IMMUNOLOGICAL ANALYSIS OF PLASMINOGEN ACTIVATORS FROM NORMAL AND TRANSFORMED HAMSTER CELLS

Evidence that the Plasminogen Activators Produced by SV40 Virus-Transformed Hamster Embryo Cells and Normal Hamster Lung Cells are Antigenically Identical*

BY JUDITH K. CHRISTMAN, SAMUEL C. SILVERSTEIN,$ AND GEORGE ACS

(From the Departments of Pediatrics and Biochemistry, Mt. Sinai School of Medicine, New York 10029 and the Department of Cellular Physiology and Immunology, The Rockefeller University, New York 10021)

Plasminogen activators, enzymes which convert the proenzyme plasminogen to its proteolytically active form (plasmin), have been recognized in a variety of cells, tissues, and body fluids (1). The best characterized of these plasminogen activators is urokinase, a serine protease of mol wt 54,000 which is found in the urine of normal adults (2). Kucinski et al. (3) prepared a guinea pig antiserum against human urokinase. This antiserum inhibited the plasminogen-activating capacity of human and primate urokinases and the plasminogen activator secreted by cultured adult kidney cells. Since the antibody had no inhibitory effects upon plasminogen activators produced by other human tissues or body fluids Kucinski et al. (3) concluded that urokinase is produced by the kidney and that it is immunologically distinct from plasminogen activators produced elsewhere in the body.

Unkeless et al. (4), Ossowski et al. (5), and Rifkin et al. (6) have reported that a variety of transformed cells and primary explants of human tumors actively secrete plasminogen activators in vitro. Pollack et al. (7) have found a strong correlation between plasminogen activator secretion and the capacity of cells to grow in semisolid medium, and Christman et al. (8) have shown that the capacity of mouse melanoma cells to secrete plasminogen activators is closely correlated with tumorigenic potential of these cells in vivo. Despite these correlations, the role of plasminogen activators in tumorigenicity remains obscure.

Recently, Christman and Acs (9) purified a plasminogen activator secreted by a line of SV40-transformed hamster embryo cells. Their most highly purified preparation had a specific activity 14,000-fold above that of the starting material, exhibited a single Coomassie blue stained band on SDS-polyacrylamide gel electrophoresis, and had a mol wt of 50,000. This enzyme had an isoelectric point of pH of 9.5 and was shown to be a serine protease composed of at least two subunits joined by disulfide linkages. The catalytic subunit, identified by labeling with $[^3]$Hdiisopropylphosphofluoridate had a mol wt of 25,000.

* Supported by grant NP636K from the American Cancer Society, grants CA16890 and AI08697 from the U. S. Public Health Service, and by The Muscular Dystrophy Association.

† Established Investigator of the American Heart Association.

THE JOURNAL OF EXPERIMENTAL MEDICINE · VOLUME 142, 1975 419
The characterization of this enzyme and the availability of several alternative methods for its purification (10) have enabled us to prepare antibodies against it. These antibodies have been used to compare the immunological properties of this particular enzyme with those of plasminogen activators from normal tissues and with plasminogen activators from other transformed cells.

In this report, we describe the preparation and specificity of antibodies against the plasminogen activator produced by a line of SV40-transformed hamster cells. The antibody inhibits the enzymatic activity of the cell-bound and secreted forms of this plasminogen activator, prevents the release of SV40-transformed hamster cells from the growth surface (4), and forms a single precipitin line on immunodiffusion using purified SV40 hamster plasminogen activator as antigen. The antibody is species specific; it does not inhibit plasminogen activators derived from normal or transformed human cells or from transformed murine cells. Most importantly, the antibody appears to discriminate between plasminogen activators from different tissues of the same organism since it inhibits the plasminogen activator(s) produced by an established line of hamster lung cells and by a primary explant of newborn hamster lung but it has no inhibitory effect upon plasminogen activator(s) secreted by an established line of hamster kidney cells or a primary explant of newborn hamster kidney.

Materials and Methods

Cell Cultures. Hamster cell lines HaK and DON were obtained from the American Type Culture Collection, Rockville, Md., and maintained in Dulbecco's Modified Eagle's Medium supplemented with 10 and 20% fetal bovine serum, respectively. Primary explants of hamster, lung, kidney, and skin were obtained by trypsinization (0.25%) of the appropriate organs of 2- to 3-day old Syrian hamsters (Lakeview Hamster Colony, Newfield, N. J.). The cells were grown in Dulbecco's medium, containing 10% fetal bovine serum. SV40 virus-transformed hamster embryo cells (5), the kind gift of Doctors E. Reich and L. Ossowski, both from The Rockefeller University, were maintained in the same medium. Mouse melanoma clones B,59 and B,9 were the kind gift of Dr. S. Silagi, Cornell University Medical College, New York. Their derivation and maintenance is described in reference 11. The mouse mammary tumor line was the gift of Dr. S. Spiegelman. Powdered media and dog serum were from Grand Island Biological Corp., Grand Island, N. Y., and fetal bovine serum from Flow Laboratories, Inc., Rockville, Md.

Enzymes. The purification of plasminogen activator from SV40-transformed hamster cells has been described in detail (9, 10). Plasminogen activator released from HaK cells was purified using the same methods. The plasminogen activator preparations utilized for these studies were as follows: (a) Harvest fluid (HF), the serum-free medium containing plasminogen activator released by cells; Tables III and IV. (b) Protein from a 50% (NH₄)₂SO₄ precipitate of HF dissolved in 0.05 M glycine-HCl, pH 4.0, and extensively dialyzed against this buffer [stage 3, (9) approximately eight-fold purification]; Tables I and IV. (c) 0.5 M (NH₄)₂SO₄ eluate from SP-C25 Sephadex, dialyzed and concentrated against 0.05 M glycine-HCl, pH 4.0 [stage 5, (9) approximately 400-fold purification]; Table IV and Figs. 2, 3, and 4, and as immunogen to prepare antiplasminogen activator antibody (APA-IgG). Purification of plasminogen activator by high-voltage liquid-phase isoelectric focusing has also been described (10). Such preparations (Figs. 2 and 4) display a single protein band of mol wt 50,000 on SDS-polyacrylamide gel electrophoresis.

