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Purification and Characterization of the Plasminogen Activator Secreted by Human Melanoma Cells in Culture*

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The plasminogen activator secreted by a cultured human melanoma cell line was purified and compared with urokinase and with tissue plasminogen activator from human uterus. The purification procedure consisted of chromatography on zinc chelate-agarose, concanavalin A-agarose, and Sephadex G-150 in the presence of 0.01% (v/v) Tween 80. The purified material was obtained from the culture medium with a yield of 46% and a purification factor of 263. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed one main band with a molecular weight of about 72,000, and in the presence of reducing agents, two bands of 33,000 and 39,000. Addition of the protease inhibitor Aprotinin to the culture media and column buffers yielded a one-chain plasminogen activator with a molecular weight of about 72,000. One molecule of activator reacted with about one molecule of [^{3}H]diisopropylfluorophosphate. The melanoma plasminogen activator and the uterine tissue plasminogen activator appeared to be very similar on dodecyl sulfate-polyacrylamide gel electrophoresis, amino acid analysis, and amidolytic properties. Both activators bound to fibrin clots, while urokinase did not. In immunodiffusion, as well as in quenching experiments of the fibrinolytic activities, the melanoma plasminogen activator appeared to be immunologically identical with the uterine tissue plasminogen activator, but unrelated to urokinase. All these findings indicate that the plasminogen activator secreted by human melanoma cells in culture is very similar to, or identical with, the plasminogen activator found in normal tissue, but different from urokinase.

The inactive proenzyme plasminogen is enzymatically converted into plasmin by plasminogen activators which are widely distributed in the body. The best known mammalian activators are urokinase, found in urine, and tissue plasminogen activator, found in tissue extracts (1). The latter activator is presumably located in the endothelial cells (2) and probably represents the same enzyme as the vascular plasminogen activator, which is found in blood vessel perfusates and in blood after certain stimuli (3, 4). Urokinase and tissue plasminogen activator are immunologically unrelated molecules (4, 5).

Plasminogen activators can also be produced by cells in culture; especially tumor cells which may produce high levels of activator (reviewed by Christman *et al.* (6)). Plasminogen activators from human tumor cells can be classified as urokinase-like and non-urokinase-like activators (7-10). The present study deals with the purification and characterization of a plasminogen activator secreted by a human melanoma cell culture. The activator is compared with urokinase, and with a tissue plasminogen activator, recently purified from human uterus (11). Our findings indicate that the melanoma plasminogen activator is very similar to, or identical with, the uterine tissue plasminogen activator. A preliminary report of these results has been published elsewhere (12).

EXPERIMENTAL PROCEDURES

Plasminogen-containing and plasminogen-free bovine fibrinogen was obtained from Poviet, Organon-Teknika, Oss, The Netherlands, and human urokinase (M_r = 54,000) from Choay. The *World Health Organization 1st International Reference Preparation of Human Urokinase* (66/46) was supplied by the National Institute for Biological Standards and Control, London, U. K. Thrombin was obtained from Leo Pharmaceuticals, Ballerup, Denmark; Aprotinin (Trasylol) from Bayer; the low molecular weight calibration kit for SDS[1]-gel electrophoresis, Sepharose 4B, Protein A-Sepharose CL-4B, concanavalin A-agarose, and Sephadex G-150 (superfine) from Pharmacia; H-D-valyl-glycyl-L-arginine-*p*-nitroanilide (S-2322) from Kabi; [^{3}H]-DFP from New England Nuclear; modified Eagle's minimum essential medium, sodium bicarbonate (7.5% solution), and L-glutamine (200 mM solution) from Flow Laboratories; and newborn calf serum from Gibco. Zinc chelate-agarose was prepared by coupling iminodiacetic acid to Sepharose 4B, according to the method of Porath *et al.* (13), and by saturating this material with zinc chloride (7.3 mM). The gel was regenerated by washing consecutively with 0.05 M EDTA, pH 8.0, 0.05 M NH_4HCO_3, pH 10.5, and water, and by resaturating with zinc chloride.

Human tissue plasminogen activator was purified from uterine tissue as described earlier (11), with a minor modification: Tween 80 (Baker), was added to all column buffers (0.01% (v/v) final concentration) to prevent adsorption to glassware. The specific activity of the purified activator, determined as described below, was 48,000 IU/mg of protein. When indicated, Aprotinin was added to all buffers used during isolation and purification to a final concentration of 25 KIU/ml, except to that used for gel filtration.

Antisera against human uterine tissue plasminogen activator and high molecular weight human urokinase (M_r = 54,000) were raised in rabbits as described earlier (5). The antiserum against the melanoma plasminogen activator was raised in a rabbit by injecting 100 μg of purified protein, dissolved in 0.5 ml of saline, and emulsified with 0.5 ml of Freund's complete adjuvant. Booster injections of 50 μg were given at 2-week intervals. Serum was collected 1 week after the second booster. The IgG fractions of the antisera were isolated by affinity chromatography on Protein A-Sepharose (14) and dialyzed against 0.15 M NaCl solution.

