THE SECRETION OF FIBRINOLYSIN BY CULTURED RAT OVARIAN TUMOR CELLS

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Summary

Rat ovarian tumor cells grown in culture secrete a fibrinolysin which can be obtained in highly purified form from conditioned serum-free medium. Increased fibrinolytic activity may be a property associated with neoplasia and is currently under active investigation.

In the course of investigations by the senior author on rat ovarian tumor cells (1, 2) in the laboratory of Dr. G. Sato (summer 1972), gross fibrinolysis was observed around cells during growth on fibrin plates. A search into the literature revealed that a similar phenomenon was observed by Fischer in 1925 (3) who found that plasma clots were rapidly lysed by chicken sarcoma tissue but not by normal connective tissue. Since this reaction was not subsequently studied and in view of its possible relevance to neoplasia, we are pursuing the problem in some detail. Our records to date indicate that the rat ovarian tumor cells secrete a fibrinolysin into the surrounding medium. The enzyme is obtained in highly purified form from serum-free conditioned medium. Assay, partial purification, and characterization are described herein.

Materials and Methods

Bovine fibrinogen type I was obtained from Sigma Chemical Co., St. Louis, Mo. Highly purified human fibrinogen was a gift from Dr. Michael Mossesson (Brooklyn, N.Y.). Sera and powdered media were purchased from Grand Island Biological Co. Disposable Falconware petri dishes were used.

Growth and maintenance of cells. The origin and maintenance of several clones of rat ovarian tumor cells were described previously (1, 2). Cells were grown routinely in Dulbecco's modified Eagle medium with 10% fetal calf serum and 2.5% horse serum. Initially, luteinizing hormone (NIH-LH) was added to each plate at a final concentration of 1 μg/ml, but in later experiments, LH was not necessary.

Preparation of fibrin plates. Fibrinogen solutions of 1% concentration were prepared in 0.005 M PO_4 - 0.15 M NaCl, pH 7.5, (phosphate buffered saline, PBS) and sterilized by filtration. Five-ml aliquots were pipetted into 5-cm petri dishes to which 5 units of thrombin had been added followed by thorough mixing. After the formation of a firm clot the plates were equilibrated in several changes of medium over a 24-hr. period before cell plating.

Fibrin-agar plates for assay of fibrinolytic activity were prepared as described by Nilsson, Tomar and Taylor (4) with the following modifications: 6 ml aliquots of buffer containing 0.5% agar, 0.3% fibrinogen, and 0.1% sodium azide, were poured into 5-cm petri dishes containing 5 units of thrombin. The contents were rapidly mixed and allowed to clot. Three mm. wide wells were punched into the agar-fibrin and filled with 10 μl of the fibrinolysin. After 24-hrs. incubation at 37°C the cleared zones of fibrinolysis around the wells were measured.

Demonstration of fibrinogenolysis by immunoelectrophoresis. Following incubation of a known concentration of highly purified human fibrinogen with the enzyme, residual fibrinogen was measured by antigen-antibody crossed electrophoresis (5) using specific antihuman fibrinogen antibody.

Results

When 31 A cells (1, 2) (Fig. 1 a) were grown on fibrin plates, areas of gross fibrinolysis were observed after 4-6 days, proceeding to lysis of the entire fibrin layer in 10 days at which time cells had covered the bottom of the plate. Microscopic observations of this process are shown in Fig. 1 b and c. Cells plated on day 0 attach to the fibrin and in two days become surrounded by a "halo" of fibrinolysis (Fig. 1 b). As a cell lyses through the fibrin layer, it attaches to the bottom of the plate and forms a colony (Fig. 1 c). After 6-8 days the plate is filled with holes (Fig. 2), corresponding to the number of colonies.

Fibrinolysis could result from a cell membrane-associated enzymatic activity or from an enzyme secreted into the medium. The relatively large area of fibrinolysis surrounding a single cell suggested the latter possibility. Cell free conditioned medium from 5 day-old plates when layered on fibrin plates resulted in gross lysis in 10 to 12 hrs. (Fig. 3). Lysis could be effected by conditioned medium, whole cells, cell homogenates or extracts. Of the 6 clones of rat ovarian tumor cells (1, 2) tested, only two exhibited strong fibrinolytic activity, 53 I and 31 A clone 1. Weak activity was exhibited by 31 A clone 2 but none was observed by clones 3, 5, or 12. Extracts prepared from these latter clones were also inactive. Conditioned medium from mouse fibroblast (3T3) had no activity.