Cell-bound enzyme was a plasma membrane-enriched fraction prepared from HaK, DON, and SV40-transformed hamster cells using the method of Atkinson and Summers (12). Cells were

---

1Abbreviations used in this paper: APA, antiplasminogen activator; CFA, complete Freund's adjuvant; FDP, fibrin degradation products; HBSS, Hanks balanced salt solution without phenol red; HF, harvest fluid; PBS, phosphate-buffered saline; SAR-IgG, sheep antibody to rabbit IgG.
harvested by scraping and lysed according to the methods used in references 10 and 12. After removal of nuclei, large membrane fragments and "ghosts" were pelleted at 6-10,000 g and suspended in buffer containing 0.01 M Tris-HCl, pH 8.0, 0.01 M NaCl, 0.001 M Na₂SO₄, and 0.003 M Mg acetate at a concentration of approximately 10⁶ cells/ml. These preparations were used for the experiments shown in Tables I and IV and as immunogen to prepare anti-SV40 hamster cell membrane antibody.

**Plasminogen Activator Assays.** All assays for plasminogen activator are based on the capacity of this enzyme to convert plasminogen to plasmin. Plasmin activity was determined by its ability to release ¹²⁵I-labeled fibrin degradation products (FDP) from ¹²⁵I-labeled fibrin-coated petri dishes. The standard 35 mm dish used for all determinations was coated with approximately 0.1 mg ¹²⁵I-labeled fibrinogen containing 100,000 cpm. Two forms of the assay were used. (a) Cells were plated directly on radioactive fibrin layers and served as the source of plasminogen activator. Dog serum (10%) was plasminogen source. (b) Purified plasminogen activator or cell-free HF was incubated in a final vol of 1 ml 0.1 M Tris-HCl, pH 8.1, containing 4 μg of dog plasminogen [purified by lysine affinity chromatography (13)]. Release of ¹²⁵I-labeled FDP was determined directly by measuring the radioactivity of the incubation mixture in a Nuclear Chicago scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.) with Hydromix (Yorktown Research, N. Y.) as fluor.

**Cytotoxicity Assays.**—Adsorbed guinea pig complement (C): SV40-transformed hamster cells in monolayer cultures were washed three times with 5 ml of phosphate-buffered saline (PBS) (pH 7.4) [solution A of Dulbecco and Vogt (14)], and removed from the monolayer by incubation at room temperature for approximately 10 min in 0.25% trypsin (GIBCO) in the same buffer. 5 ml of Dulbecco's medium containing 10% fetal bovine serum were added to inhibit further proteolysis, the cells were pelleted at 250 g for 10 min at 4°C, and resuspended in Hanks' balanced salt solution without phenol red (HBSS), pH 7.4, (GIBCO) at a concentration of 2 x 10⁶ cells/ml (>97% viable by trypan blue exclusion). Whole C (lot no. TO 933) from Cordis Laboratories, Miami, Fla., was resuspended in 5 ml HBSS. 0.5 ml of this C solution was incubated for 30 min at 4°C with 1 x 10⁶ hamster cells in 0.5 ml of HBSS. The cells were removed by centrifugation (250 g, 10 min at 4°C) and the clear supernate was diluted with an equal volume of HBSS. This final solution is referred to as adsorbed C.

**Antisera.** Sheep antibody to rabbit IgG (SAR-IgG) was the IgG fraction of sheep serum obtained after DEAE-cellulose column chromatography as described previously (15). 5 x 10⁶ SV-40 hamster cells in monolayer culture were trypsinized, pelleted by centrifugation, and incubated in 1 ml of SAR IgG (40 mg/ml in PBS) for 2 h at 4°C. The cells were pelleted (250 g, 10 min at 4°C) and the supernatant solution is the adsorbed SAR-IgG used in the experiment described in Table V.

Rabbit anti-SV40 hamster cell plasminogen activator IgG (APA-IgG) was prepared as follows: 2 ml of plasminogen activator (stage 5 purification) containing in each milliliter an amount of plasminogen activator activity equivalent to that found in approximately 26 μg of the isoelectrically focused purified enzyme was mixed with 1 ml of complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, Mich.). A brown rabbit was injected intramuscularly and subcutaneously with this material. 18 days later 0.5 ml of the same plasminogen activator solution (without Freund's adjuvant) was injected into each hind foot pad, and the rabbit was bled 2 wk later. The IgG fraction from 30 ml of serum was obtained by DEAE-cellulose column chromatography (15), brought to a final concentration of 30 mg/ml in PBS and used for the studies described in Tables I, II, III, IV, and V, and Fig. 2. 4 mo after the initial bleeding the rabbit was given a second injection of 3.5 μg of isoelectrically purified plasminogen activator in CFA, bled 7 days later, and the IgG fraction purified as above. This preparation (45 mg/ml) was used for the experiments described in Figs. 2 to 4.

Rabbit anti-SV40 hamster cell membrane IgG (30 mg/ml in PBS) was prepared as follows: A white New Zealand rabbit was injected subcutaneously and intramuscularly with a cell membrane-enriched fraction (prepared as described above from approximately 2 x 10⁵ SV40 hamster cells) in CFA, and 18 days later in the foot pads with the same membrane fraction (from 1 x 10⁵ cells) in PBS. 2 wk later the rabbit was bled and an IgG fraction obtained by DEAE-cellulose chromatography (15).