An established human melanoma cell line (Bowes) was obtained from Dr. D. B. Rifkin, Rockefeller University, New York. The cells were grown to confluent monolayers in plastic tissue culture flasks (175 cm^2, style 3028, Falcon Labware) at 37 °C in atmospheric air

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[1] The abbreviations used are: SDS, sodium dodecyl sulfate; DPF, diisopropyl phosphofluoridate; IgG, immunoglobulin G; KIU, kallikrein inhibitor units.

supplemented with 6% of CO_2. The growth medium consisted of 100 ml of modified Eagle's essential medium supplemented with sodium bicarbonate (16 ml of a 7.5% solution per liter of medium), L-glutamine (10 ml of a 200 mM solution per liter of medium), and heat-inactivated newborn calf serum (final concentration, 10%). Then the cells were washed with medium without calf serum and incubated with 25 ml of serum-free medium. The medium was harvested and replaced on 3 consecutive days, centrifuged at 7,000 × *g* for 30 min, and stored at −20 °C until use. When indicated, Aprotinin was added, both to the serum-containing and to the serum-free medium (20 KIU/ml, final concentration).

The fibrinolytic activities of plasminogen activator solutions were determined on plasminogen-containing bovine fibrin plates (15) and expressed in IU using the *World Health Organization 1st International Reference Preparation of Urokinase*, as described earlier (11). Aprotinin, when present in the plasminogen activator solutions, reduced the response on the fibrin plates. The same amount of Aprotinin was therefore added to the standard urokinase solutions.

SDS-polyacrylamide gel electrophoresis was performed according to Weber and Osborn (16) using 7% gels. For the electrophoresis of ^{3}H-labeled compounds, the cross-linking reagent *N,N'*-methylenebisacrylamide (0.23%) was replaced by *N,N'*-diallyltartardiamide (1.36%), which allowed dissolution of gel slices in 2% periodic acid (17). Plasminogen activator preparations were concentrated by dialysis against distilled water containing 0.001% (v/v) Tween 80 followed by lyophilization. Molecular weights were determinated with the use of a low molecular weight calibration kit (Pharmacia, Uppsala, Sweden).

Amino acid compositions were determined with a 1911 CL Beckman amino acid analyzer after hydrolysis of the protein samples with 6 M HCl *in vacuo* at 110 °C for 20 h.

Protein concentration was measured by the method of Lowry *et al.* (18), using bovine albumin as the reference.

Active site titration with [^{3}H]DFP was performed on two melanoma plasminogen activator preparations with specific activities of 96,000 and 115,000 IU/mg. The preparations were dissolved in 1 ml of 0.15 M NaCl, 0.01 M phosphate buffer, pH 8.0, containing 0.001% (v/v) Tween 80 to a concentration of 64 or 70 μg of protein per ml. These solutions were mixed with 100 μl of 3 mM [^{3}H]DFP (specific activity, 0.33 Ci/mmol) and incubated overnight at room temperature. After extensive dialysis against 0.15 M NaCl containing 0.001% (v/v) Tween 80, the incorporation of [^{3}H]DFP in the activator was measured in a liquid scintillation counter.

The adsorption of plasminogen activators (melanoma activator, uterine activator, and urokinase) to fibrin clots was determined by mixing 1 ml of plasminogen-free fibrinogen (1.5 mg/ml), 25 μl of plasminogen activator (3,000 IU/ml), and 50 μl of thrombin (20 NIH units/ml). The final mixtures consisted of 0.05 M sodium diethylbarbiturate buffer, pH 7.8, containing 0.1 M NaCl and 0.001% (v/v) Tween 80, and were incubated at 37 °C for 30 min. The clots were removed by centrifugation at 48,000 × *g* for 15 min and washed with 1 ml of the diethylbarbiturate buffer. After centrifugation, the clots were extracted with 1 ml of 0.05 M sodium diethylbarbiturate buffer, pH 7.8, containing 1 M KSCN and 0.001% (v/v) Tween 80 at 4 °C for 4 h, and then centrifuged again. Aliquots of the clot supernatants and of the clot extracts were tested on fibrin plates after mixing with equal volumes of sodium diethylbarbiturate buffer containing KSCN or NaCl in order to obtain the same salt composition. To 0.5 ml of the clot supernatants, 50 μl of fibrinogen (15 mg/ml) was added and the adsorption procedure was repeated as described above.

The amidolytic activities of the melanoma and uterine tissue plasminogen activators were determined with the chromogenic substrate H-D-valyl-glycyl-L-arginine-*p*-nitroanilide (S-2322). Melanoma plasminogen activator (160 IU/ml) and uterine tissue plasminogen activator (117 IU/ml) were incubated in 0.05 M Tris/HCl/NaCl, pH 9.0, I = 0.05, containing 0.001% (v/v) Tween 80 and varying concentrations of S-2322. The initial release of *p*-nitroaniline was measured at 37 °C at 405 nm. A molar extinction coefficient of 10,500 liters·mol^{-1}·cm^{-1} was used for *p*-nitroaniline.

