate slab gel with proteins of known molecular weight.

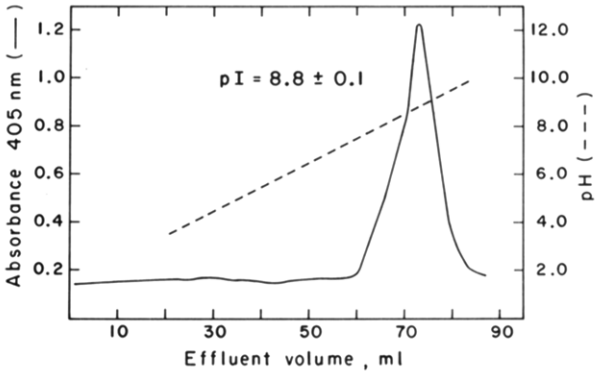

FIGURE 5: Isoelectric focusing profiles of the rat adenocarcinoma cell plasminogen activator focused at 4 °C on a pH 3–10 gradient. Fractions of approximately 1 mL were collected and assayed for their pH (---) and ability to activate plasminogen (—) by the screening assay described under Materials and Methods. Activations were carried out for 1 h at 37 °C. The absorbance at 405 nm represents the extent of plasmin hydrolysis of the substrate, S-2251, after a 5-min time period.

192 000 units/mg. A nonreduced sodium dodecyl sulfate gel, stained for protein, demonstrated that the activator was approximately 90–95% pure.

In order to determine the molecular weight of the purified activator, and to establish its relationship to urokinase or to tissue activator, comparative fibrin autographs were performed on the tissue activator, on high and low molecular weight urokinases, and on the Sepharose 4B–benzamidine eluted prostate activator. The results are shown in Figure 4. The single zone obtained for the activator corresponds to a molecular weight of approximately 45 000, identical with that observed by staining the sodium dodecyl sulfate gel, a size intermediate to low and high molecular weight urokinase. When rabbit antibodies to human low molecular weight urokinase are included in the fibrin gel, the lysis zones of the low and high molecular weight urokinases as well as the purified prostate cell activator disappear. However, the tissue activator lytic zone is not affected. Similar results are obtained when rabbit antibodies to high molecular weight urokinase are added to the fibrin gel. These same findings occur when the anti-urokinase antibodies are added to the fibrin plate after sodium dodecyl sulfate gel electrophoresis of the crude culture supernates (Figure 1B). Here, fibrin autography also shows the disappearance of the lytic zone of the 45 000-dalton activator. This shows that the purified activator possesses properties similar to the same activator in the culture supernate.

The neutralizing effects of the anti-urokinase antibodies toward the urokinase-like activator have been employed to estimate the levels of each activator in the prostrate supernates. When the area on the lytic zone on a fibrin gel was measured on an aliquot of the culture supernates, before and after neutralization of the urokinase-like activator with anti-urokinase antibodies, it was found that approximately 50–70% of the total activator activity was of the tissue activator type.

The purified activator was subjected to preparative isoelectric focusing, and the profile obtained is shown in Figure 5. Here, the activity of enzyme toward Glu_1-plasminogen activation was determined in each column fraction, as described above, and correlated to the pH of the same fraction. The activator appears to focus in a major peak, with a p*I* of 8.8 ± 0.1. Another activator isoelectric form may be present on the ascending portion of the peak, with a slightly lower p*I* value.

Next, we examined the activation of human Glu_1-plasminogen by the prostate adenocarcinoma activator. Molecular

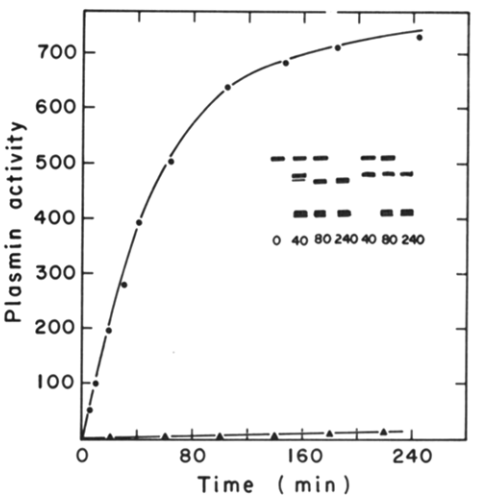

FIGURE 6: Activation of Glu-plasminogen by the purified plasminogen activator. The activation was carried out at 37 °C in a total volume of 400 μL. The final concentration of plasminogen was 1 μM, while the concentration of plasminogen activator was 10 units/mL. The buffer used consisted of 50 mM Tris/50 mM lysine/0.01% Triton X-100, pH 7.4. At various time intervals, 40-μL aliquots were removed and assayed for plasmin activity by using the substrate D-Val-Leu-Lys, PNA (S-2251), while 50-μL aliquots were removed for sodium dodecyl sulfate–polyacrylamide gel electrophoresis, in the absence (insert, left four lanes) of trasylol. (●) Glu_1-plasminogen and activator; (▲) Glu_1-plasminogen. The three right lanes of the inset (times of 40, 80, and 240 min) represent sodium dodecyl sulfate gel electrophoretograms of the same activation mixture in which trasylol (100 units/mL) was added prior to initiation of the assay.