Rabbit anti-SV40 hamster cell IgG (13 mg/ml in PBS) was similarly prepared by injecting a white New Zealand rabbit with a total of approximately 5 x 10⁶ viable SV40 hamster cells both intravenously and intramuscularly following the immunization schedule described above. A control IgG fraction (9 mg/ml) was similarly prepared from an unimmunized rabbit which had not been actively challenged with any hamster protein (9 mg/ml).

**Immunodiffusion.** The studies described in Fig. 2 were performed using immunodiffusion plates
IMMUNOLOGICAL ANALYSIS OF PLASMINOGEN ACTIVATORS

from Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif. Immunodiffusion in Noble agar (Difco Laboratories) containing bovine fibrinogen (Pentex Biochemical, Kankakee, Ill.) (Fig. 4) was performed exactly as described by Clausen (16), except that 4.2 μg of purified dog plasminogen were added for each ml of fibrinogen-containing agar.

Results

Inhibition of Cell-Bound and Secreted SV40 Hamster Plasminogen Activator by APA-IgG. APA-IgG effectively inhibited the action of both the released and cell-associated forms of plasminogen activator from SV40-transformed hamster cells. IgG from the serum of normal rabbits or rabbits immunized with SV40-transformed hamster cells had no inhibitory effect on either enzyme preparation (Table I). IgG from rabbits immunized with a plasma membrane-enriched fraction of SV40-transformed hamster cells (containing cell-associated plasminogen activator) had no effect on activation of plasminogen by released enzyme but did inhibit the cell-associated enzyme at high antibody concentrations (Table I). This inhibition of the cell-bound but not the released form of plasminogen activator by anti-SV40 hamster cell membrane IgG is unexplained. The anti-SV40 hamster cell membrane IgG does not cross react with purified plasminogen activator as measured by immunodiffusion in agar gels. Since the intracellular form of the enzyme is tightly bound to cellular membranes (10), it is possible that the interaction of antibody molecules with nearby membrane antigens sterically limits access of plasminogen to the enzyme. It is also possible that the intracellular form of plasminogen activator contains additional antigenic determinants which are not present on the secreted enzyme. This is indeed the case for insulin (17). Resolution of this question awaits a reliable method for solubilizing the intracellular form of the enzyme.

The release of FDP by SV40-transformed hamster cells plated on 125I-labeled fibrin-coated Petri dishes in the presence of suitable serum was also inhibited by APA-IgG (Table II). The data in Table II are from a typical experiment where the number of cells plated and the incubation time are such that the release of FDP by the control culture is between 50-80% of maximal release. If the incubation time is extended, all of the cultures, even those which are totally inhibited at 24 h, eventually release the maximal amount of 125I-labeled fibrin.

When SV40-transformed hamster cells were grown in monolayer culture supplemented with dog serum they activated sufficient plasmin to cause release of the cells from the growth surface (Fig. 1a and reference 5). Addition of APA-IgG to the culture medium did not slow cellular growth or inhibit the “piled up” appearance of these cells, but did prevent the release of the cells from the surface of the Petri dish (Fig. 1b). The duration of this inhibition was dependent upon the quantity of added APA-IgG. Once the inhibitory capacity of the antibody was saturated, plasmin was generated and the cells were released from the Petri dish. Two conclusions may be drawn from these experiments: (a) APA-IgG does not inhibit release of plasminogen activator from SV40-transformed hamster cells since cells treated with this antibody are able to secrete sufficient plasminogen activator to exhaust the neutralizing capacity of the antibody. (b) The cell-bound and secreted forms of plasminogen activator
Table 1
Effect of Several IgG Preparations on the Activation of Plasminogen by Enzyme(s) from SV40 Virus-Transformed Hamster Fibroblasts

| Source of enzyme | IgG preparation | IgG concentration | FDP cpm released 2 h, 37°C | Control activity |
|-----------------|----------------|-------------------|---------------------------|-----------------|
|                 |               | µg protein/ml      |                           |                 |
| Released        | Antiplasminogen activator IgG | 0                 | 17,700                    | 100             |
|                 |                 | 3                 | 7,330                     | 42              |
|                 |                 | 6                 | 4,828                     | 27              |
|                 |                 | 14.5              | 1,340                     | 8               |
|                 |                 | 29                | 0                         | 0               |
|                 |                 | 145               | 0                         | 0               |
|                 | Antihamster cell membrane IgG | 0                 | 14,300                    | 100             |
|                 |                 | 29                | 18,900                    | 132             |
|                 |                 | 58                | 23,450                    | 163             |
|                 |                 | 145               | 14,000                    | 90              |
|                 |                 | 290-2,900         | 14,000-15,730             | 100-110         |
|                 | Antihamster cell IgG | ≤145              |                           | 100-150         |
|                 | Control rabbit IgG | ≤450              |                           | 100-110         |
| SV-40 hamster cell membrane enriched fraction | Antiplasminogen activator IgG | 0                 | 19,800                    | 100             |
|                 |                 | 3                 | 4,300                     | 22              |
|                 |                 | 6                 | 2,700                     | 14              |
|                 |                 | 14.5              | 270                       | 1               |
|                 |                 | 29                | 0                         | 0               |
|                 | Antihamster cell membrane IgG | 0                 | 15,000                    | 100             |
|                 |                 | 29                | 12,800                    | 85              |
|                 |                 | 145               | 6,770                     | 45              |
|                 |                 | 290               | 2,864                     | 19              |
|                 |                 | 580-2,900         | 0                         | 0               |
|                 | Antihamster cell IgG | ≤1,300            |                           | 90-110          |
|                 | Control rabbit IgG | ≤360              |                           | 100             |