Immunological studies were performed using the immunodiffusion technique according to Ouchterlony (19). Tween 80 was added to the agarose (0.1% (v/v) final concentration). Quenching of the fibrinolytic activities of the plasminogen activators by specific antibodies was measured by incorporation of the IgG fractions of the antisera in varying amounts into the fibrin plates and by measuring the residual activities.

RESULTS

Purification of the Melanoma Plasminogen Activator

The results of the purification of the plasminogen activator secreted by human melanoma cells in culture are summarized in Table I. The whole purification procedure was performed at 4 °C. The serum-free conditioned medium used as starting material had a protein content of about 60 μg/ml and a plasminogen activator activity of about 20 IU/ml. This activity was completely plasminogen-dependent since no lysis was observed on plasminogen-free fibrin plates. In the first purification step, the plasminogen activator was adsorbed to zinc chelate-agarose. Using a batch-wise adsorption procedure, only 70% of the activity was bound to the gel, while column chromatography resulted in a complete binding of the activity. The latter procedure was performed by applying 10 liters of medium to a zinc chelate-agarose column (5 × 10 cm), equilibrated with 0.02 M Tris/HCl buffer, pH 7.5, containing 1 M NaCl and 0.01% (v/v) Tween 80 at a flow rate of 200 ml/h. After washing the column with approximately 1 liter of equilibration buffer, a linear gradient from 0 to 0.05 M imidazole in the same buffer (total volume 11) was applied. Fractions of 10 ml were collected at a flow rate of 120 ml/h. Plasminogen activator eluted as a single peak, partially separated from the main protein peak (Fig. 1).

In the next step, the pooled plasminogen activator fractions were applied to a concanavalin A-agarose column (0.9 × 25 cm), equilibrated with 0.01 M phosphate buffer, pH 7.5, containing 1 M NaCl and 0.01% (v/v) Tween 80. Fractions of 3.2 ml were collected at a flow rate of 6.4 ml/h. After washing the column with equilibration buffer a linear gradient of the equilibration buffer (100 ml) to 0.01 M phosphate buffer, pH 7.5, containing 0.01% (v/v) Tween 80, 0.4 M α-D-methylmannoside, and 2 M KSCN (100 ml) was applied. The plasminogen activator eluted at about 0.2 M α-D-methylmannoside and 1 M potassium thiocyanate (Fig. 2).

In the last purification step, plasminogen activator was gel-filtered on a Sephadex G-150 (superfine) column (2.5 × 90 cm). Solid KSCN was added to the pooled fractions from the concanavalin A-agarose column in order to increase the KSCN concentration from 1 M to 1.6 M and the solution was concentrated to about 10 ml by dialysis against solid polyethylene glycol 20,000. A small precipitate was removed by centrifugation at 10,000 × *g* for 15 min. Gel filtration was performed in 0.01 M phosphate buffer, pH 7.5, containing 1.6 M KSCN and 0.01% (v/v) Tween 80. Fractions of 3.4 ml were collected at a flow rate of 6.8 ml/h. Plasminogen activator eluted as a single peak which coincided with a small protein peak (Fig.

TABLE I

Purification of human melanoma plasminogen activator

The figures represent the mean value with the standard error of the mean of (the first) three preparations.

	Volume	Total protein	10^{-4} × total activity	10^{-4} × specific activity	Yield	Purification factor
	ml	*mg*	*IU*	*IU/mg*	%	
Conditioned medium	10,000	610 ± 40	20 ± 2	0.032 ± 0.002	100	1
Zinc chelate-agarose	151	46 ± 2	17 ± 4	0.36 ± 0.08	83	11
Concanavalin A-agarose	84	4.5 ± 0.2	11 ± 2	2.5 ± 0.7	56	77
Sephadex G-150	37	1.0 ± 0.2	9 ± 3	9 ± 2	46	263

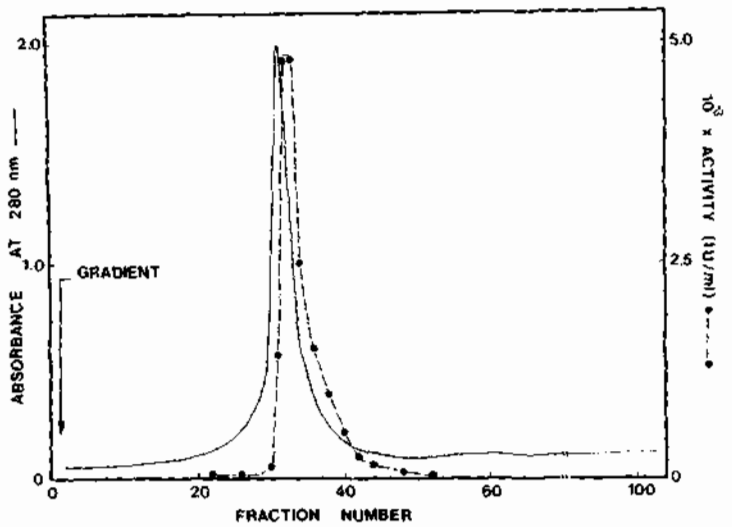

FIG. 1. **Zinc chelate-agarose chromatography of the melanoma plasminogen activator.** The serum-free medium was applied and the column was washed as described in the text. The figure shows the elution pattern during the imidazole gradient. Fractions 31 to 46 were pooled.