events which occur in this activation are shown in Figure 6. Here, activator was added to Glu_1-plasminogen, and aliquots were removed and assayed for amidolytic activity. It is seen that little plasmin activity is generated in the absence of activator, whereas high levels of plasmin activity were generated in its presence. Samples at various time points were also analyzed by sodium dodecyl sulfate gel electrophoresis, shown in the insets of Figure 6. At times of 40 min (lane 2), plasminogen is converted to its component heavy and light chains. At longer times of 80 and 240 min (lanes 3 and 4, respectively), progressively more plasmin is generated, and the heavy chain is converted to a lower molecular weight form. This latter phenomena is prevented when trasylol (basic pancreatic inhibitor) is added to the activation mixture and samples are removed at times of 40 (lane 5), 80 (lane 6), and 240 min (lane 7). Here, of course, no plasmin activity is generated since

trasylol blocks plasmin activity. The activity of the activator is not affected by trasylol.

Lineweaver–Burk analyses of the initial rates of activation of Glu_1-plasminogen both with low molecular weight urokinase and with the isolated rat prostate adenocarcinoma cell plasminogen activator were performed. The K_m values obtained for Glu_1-plasminogen activation were similar; 2.7 ± 0.5 μM for urokinase and 1.7 ± 0.2 μM for the prostate activator. The V_{max} for this activation was 0.33 ± 0.05 pmol of plasmin min^{-1} $unit^{-1}$ with urokinase and 0.53 ± 0.1 pmol of plasmin min^{-1} $unit^{-1}$ with the prostate activator.

In examining various chromogenic peptides, as possible substrates for the isolated activator, we found that H-D-Ile-Pro-Arg-*p*-nitroanilide (S-2288) was hydrolyzed by this enzyme. The reaction rate, as a function of substrate concentration, was analyzed by a Lineweaver–Burk plot. The kinetic constants characterizing this amidolytic reaction were found to be 0.33 ± 0.04 mM for the K_m and 55 ± 3 s^{-1} for the k_{cat}.

We have also demonstrated (data not shown) that the rat prostate adenocarcinoma cell activator is a serine protease, inhibited by diisopropyl fluorophosphate (iPr_2PF). At 30 nM activator and 1 mM iPr_2PF, the half-life of the enzyme activity is 3.8 min. A control experiment, in the absence of iPr_2PF, in which 2-propanol was added at a concentration equivalent to that present in the iPr_2PF solution, demonstrated that the activator was stable under the conditions of the assay and that inhibition was the result of iPr_2PF inactivation.

Finally, the binding of urokinase and the prostate activator to fibrin has been assessed, as described under Materials and Methods. Employing this procedure, we have recovered approximately 60–67% of each activator in the clot supernates and clot extracts. Of the material recovered, only 3–7% of both urokinase and prostate activator were extracted from the clot, suggesting that the clot binding properties of the activator are very similar to those of urokinase.

Discussion

Previous reports have documented the existence of spontaneous prostate adenocarcinomas, of unknown etiology, in nine aged germ-free Lobund Wistar (LW) rats (Pollard, 1973, 1975). Of these, transplantable tumor lines have been derived from three such rats and have been propagated as cell monolayers (Chang & Pollard, 1977). The tumors, and the corresponding cell lines, have been designated PA-I, PA-II, and PA-III and can be differentiated by means of differences in their morphology, histology, and metastatic patterns (Pollard, 1980). Cell line PA-III, which has been employed in this study, has been shown to spread in LW rats after subcutaneous injection, in all animals and via ipsilateral lymphatics to the lungs (Pollard, 1980). The fact that this tumor line is a true carcinoma, that it spread without artificial manipulation and with high frequency to a predetermined distant organ, and that it spread completely and reproducibly suggests that the PA-III line is an excellent animal tumor model for its counterpart disease in man.

In an effort to discover features of the PA-III line which may be common and specific to metastatic tumors, studies on enzymes produced by these cells have been undertaken. Since there are indications in the literature that changes in hydrolytic enzymes may be associated with neoplasia (Wallach, 1976; Dano & Reich, 1978; Quigley, 1979), and since it has been shown in many studies that alterations in plasminogen activator content occur in tumor cells and their culture supernates [e.g., see Wilson et al. (1980)], we have examined the plasminogen activator secreted by PA-III cells in culture. Previously, it has been shown that the culture supernate of these cells produces a substance responsible for the plasminogen-dependent fibrinolytic activity exhibited by PA-III (Pollard & Chan, 1981).