The indicated IgG and enzyme preparations were incubated on 125I-labeled fibrin-coated assay plates at 23°C in 0.5 ml of 0.1 M Tris, pH 8.1. After 15 min, 4 µg of dog plasminogen in 0.5 ml of the same buffer were added and incubation continued at 37°C for 2 h. The IgG concentration given is that present during the final incubation. Released enzyme was at stage 3 of purification (9) and had been concentrated and dialyzed against 0.05 M glycine-HCl, pH 4.0, in a Prodicon vacuum dialyzer (Bio-Molecular Dynamics, Beaverton, Oreg.). (Degree of purification had no effect on the inhibition of plasmin formation by APA-IgG when the enzyme preparation was properly desalted.) The cell membrane-enriched fraction was prepared as described in the Materials and Methods. All data have been corrected for 125I released into medium containing plasminogen alone. Enzyme or IgG without added plasminogen released fewer counts than plasminogen.
TABLE II
Effect of Antiplasminogen Activator IgG on the Release of Fibrin Degradation Products by SV40-Transformed Cells

| IgG concentration | Released $^{125}$I-labeled FDP cpm 24 h, 37°C* | Control activity % |
|-------------------|---------------------------------------------|-------------------|
| $\mu$g protein/10^6 cells |                              |                   |
| 0                 | 39,700                                      | 100               |
| 116               | 31,800                                      | 80                |
| 230               | 11,910                                      | 30                |
| 580               | 0                                           | 0                 |

Cells ($5 \times 10^5$) were plated on sterile $^{125}$I-labeled fibrin-coated dishes in modified Eagle's medium. After 4-5 h at 37°C, the indicated amount of IgG was added, incubation was continued for 30 min, and then dog serum was added to a final concentration of 10%. The final vol was 3 ml.

*Corrected for $^{125}$I released into medium containing dog serum without added cells. Cells without added serum released fewer counts than dog serum alone.

contain similar immunological determinants since both are inhibited by APA-IgG.

**Immunological Characterization of Plasminogen Activator.** APA-IgG was prepared using SV40 hamster cell plasminogen activator (purified to stage 5, see Materials and Methods) as the immunogen. As a consequence, the APA-IgG contained antibodies to several antigens which are present in this partially purified material. Analysis of two such partially purified plasminogen activator preparations by double diffusion in agar gels against APA-IgG revealed several distinct precipitin lines (Fig. 2 a, well nos. 2 and 4). Further purification of the plasminogen activator by high-voltage liquid-phase isoelectric focusing resulted in a highly purified (one band on SDS gel electrophoresis) and enzymatically active preparation which gave a single precipitin line on immunodiffusion (Fig. 2 b). This precipitin line is identical to one of the lines found in the partially purified plasminogen activator (Fig. 2 a, well no. 3). Immunoelectrophoretic analysis of partially purified plasminogen activator showed two precipitin lines, only one of which corresponds to the electrophoretic mobility of plasminogen activator (Fig. 3). Confirmation that the lighter precipitin band is formed by precipitation of the plasminogen activator by APA-IgG was achieved by diffusion in gels containing fibrinogen and plasminogen. As shown in Fig. 4, there is good correspondence between the zone of inhibition of fibrinolysis and the single precipitin line formed between isoelectrically focused purified plasminogen activator and APA-IgG. Partially purified plasminogen activator exhibits two precipitin lines in this assay; a dense precipitin line whose position does not correspond to the zone of inhibition of fibrinolysis, and a light precipitin line whose position corresponds to the zone of inhibition of fibrinolysis but is obscured by it. The inhibition of fibrinolysis is immunologically specific. Control rabbit IgG preparations did not inhibit fibrinolysis mediated by SV40 hamster cell-secreted plasminogen activator and APA-IgG did not inhibit fibrinolysis mediated by hamster kidney cell-secreted plasminogen activator.

**Species Specificity of APA-IgG.** Table III summarizes the results of a number of experiments demonstrating the lack of effect of APA-IgG on the ability of
Fig. 1. Effect of APA-IgG on the ability of SV40-transformed hamster cells to remain attached to the growth surface in the presence of a plasminogen source. Cells were plated at a density of $5 \times 10^5$ on 35 mm Petri dishes coated with fibrin. (Procedure exactly as described in Table II.) (a) No APA-IgG added. (b) 300 $\mu$g/ml of APA-IgG added. Both cultures contain 10% dog serum and have been incubated at 37°C for approx 18 h. × 500.
IMMUNOLOGICAL ANALYSIS OF PLASMINOGEN ACTIVATORS

Fig. 2. Ouchterlony immunodiffusion analysis of SV40 hamster cell plasminogen activator preparations. (a) Center well, 10 μl APA-IgG. Well 1, 10 μl 0.05 M glycine-HCl buffer, pH 4.0; wells 2 and 4, 10 μl stage 5 plasminogen activator in the same glycine-HCl buffer; and well 3, 10 μl isoelectrically focused plasminogen activator. (b) Center well, 10 μl isoelectrically focused plasminogen activator. Well 1, 10 μl anti-SV40 cell membrane IgG. Well 2, 10 μl APA-IgG (first preparation, 30 mg/ml) and wells 3 and 4, APA-IgG (second preparation, 45 mg/ml). Well 5, 10 μl control IgG. Both APA-IgG preparations give a precipitin line of identity against the highly purified plasminogen activator. Note the absence of spurring which is seen in figure 2a using the partially purified enzyme as antigen.