3). The pooled fractions were dialyzed against 0.15 M NaCl containing 0.01% (v/v) Tween 80 and stored at −80 °C. When the plasminogen activator concentration was above 100 μg/ml, the NaCl concentration was increased to 0.30 M. The specific activity amounted to about 90,000 IU/mg of protein (Table I). The final yield was about 1 mg of protein (Table I). In subsequent studies, the production of plasminogen activator by the melanoma cells appeared to have increased and about 4 mg of purified plasminogen activator were obtained repeatedly from 10 liters of conditioned medium.

Plasminogen activator produced by the melanoma cells in the presence of Aprotinin was purified as described above; however, with the addition of Aprotinin to the buffers of the zinc chelate-agarose and the concanavalin A-agarose columns (10 KIU/ml, final concentration). The inhibitor was omitted during the gel filtration step to obtain a final product without Aprotinin. The presence of Aprotinin did not influence the chromatographic behavior nor the yield and specific activity of the plasminogen activator.

Characterization of the Melanoma Plasminogen Activator; Comparison with Uterine Tissue Plasminogen Activator and Urokinase

Physicochemical Properties—SDS-gel electrophoresis of purified melanoma plasminogen activator usually showed one main band with a molecular weight of about 72,000, while in some preparations a small contaminant could be observed (Figs. 4 and 5). After reduction with dithiothreitol, the melanoma plasminogen activator purified in the absence of Aprotinin showed two bands with molecular weights of about 33,000 and 39,000, respectively (Fig. 4), suggesting a two-chain structure for this plasminogen activator. In contrast, the melanoma plasminogen activator purified in the presence of Aprotinin showed only one band after reduction (Fig. 5), which is indicative of a one-chain structure. Mixtures of the one-chain form, and the two-chain form were occasionally obtained when Aprotinin was not added during the purification procedure (see, for instance, Fig. 6).

SDS-gel electrophoresis of the uterine tissue plasminogen activator indicated also a one-chain structure (Fig. 5) or a two-chain structure (Fig. 4) depending on whether Aprotinin was used during the purification or not. The mobilities of the uterine tissue plasminogen activator preparations were similar

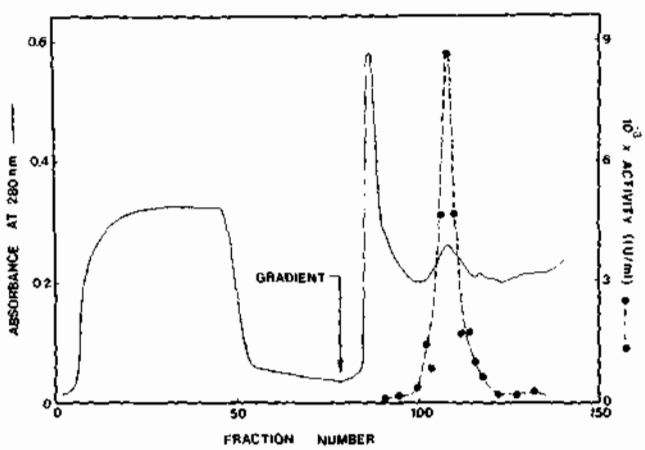

FIG. 2. **Concanavalin A-agarose chromatography.** The melanoma plasminogen activator recovered from the zinc-chelate agarose column (Fig. 1) was used. Fractions 100 to 125 were pooled.

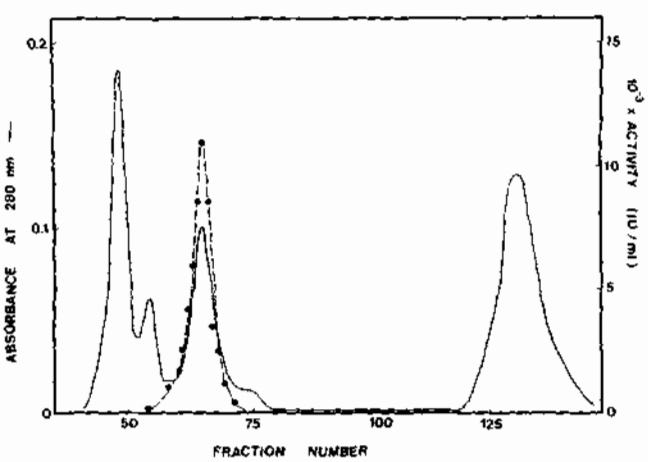

FIG. 3. **Gel filtration on Sephadex G-150.** The melanoma plasminogen activator recovered from the concanavalin A-agarose column (Fig. 2) was used. Fractions 60 to 69 were pooled.