Fibrin autographs of the culture supernates of PA-III, after sodium dodecyl sulfate gel electrophoresis, clearly establish the presence of two types of secreted plasminogen activator. An 80 000 molecular weight form and a 45 000 molecular weight form, both dependent on plasminogen for expression of their fibrinolytic activity, are present in the supernate 72 h after confluency of the cells is achieved. We have not extensively studied the time course of production of each activator, at this stage, since our current objective was to characterize the types of activators present. Interestingly, it has recently been shown that activators of the same molecular weight are present in rat plasma, in rat liver perfusates, and in culture supernates of rat hepatocytes (Sharoni et al., 1982).

The two types of plasminogen activators which exist in the culture supernates of the PA-III cell lines can be separated by gel filtration on Sephacryl S-200, and the low molecular weight form has been isolated by affinity chromatography on Sepharose–benzamidine. The isolated activator possessed a specific activity as high as any purified to date, and greater than the two molecular weight forms of urokinase. The data of Figure 4 show that its mobility in sodium dodecyl sulfate gels is intermediate to those for the low and high molecular weight forms of urokinase. Under our harvesting conditions, the activator was not present as a zymogen, but as a serine protease, inhibited by diisopropyl fluorophosphate. Its activity was not enhanced by proteases such as plasmin, which has been shown to activate zymogen forms of human urokinase (Wun et al., 1982).

From the data presented herein, it is clear that the isolated 45 000 molecular weight form of the activator shares common properties with urokinase and should be classified as a "urokinase-like" activator. While the molecular weight and isoelectric point of the isolated activator are similar to those for human urokinase, the most convincing evidence supporting the view that the activator is a urokinase-like enzyme is that the plasminogen-dependent fibrinolytic properties of the prostate activator were neutralized by antibodies to human urokinase. Excellent confirmation of the urokinase-like properties of the activator is obtained from the lack of binding of the activator to fibrin clots, a property shared by urokinase (Thorsen et al., 1972) and other activators similar to urokinase (Aasted, 1981; Wilson et al., 1980), but not by tissue activators (Rijken & Collen, 1981). Further, the similarity of the K_m and k_{cat} values of the prostate activator and urokinase toward activation of human Glu_1-plasminogen, and their large differences from the same properties of the tissue plasminogen activator (Hoylaerts et al., 1981), lends additional credence to the identification of the 45 000 molecular weight prostate activator with urokinase.

The molecular mechanism of activation of Glu_1-plasminogen also appears very similar to that of urokinase (Violand & Castellino, 1976). The data of Figure 6 show that the predicted heavy and light chains of plasmin are produced in parallel to the expression of plasmin activity, that the heavy chain is degraded to a lower molecular weight form, and that this latter process is inhibited by trasylol, a plasmin inhibitor with little effect on the activator. These same properties are common to activations carried out with urokinase as the activator (Violand & Castellino, 1976).

We have not currently undertaken an extensive investigation with the 80 000 molecular weight activator seen in the culture supernates of the PA-III cell line. We do believe, however,

that this activator is of the tissue activator type due to the similarity of its molecular weight with other activators thus classified (Wilson et al., 1980), due to the fact that fibrin stimulates its activity, and due to the observation that the plasminogen-dependent fibrinolytic activity of the material present in the culture supernatant is neutralized by antibodies to the human melanoma activator, an enzyme previously classified as a tissue-type activator (Rijken & Collen, 1981).

The fact that neoplastic cells produce plasminogen activators at levels in excess of normal cells has been conclusively demonstrated in many past studies; however, most of these have been qualitative in nature, and little work has appeared on systematic characterization of the biochemical properties of these activators. Most neoplastic cell lines produce, almost exclusively, urokinase-like activators, and the presence of the tissue-type activator in the PA-III prostate adenocarcinoma cell line is an important occurrence. In order to understand the relationship between the production of the two types of activators and neoplastic and metastatic phenomena, and the influence of fibrinolysis on this phenomena, and in order to understand the regulation of activator production at the geneomic level, it is important to define the biochemical characteristics of activators produced in a variety of cell types. The particular animal model cell line employed here, rat prostate adenocarcinoma PA-III, secretes at least two types of plasminogen activators, related both to urokinase and to tissue activator. Studies under way are aimed at attempting to correlate the production of each type of activator with the metastatic behavior of these cells.

Registry No. S-2288, 77672-35-6; plasminogen, 9001-91-6; urokinase, 9039-53-6.

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