Fig. 3. Immunoelectrophoretic analysis of stage 5 plasminogen activator. Glass slides (25 x 75 mm) were coated with 3 ml of 2% Noble agar in 0.05 ionic strength barbital buffer (pH 8). 20 μl of a partially purified plasminogen activator preparation (stage 5, reference 9) was placed in the center well and electrophoresed at room temperature at 5 V/cm for 2 h. The agar strip was then divided longitudinally. 50 μl of APA-IgG (45 mg/ml) was allowed to diffuse into one-half of the gel (shown at the bottom) for 2 days at 4°C. The other half of the gel was cut into 15 equal sections, homogenized in 1.5 ml 0.1 M Tris buffer, pH 8, using a 1 ml syringe equipped with a no. 22 gauge needle and the entire solution was incubated on 125I-labeled fibrin-coated plates for 2 h at 37°C. The final mixture (total incubation vol of 2 ml) contained 8 μg of dog plasminogen. The amount of 125I-labeled FDP released from the plate by the plasminogen activator contained in each gel section was assayed as described in the Materials and Methods and is plotted in the top half of the figure.
Fig. 4. Immunodiffusion of plasminogen activator in fibrin and plasminogen containing agar. Center well, 20 \( \mu \)l APA-IgG. Well 1, 10 \( \mu \)l stage 5 plasminogen activator. Well 2, 10 \( \mu \)l isoelectrically purified plasminogen activator. Immunodiffusion was allowed to proceed at 4°C for 2 days after which the slide was incubated at 37°C for 12 h in a humidified atmosphere to allow development of the zones of fibrin lysis.

### Table III

Species Specificity of Antibody to SV40-Transformed Hamster Fibroblast Plasminogen Activator

| Protein Source | APA-IgG Protein/10⁵ Cells | Control Activity* |
|---------------|--------------------------|------------------|
| SV40-transformed hamster fibroblasts | 175 | 0–11 |
| B,59 mouse melanoma | 175 | 116–167 |
| B,4 mouse melanoma | 175 | 95–100 |
| Mouse mammary tumor | 250 | 100 |

* For each set of assays sufficient cells of the indicated type were plated to release approximately 80% of the available radioactive fibrin as FDP after 24 h incubation at 37°C. The number of cells plated on each assay dish were as follows: SV40 hamster cells, 2 \( \times \) 10⁵; B,59 melanoma cells, 5 \( \times \) 10⁵; B,4 melanoma cells, 5 \( \times \) 10⁵; and mouse mammary tumor cells, 2 \( \times \) 10⁴. All other details as in Materials and Methods or Fig. 2.

| Protein Source | APA-IgG Protein/ml | Control Activity‡ |
|---------------|--------------------|------------------|
| Urokinase     | 580                | 95–100           |
| Released enzyme, melanoma (RPMI-8352) | 580 | 95–123 |

‡ For each set of assays, the incubation mixture contained sufficient enzyme to release 50–80% of available radioactivity in 2 h at 37°C. Human urokinase was purchased from Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio, and HF (Methods) from a line of human melanoma cells (6) was the kind gift of Doctors D. B. Rifkin and E. Reich. All details as in Fig. 1.

plasminogen activators from murine and human cell lines to activate plasminogen. In each experiment, the number of cells plated was sufficient to release approximately 80% of the \(^{125}\)I-labeled fibrin bound to the dish in 24 h at 37°C. The maximal amount of APA-IgG added to each assay plate is indicated.
In no case were lower levels of APA-IgG inhibitory. Addition of APA-IgG did not alter the appearance, slow the growth, or inhibit the capacity of these tumor cells to convert plasminogen to plasmin. Similarly, APA-IgG had no inhibitory effects on plasminogen activators obtained from normal (urokinase) or transformed (melanoma) human cells (Table III B). These data indicate that the antigenic determinants recognized by APA-IgG are not shared by plasminogen activators from other species. They also provide conclusive evidence that APA-IgG directly inhibits the enzymatic function of the plasminogen activator released by SV40-transformed hamster cells and that APA-IgG does not inhibit plasmin degradation of the fibrin substrate.

**Effect of APA-IgG on Plasminogen Activators from Cell Lines and Primary Explants of Hamster Tissues.** Having established that APA-IgG does not inhibit plasminogen activators from species other than hamster, we examined the effect of APA-IgG upon plasminogen activators produced by two hamster cell lines. The cell lines chosen were DON [a diploid line derived from normal Chinese hamster lung (18)] and HaK [derived from normal golden Syrian hamster kidney (18)]. When these cell lines were plated on 125I-labeled fibrin-coated tissue culture dishes in the presence of a suitable source of plasminogen (dog serum), 125I-labeled FDP were released into the medium. Continued incubation of the cells under these conditions stimulated the formation of sufficient plasmin to cause release of the cells from the surface (see for example Fig. 1 a). Addition of APA-IgG to the culture medium completely inhibited the fibrinolytic activity of DON lung cells (Table IV A), and the cells remained attached to the dish (see for example Fig. 1 b). In contrast, APA-IgG had no inhibitory effect on either the fibrinolytic activity of HaK kidney cells (Table IV A) or on their subsequent release from the culture dish. Similar results were obtained when the plasminogen activator(s) from DON and HaK were tested in cell-free assays (Table IV B). APA-IgG did not block plasminogen activation by either the released or cell-bound enzyme from kidney but effectively inhibited the activity of the cell-bound enzyme from lung.