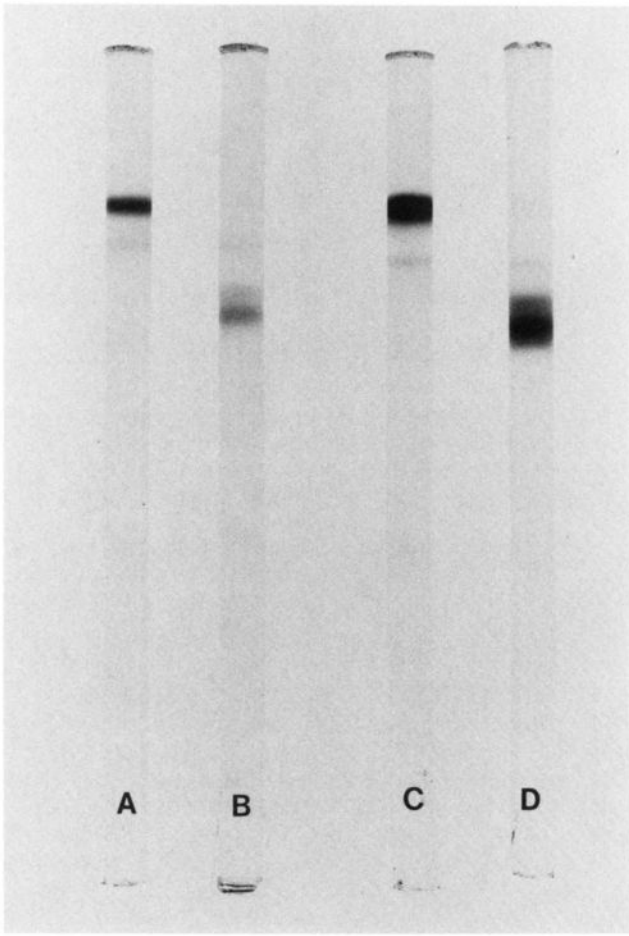

FIG. 4. **SDS-polyacrylamide gel electrophoresis of plasminogen activators purified in the absence of Aprotinin.** *A* and *B* represent uterine tissue plasminogen activator (30 μg), nonreduced and reduced, respectively; *C* and *D* represent melanoma plasminogen activator (50 μg), nonreduced and reduced, respectively.

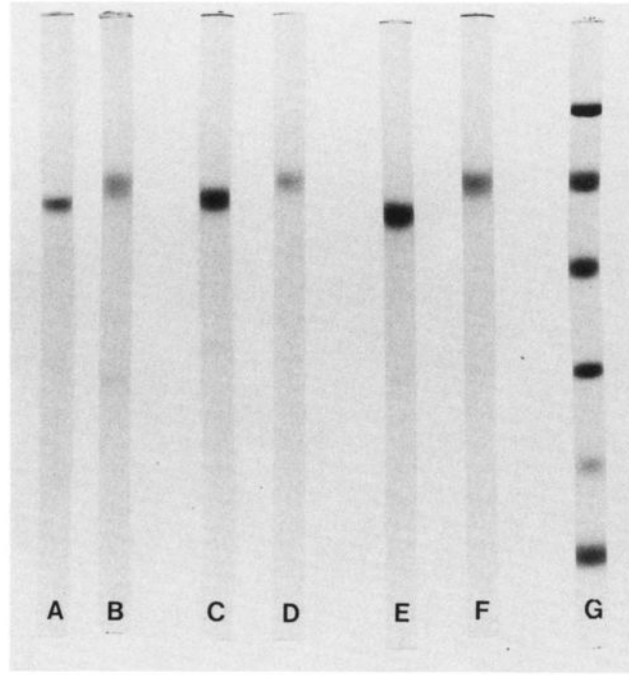

FIG. 5. **SDS-polyacrylamide gel electrophoresis of plasminogen activators prepared in the presence of Aprotinin.** *A* and *B*, nonreduced and reduced uterine tissue plasminogen activator (about 25 μg); *C* and *D*, nonreduced and reduced melanoma plasminogen activator (about 25 μg); *E* and *F*, nonreduced and reduced mixtures of uterine tissue plasminogen activator (about 25 μg) and melanoma plasminogen activator (about 25 μg); *G*, mixture of reduced standard proteins from top to bottom: phosphorylase *b* (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400).

to those of the melanoma plasminogen activator preparations (Figs. 4 and 5), and mixtures of both showed only single bands (Fig. 5). This finding indicates that the two plasminogen activators have very similar, or identical molecular weight.

Amino acid analyses of the melanoma plasminogen activator preparations and the uterine tissue plasminogen acitvator preparations are summarized in Table II. No significant differences were found, neither between the melanoma plasminogen activator and the uterine tissue plasminogen activator, nor between the one-chain and two-chain forms of the plasminogen activators.

Active Site Titration of Melanoma Plasminogen Activator—Two melanoma plasminogen activator preparations (mixtures of one-chain and two-chain activator) were treated with [^{3}H]DFP (0.27 mM, final concentration). The fibrinolytic activities were inhibited 95 and 97%, respectively, while control solutions, incubated without [^{3}H]DFP, did not lose activity. Using the protein concentrations as determined by the Lowry method and a molecular weight of 72,000, 1.32 and 1.08 mol of [^{3}H]DFP, respectively, was incorporated per mol of plasminogen activator. These values are compatible with one active site per enzyme molecule. SDS-gel electrophoresis of ^{3}H-labeled melanoma plasminogen activator confirmed that all radioactivity was located in the protein bands (Fig. 6). The reduced gel suggested that the active site serine is located in the M_r-33,000 chain of the two-chain activator (Fig. 6).

Amidolytic Activities—The amidolytic activities of melanoma plasminogen activator and uterine tissue plasminogen activator (both two-chain forms) were determined with the chromogenic substrate D-H-valyl-glycyl-L-arginine-paranitroanilide (S-2322). The Lineweaver-Burk plots of Fig. 7 show that the amidolytic activities of the enzymes were similar. The kinetic constants, derived from these plots by the least

TABLE II

Amino acid compositions of melanoma plasminogen activator and uterine tissue plasminogen activator preparations

Figures are given as the number of residues per 100 residues and represent the mean of two analyses of different preparations (one-chain melanoma plasminogen activator and two-chain uterine plasminogen activator) or of one preparation (two-chain melanoma plasminogen activator and one-chain uterine plasminogen activator).

	Melanoma plasminogen activator		Uterine plasminogen activator	
	One-chain	Two-chain	One-chain	Two-chain
Aspartic acid	9.8	10.5	11.2	10.0
Threonine	5.4	5.8	6.2	5.6
Serine	9.2	8.8	11.0	10.9
Glutamic acid	13.1	12.0	9.9	12.4
Proline	7.1	6.9	6.3	5.8
Glycine	10.4	9.4	10.8	13.0
Alanine	6.6	7.1	7.4	6.2
Cysteine	ND[a]	ND	ND	ND
Valine	4.1	4.4	4.6	4.2
Methionine	0.9	1.1	1.0	1.0
Isoleucine	3.0	3.2	3.3	2.8
Leucine	8.1	8.5	7.7	7.2
Tyrosine	4.0	3.9	3.7	3.9
Phenylalanine	3.7	3.8	4.0	3.4
Histidine	3.3	3.2	3.1	3.2
Lysine	5.5	5.4	5.2	5.4
Arginine	5.9	6.1	4.6	5.2
Tryptophan	ND	ND	ND	ND

[a] ND, not determined.

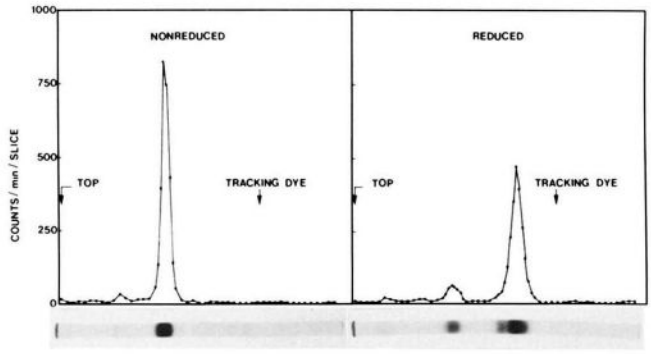

FIG. 6. **Incorporation of [^{3}H]DPF in a mixture of one-chain and two-chain melanoma plasminogen activator.** Distribution of the radioactivity after SDS-polyacrylamide gel electrophoresis of [^{3}H]DPF-treated melanoma plasminogen activator mixed with untreated plasminogen activator (40 μg in total). After staining, the gels were sliced and each slice was dissolved in perchloric acid and counted for radioactivity.

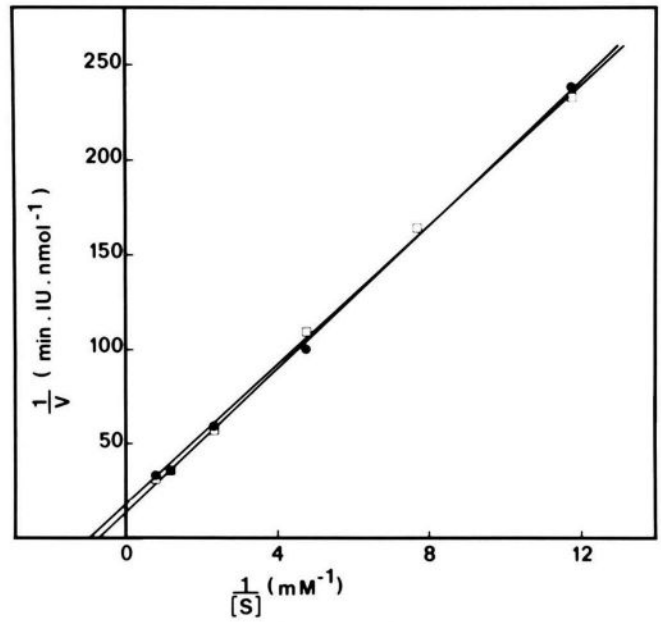

FIG. 7. **Lineweaver-Burk plots of the amidolytic activities of melanoma plasminogen activator (□) and uterine tissue plasminogen activator (●).** The chromogenic tripeptide substrate D-Val-Gly-Arg-Nan (S-2322) was used.

squares method are K_m = 1.0 mM and V_{max} = 0.053 nmol per min for an amount of melanoma plasminogen activator corresponding to 1 IU of fibrinolytic activity and K_m = 1.3 mM and V_{max} = 0.071 nmol per min for an amount of uterine tissue plasminogen activator corresponding to 1 IU of fibrinolytic activity. Based on a specific activity of 90,000 IU/mg and a molecular weight of 72,000, the catalytic rate constant k_2 of the melanoma plasminogen activator for the synthetic substrate is 5.7 s^{-1}.