These results indicate that hamster cells can make at least two immunologically distinct forms of plasminogen activators and that the DON lung cells produce plasminogen activator(s) which share antigenic determinants with the enzyme produced by SV40-transformed hamster cells. However, these data do not resolve whether the plasminogen activator produced by the DON lung cell line is immunologically similar to the enzyme produced by normal hamster lung cells. To clarify this point we prepared primary cell cultures from the lungs, skin, and kidneys of golden Syrian hamster neonates. These cells were assayed for plasminogen activator after their first passage in culture. Skin fibroblasts produced no detectable plasminogen activator. In both the lung and the kidney explants, however, plasminogen-dependent fibrinolytic activity was readily detected. Addition of APA-IgG to the lung cells markedly inhibited their fibrinolytic activity while similar amounts of APA-IgG had no inhibitory effect upon the fibrinolytic activity of the kidney cells (Table IV A). It should be noted that the enzyme released by the kidney cells does not destroy the activity of APA-IgG; APA-IgG which has been incubated with kidney cells retains its ability to inhibit the plasminogen activator from SV40-transformed hamster cells. These
TABLE IV
Effect of Anti-SV40 Plasminogen Activator IgG on the Capacity of Hamster Cells and Enzymes to Activate Plasminogen

| Cells                                      | µg APA-IgG protein/10⁵ cells | % Control activity |
|--------------------------------------------|----------------------------|--------------------|
| A. Effect on the release of fibrin degradation products by cells: |
| DON (Chinese hamster lung)                 | 175                        | 20                 |
| HaK (Golden Syrian hamster kidney)         | 580                        | 120                |
| SV40 virus-transformed hamster fibroblast  | 175                        | 0                  |
| 1° Golden Syrian hamster lung              | 75–241                     | 0–20               |
| 1° Golden Syrian hamster kidney            | 175                        | 90–100             |
| 1° Golden Syrian hamster fibroblast        | 0                          | No activity        |

| Enzyme                                    | APA-IgG Concentration (µg protein/ml) | % Control activity |
|--------------------------------------------|--------------------------------------|--------------------|
| B. Effect on the enzymatic activity of plasminogen activators from HaK and DON cells: |
| and DON cells:                             |                                       |                    |
| Soluble HaK plasminogen activator          | 290                                   | 110–290            |
| HaK cell membrane enriched fraction        | 120                                   | 100                |
| DON cell membrane enriched fraction        | 30                                    | 0–5                |

All procedures and assays were performed as outlined in the legends of Tables I and II. For each assay, sufficient cells of the indicated type were plated to release approximately 80% of the available radioactive fibrin as FDP after 24 h incubation at 37°C.

(A) Incubations were for 18–24 h except for the primary lung cells and skin fibroblasts which were incubated for 48 h. The number of cells plated on each assay dish were as follows: DON, 5 × 10⁵; HaK, 1–2 × 10⁶; SV40 hamster embryo, 2 × 10⁵; primary hamster lung, 3 × 10⁵; primary hamster kidney, 5 × 10⁵; and primary hamster skin fibroblasts, 1 × 10⁵.

(B) HaK-secreted enzyme was tested (a) as the unpurified harvest fluid from confluent cell monolayers, (b) as the stage 3 purified enzyme, and (c) as the stage 5 purified enzyme (see Materials and Methods and reference 9). None of these enzyme preparations were inhibited by APA-IgG. IgG concentration represents the highest concentration used.

findings confirm the immunological cross-reactivity of plasminogen activator produced by the SV40-transformed hamster cell line with plasminogen activator from normal hamster lung cells.

Is Plasminogen Activator Present on the Cell Surface? A major proportion of SV40-transformed hamster cell-bound plasminogen activator activity cosediments with a plasma membrane-enriched fraction from these cells (10). To examine the possibility that plasminogen activator is a surface component of these transformed hamster cells we took advantage of the capacity of antibodies directed against cell membrane antigens to initiate C-mediated cytotoxic effects upon cells displaying these antigens. No deleterious effect on cell viability as measured by trypan blue exclusion was noted when a suspension of SV40 hamster cells was incubated with APA-IgG and C. Control experiments showed that these SV40 hamster cells become permeable to trypan blue when they are incubated with C in the presence of anti-SV40 hamster cell membrane or anti-SV40 hamster cell immunoglobulin preparations, and that both C and immune IgG are required (Table V, method 1). Cell monolayers treated with
IMMUNOLOGICAL ANALYSIS OF PLASMINOGEN ACTIVATORS

TABLE V
Complement-Mediated Cytotoxic Effects of Immunoglobulin Preparations*

| Immunoglobulin                  | Complement | Viable cells | Method 1 | Method 2 | Method 3 |
|---------------------------------|------------|--------------|----------|----------|----------|
| Antiplasminogen activator IgG   | +          | >95          | >95      | >95      |          |
|                                 | -          | >95          | >95      |          | >95      |
| Antihamster membrane IgG        | +          | <5           | 6        | Cells lysed |         |
|                                 | -          | >95          | >95      | >95      |          |
| Antihamster cell IgG            | +          | <5           | 2        | cells lysed |         |
|                                 | -          | >90          | >90      | >95      |          |
| Control rabbit IgG              | +          | >95          | >95      | >95      |          |
|                                 | -          | >95          | >95      | >95      | ND       |

* This assay was performed by three methods, all of which gave similar results. (Method 1) SV40 hamster cell monolayers in 100 mm petri dishes were washed with PBS, and removed from the dish with trypsin (0.25% in PBS). The cells were mixed with an equal volume of Dulbecco’s medium containing 10% fetal bovine serum, pelleted by centrifugation (250 g, 4°C, 10 min) and resuspended in HBSS at a concentration of 1.0 x 10⁵ cells/ml. Each assay contained 5 x 10⁵ cells, 50 µl of the indicated antibody preparation, and 50 µl of adsorbed guinea pig C when appropriate. Samples in which C or antibody were omitted were brought to a final vol of 150 µl with HBSS. The samples were incubated 30 min at 37°C, immediately added to an equal volume of 0.08% trypan blue in HBSS, and assayed for cell viability by phase-contrast microscopy. (Method 2) 35 mm Petri dishes containing 5 x 10⁵ SV40 hamster cells in monolayer culture were washed with PBS and incubated at 37°C for 30 min with 0.1 ml HBSS, 0.1 ml of the indicated antibody preparation, and 0.1 ml of adsorbed guinea pig C as indicated. Samples in which C or antibody were omitted were brought to a final vol of 1 ml with HBSS. At the end of the incubation period the medium was removed, and the cells were incubated for 5 min at room temperature with 1 ml HBSS containing 0.04% Trypan blue. The medium was removed, a cover slip was placed over the monolayers and cell viability was measured as above. (Method 3) SV40 hamster cells were incubated as in method 2 above with HBSS and the indicated antibody preparation. The monolayers were then washed twice with HBSS, incubated an additional 30 min at 37°C with 0.8 ml HBSS, 0.1 ml adsorbed sheep antirabbit IgG, and 0.1 ml adsorbed C as indicated, and assayed for viable cells as in method 2 above.