Binding to Fibrin Clots—Table III summarizes the results of the binding experiments of the melanoma plasminogen activator, the uterine tissue plasminogen activator (both two-chain forms), and urokinase to fibrin. Both the melanoma plasminogen activator and the uterine tissue plasminogen activator bound to a great extent. The unadsorbed portions bound almost completely to a second fibrin clot. Urokinase, on the other hand, did not bind significantly to fibrin.

Immunological Characterization—Antisera against the melanoma plasminogen activator, the uterine tissue plasminogen activator, and the high molecular weight form of urokinase were used. Immunodiffusion analyses showed that the melanoma plasminogen activator was immunologically identical with the uterine tissue plasminogen activator (Fig. 8, *left*), while no relationship was found between melanoma plasminogen activator and urokinase (Fig. 8, *right*). Quenching experiments of the fibrinolytic activities of the activators by the IgG fractions of the antisera revealed that both the melanoma plasminogen activator and the uterine tissue plas-

TABLE III

Binding of different plasminogen activator preparations to fibrin clots

The figures represent the mean value with the standard error of the mean of three separate experiments.

	Melanoma plasminogen activator	Uterine plasminogen activator	Urokinase
		% *activity*	
Unbound	18 ± 1	23 ± 1	98 ± 1
Clot extract	61 ± 3	66 ± 4	8 ± 2

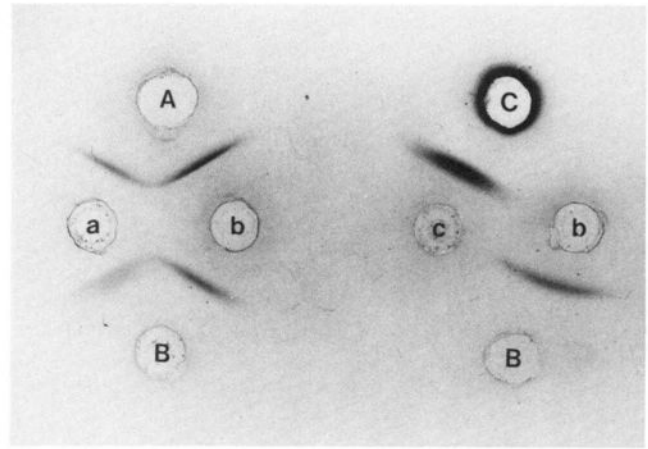

FIG. 8. **Ouchterlony double immunodiffusion analysis of various plasminogen activators.** *Well a*, two-chain uterine tissue plasminogen activator (0.35 mg/ml); *well b*, two-chain melanoma plasminogen activator (0.37 mg/ml); *well c*, urokinase (10^4 IU/ml); *well A*, anti-uterine tissue plasminogen activator IgG (3.5 mg of protein/ml); *well B*, anti-melanoma plasminogen activator IgG (5.7 mg of protein/ml); *well C*, anti-urokinase IgG (16 mg of protein/ml).

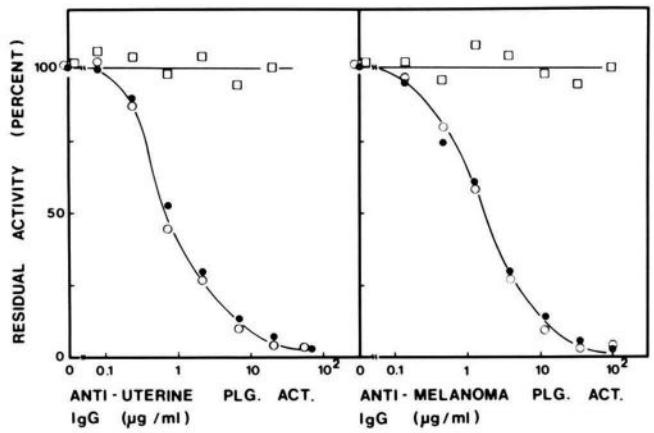

FIG. 9. **Immunological quenching of the fibrinolytic activities of two-chain uterine tissue plasminogen activator (●), two-chain melanoma plasminogen activator (○) and urokinase (□).** Antibodies against the uterine tissue plasminogen activator and the melanoma plasminogen activator were incorporated into the fibrin plates. The plasminogen activators (*PLG. ACT.*) (140 IU/ml) were dissolved in 0.02 M Tris/HCl, pH 7.5, containing 0.15 M NaCl and 0.01% Tween 80.

minogen activator were quenched completely by anti-melanoma plasminogen activator IgG (Fig. 9, *right*), as well as by anti-uterine tissue plasminogen activator (Fig. 9, *left*). A urokinase solution with the same fibrinolytic activity was not quenched by these antibodies (Fig. 9). The IgG fraction of a nonimmune rabbit serum did not quench any fibrinolytic activity in the concentration range of the experiments.

DISCUSSION

Oncogenic transformation of a variety of cells in culture is associated with the appearance of plasminogen activators (20). Human cell lines of neoplastic origin may produce substantial amounts of plasminogen activator (21). Moreover, several human tumors contain markedly more plasminogen activator than the corresponding normal tissues (22), confirming the correlation between neoplasia and plasminogen activator production. Plasminogen activators have been purified from several malignant cell cultures such as Rous sarcoma virus-transformed chicken embryo fibroblast cells (23), SV40-transformed hamster cells (24), human ovarian carcinoma cells (25), human pancreatic carcinoma cells (26), and from mouse sarcoma virus-transformed 3T3 cells (27). The plasminogen activators thus far purified from malignant human cell cultures are urokinase-like (25, 26). In the present study, a method was developed for the purification of the plasminogen activator secreted by human melanoma cells, which appeared to be a tissue plasminogen activator. The purification method involves three column chromatographic steps, zinc chelate-agarose, concanavalin A-agarose, and Sephadex G-150. It is a simplified version of a procedure, recently described for the purification of a plasminogen activator from human uterine tissue (11). The detergent Tween 80, which prevents the adsorption of tissue plasminogen activators (11), was added to all buffer solutions.

SDS-gel electrophoresis indicated that the melanoma plasminogen activator, prepared in the absence of Aprotinin consists of two chains of M_r = 33,000 and 39,000 respectively, thus having a molecular weight of about 72,000. This is comparable to the molecular weight of 69,000 and chains of 31,000 and 38,000, respectively, reported for the uterine plasminogen activator (11). However, some melanoma plasminogen activator preparations contain a mixture of a two-chain activator and a one-chain activator having the same molecular weight. This could be explained by recent findings of Wallén *et al.* (28), who showed that a two-chain tissue plasminogen activator may be the result of proteolytic degradation of a one-chain molecule, and that this conversion could be blocked by the addition of Aprotinin during the purification. This was confirmed in the present study, indicating that the melanoma plasminogen activator is secreted as a one-chain molecule.

The purified melanoma plasminogen activator appears to be very similar to the uterine tissue plasminogen activator by amino acid composition and amidolytic properties on a chromogenic tripeptide substrate. However, the specific activity of the purified melanoma plasminogen activator preparations is about 90,000 IU/mg of protein (Table I), while that of the purified uterine plasminogen activator measured under the same conditions is only about 48,000 IU/mg of protein. Since both preparations are nearly homogeneous on SDS-gel electrophoresis, this could point to a difference in fibrinolytic properties of the two enzymes. Alternatively, the uterine tissue plasminogen activator preparation might contain inactivated enzyme. In view of the longer purification procedure this seems plausible.

A striking difference between tissue plasminogen activators and urokinase is that the former activators adsorb to fibrin (29), which results in a marked enhancement of the activation of plasminogen (30). The melanoma plasminogen activator appears to adsorb to fibrin clots to the same extent as the uterine tissue plasminogen activator, whereas urokinase does not.

Both immunodiffusion and quenching experiments of the fibrinolytic activities revealed that the melanoma plasminogen activator is immunologically identical with the uterine tissue plasminogen activator and unrelated to urokinase. These findings are in line with earlier reports that melanoma cell lines mainly produce a plasminogen activator which does not react with antibodies against urokinase (8–10). Although a HeLa cell line was also reported to produce tissue plasminogen activator (31), this is not a general phenomenon for cultured human tumor cells. In fact, most human tumor cells produce mainly, or exclusively, urokinase-like activators (8–10, 25, 26). Apparently, malignancy of human cells may be associated with the production of either tissue plasminogen activator, urokinase, or both.

Although the present study may be relevant to some aspects of tumor biology, our main interest originated from the study of the human fibrinolytic system. The fibrinolytic activity in blood seems to be regulated by the vascular plasminogen activator which is released by the vascular wall. Studies on this activator are hampered however by the very small amounts which can be obtained either by the perfusion of blood vessels of human cadavers (32–35) or from blood after certain stimuli (36, 37). Since tissue plasminogen activator most likely represents the same enzyme (3), tissue extracts can be used as an alternative source. Although this offers the possibility of obtaining larger quantities of purified activator, still about 100 human uteri were necessary for 1 mg of purified protein (11). Our finding that the melanoma plasminogen activator is identical, or nearly identical, with the uterine tissue plasminogen activator makes it possible to produce substantial amounts of activator independent of human donors. This has allowed us to initiate studies on the molecular interactions which regulate fibrinolysis and on the thrombolytic properties of tissue plasminogen activator in animal models.

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