In the course of these experiments we noted that although DON lung cells activate plasminogen and contain a cell-bound form of plasminogen activator which is inhibited by APA-IgG, they do not secrete detectable plasminogen...
activator into serum-free medium. To determine whether plasminogen activation by lung cells might result from the action of enzyme bound to the cell surface we incubated DON cells with APA-IgG and C (using methods 2 and 3 of Table V); no cytotoxic effects were noted. In contrast, over 95% of DON cells incubated with anti-SV40 hamster cell antibody and C were rendered trypan blue positive by this treatment.

The failure of APA-IgG and C to damage the SV40-transformed hamster cells is consistent with the hypothesis that plasminogen activator is a secretory product which is not displayed on the cell's surface. This is the case for most IgG-secreting plasma cells which do not contain these immunoglobulins on their surface membranes and are not lysed by C and anti-immunoglobulin antibodies (19). However, it is also possible that the amount of enzyme contained on the cell's surface may be too small to be detected by the cytotoxicity tests employed.

Discussion

The basic goal of these studies is the elucidation of the role, if any, of plasminogen activators in malignant transformation and tumorigenesis. For this purpose we purified the plasminogen activator from SV40 virus-transformed hamster cells, prepared antibodies against it, and using these antibodies as probes, compared the immunological determinants of the SV40 hamster cell enzyme with those of plasminogen activators produced by normal and malignant cells.

Our results allow three major conclusions to be drawn: (a) Multiple isozymes\(^2\) of plasminogen activator exist in normal hamster tissues. Antibodies prepared against SV40 hamster cell plasminogen activator inhibited the enzymes produced by primary hamster lung cells and by the DON diploid hamster lung cell line but had no effect on the plasminogen activator(s) of HaK heteroploid hamster kidney cells or of primary hamster kidney cells. These data are consistent with the findings of Kucinski et al. (3) and Aoki and von Kaulla (20). They found that antibodies to human urokinase were specific for the plasminogen activators from other human tissues.

(b) A plasminogen activator produced by an SV40 virus-transformed hamster cell line shares immunological determinants with the isozyme produced by normal hamster lung cells. Plasminogen activators produced by lung cells were inhibited by antibodies directed against the enzyme produced by a malignantly transformed cell line. Since this SV40 virus-transformed cell line was cloned from a culture of randomly transformed hamster embryo cells, we do not know the cell type or tissue from which the original transformant arose. It is possible that the original SV40 hamster cell transformant was a lung cell and that the tissue specificity of the APA-IgG reflects this fact. Alternatively, the tissue of origin of the original transformant may be irrelevant. The transforming virus may activate the production of one of several isozymes encoded in the cellular genome. Such activation could be random (SV40 transformants of the same cell

\(^2\) We use the term isozyme in its most general context as originally proposed by Markert and Moller (21), namely enzymes occurring in a single species in more than one structural form.
type would produce any one of several possible isozymes of plasminogen activator, or specific (all SV40 transformants of cells from a given species would produce the same isozyme regardless of the tissue of origin of the cell transformed). Convincing evidence has already been presented (4, 5) that the plasminogen activators produced by Rous sarcoma virus-transformed cells are not encoded by the viral genome. It seems likely that a similar situation will prevail in the case of SV40.

(c) Different malignant cell lines derived from the same species produce immunologically distinct plasminogen activators. HaK cells, a heteroploid line derived from adult kidney tissue, has been reported to cause tumors when injected intracerebrally into hamsters (18). Thus these cells, which are derived from the same species as the SV40-transformed hamster cells and are by definition a transformed line, produce a plasminogen activator which is immunologically different from the enzyme secreted by SV40 hamster cells. Recently, we have found that two independently derived lines of Rous sarcoma virus-transformed hamster cells and one line of benzoapyrene-transformed hamster embryo cells produce plasminogen activators which are not inhibited by the antibody (APA-IgG) directed against the plasminogen activator secreted by SV40 hamster cells. Thus, no one isozyme of plasminogen activator is characteristic of transformed cells. As discussed above, we have not yet established whether the isozyme expressed by a given clone of transformed cells is characteristic of the tissue or cell type from which the original transformant is derived or of the agent which initiates the transforming event. Moreover, we do not yet know how many different isozymes of plasminogen activator are produced in a single species and whether each tissue produces a specific isozyme. If in fact each tissue does produce a characteristic plasminogen activator and if the isozyme produced by a given tumor is determined by the cell type or tissue in which the tumor originated, not by the nature of the transforming agent, isozymes of plasminogen activators may be useful for identifying the tissue origin of tumors. In addition, if these enzymes are released into the circulation of tumor-bearing individuals, antibodies directed against specific isozymes may have diagnostic value.

Summary

Rabbits were immunized against the plasminogen activator released by SV40 virus-transformed hamster embryo cells. The resulting antiplasminogen activator immunoglobulin (APA-IgG) inhibited the enzymatic activity of the plasminogen activator produced by SV40-transformed hamster cells, and the plasmin-catalyzed release of these cells from the tissue culture dish. APA-IgG was not cytotoxic for these cells even in the presence of complement and did not inhibit their release of plasminogen activator. APA-IgG formed a single precipitin line in immunodiffusion plates using highly purified plasminogen activator as antigen. APA-IgG inhibited the plasminogen activator produced by newborn hamster lung cells and by an established diploid line (DON) of hamster lung cells, but did not inhibit plasminogen activators produced by normal or transformed hamster kidney cells or by cells of other species (mouse and human). We derive three major conclusions from these data: (a) There are several
immunologically distinguishable forms (isozymes) of plasminogen activators in normal hamster tissues. (b) The plasminogen activators produced by normal hamster lung cells and by SV40 virus-transformed hamster embryo cells share antigenic determinants and are presumably the same isozyme. (c) The plasminogen activators produced by different hamster tumor cells do not share antigenic determinants and are presumably different isozymes.

We would like to thank Mr. Cornelius Whalen for his dedicated aid in preparation of plasminogen activator from SV40-transformed hamster cells and Diane Ohlbaum for preparation of IgG fractions. We are grateful to Doctors L. Ossowski, E. Reich, D. B. Rifkin, S. Silagi, and S. Speigelman for their generous gifts of cell lines.

Received for publication 21 April 1975.

Bibliography

1. Astrup, T. 1966. Tissue activators of plasminogen. Fed. Proc. 25:42.
2. White, W. F., G. H. Barlow, and M. M. Mozen. 1966. The isolation and characterization of plasminogen activators (urokinase) from human urine. Biochemistry. 5:2160.
3. Kucinski, C. S., A. D. Fletcher, and S. Sherry. 1968. Effect of urokinase antiserum on plasminogen activators: demonstration of immunologic dissimilarity between plasminogen activator and urokinase. J. Clin. Invest. 47:1238.
4. Unkeless, J., A. Tobia, L. Ossowski, J. P. Quigley, D. B. Rifkin, and E. Reich. 1973. An enzymatic function associated with transformation of fibroblasts by oncogenic viruses I. Chick embryo fibroblast cultures transformed by avian RNA tumor viruses. J. Exp. Med. 137:85.
5. Ossowski, L., J. Unkeless, A. Tobia, J. P. Quigley, D. B. Rifkin, and E. Reich. 1973. An enzymatic function associated with transformation of fibroblasts by oncogenic viruses II. Mammalian fibroblast cultures transformed by DNA and RNA tumor viruses. J. Exp. Med. 137:112.
6. Rifkin, D. B., J. N. Loeb, G. Moore, and E. Reich. 1974. Properties of plasminogen activators formed by neoplastic human cell cultures. J. Exp. Med. 139:1317.
7. Pollack, R., R. Risser, S. Conlon, and D. Rifkin. 1973. Plasminogen activator production accompanies loss of anchorage regulation in transformation of primary rat embryo cells by simian virus 40. Proc. Natl. Acad. Sci. U. S. A. 71:4792.
8. Christman, J. K., S. Silagi, E. Newcomb, S. C. Silverstein, and G. Acs. 1975. Correlated suppression by 5-bromodeoxyuridine of tumorigenicity and plasminogen activator in mouse melanoma cells. Proc. Natl. Acad. Sci. U. S. A. 72:47.
9. Christman, J. K., and G. Acs. 1974. Purification and characterization of a cellular fibrinolytic factor associated with oncogenic transformation: the plasminogen activator from SV40 transformed hamster cells. Biochim. Biophys. Acta 340:339.
10. Christman, J. K., G. Acs, S. Silagi, and S. Silverstein. 1975. Plasminogen activator: biochemical characterization and correlation with tumorigenicity. In Proteases and Biological Control. E. Reich, D. Rifkin, and E. Shaw, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y. In press.
11. Silagi, S., P. Beju, J. Marshall, and E. de Harven. 1972: Tumorigenicity and virus production in mouse melanoma cells treated with 5-bromodeoxyuridine. Proc. Natl. Acad. Sci. U. S. A. 69:3443.
12. Atkinson, D. H., and D. F. Summers. 1971. Purification and properties of HeLa plasma membranes. J. Biol. Chem. 246:5162.
13. Deutsch, D., and E. T. Mertz. 1970. Plasminogens: purification from human plasma by affinity chromatography. Science (Wash., D. C.). 170:1095.
14. Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. J. Exp. Med. 99:167.
15. Griffin, F. M. Jr., and S. C. Silverstein. 1974. Segmental response of the macrophage plasma membrane to a phagocytic stimulus. J. Exp. Med. 139:323.
16. Clausen, J. 1971. Immunochemical Techniques for the Identification and Estimation of Macromolecules. North-Holland Publishing Co., Amsterdam, The Netherlands. p. 252.
17. Chance, R. E. 1972. Amino acid sequences of proinsulins and intermediates. Diabetes. 21:461.
18. Shannon, J. E., and M. L. Macy, editors. 1972. Second Edition, Registry of Animal Cell Lines. The American Type Culture Collection, Rockville, Md.
19. Takahashi, T., L. J. Old, K. R. McIntire, and E. A. Boyse. 1971. Immunoglobulin and other surface antigens of cells of the immune system. J. Exp. Med. 134:815.
20. Aoki, N., and K. N. von Kaulla. 1971. Dissimilarity of human vascular plasminogen activator and human urokinase. J. Lab. Clin. Med. 78:364.
21. Markert, C. L., and F. Moller. 1959. Multiple forms of enzymes: tissue, ontogenetic and species specific patterns. Proc. Natl. Acad. Sci. U. S. A. 45:753.