Secretion of fibrinolysin in serum-free medium. The secretion of fibrinolysin continued for at least 48 hrs. in serum-free medium without apparent

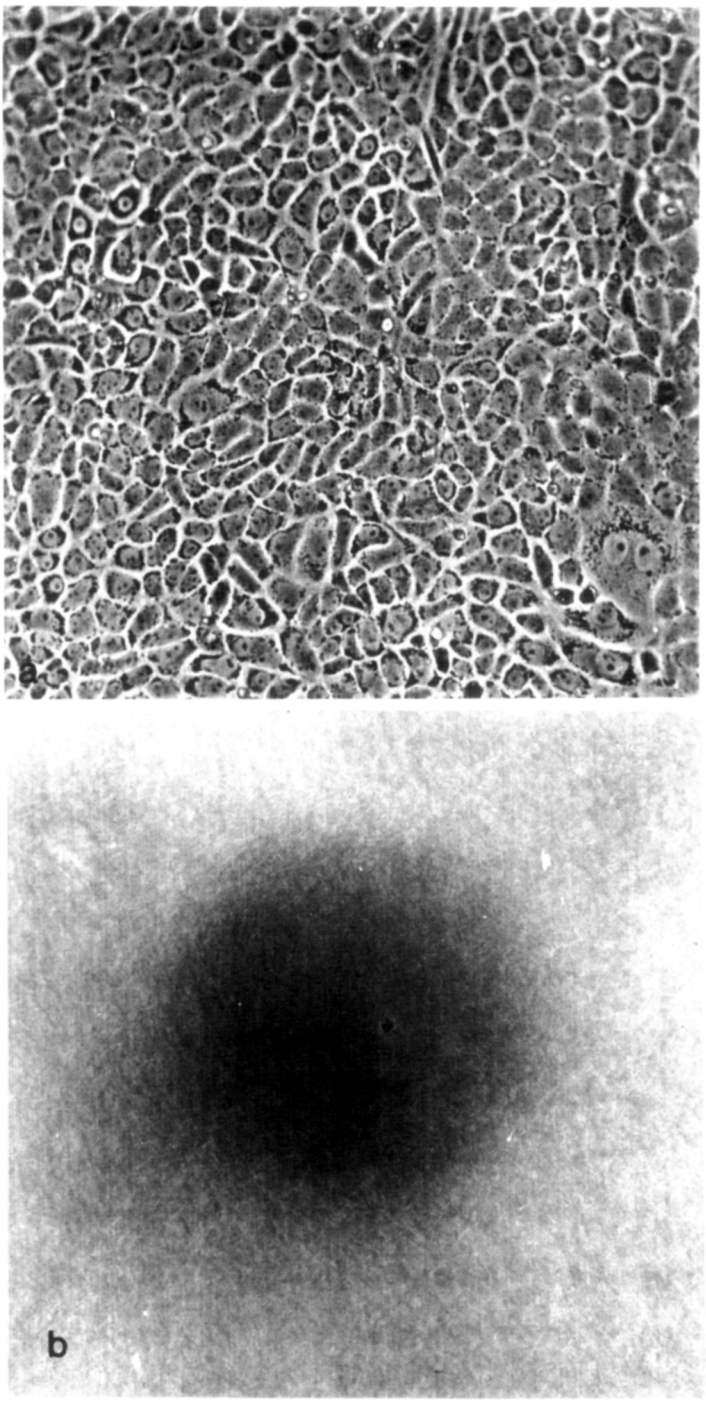


Figure 1 a. Rat ovarian tumor cells (31 A) grown in Dulbecco's modified Eagle medium with 10% fetal calf serum and 2.5% horse serum; 10^5 cells were plated in 10 cm plates. The photograph was taken on day 10.

b. Ovarian tumor cell attached to fibrin on the second day of plating surrounded by a "halo" of fibrinolysis.

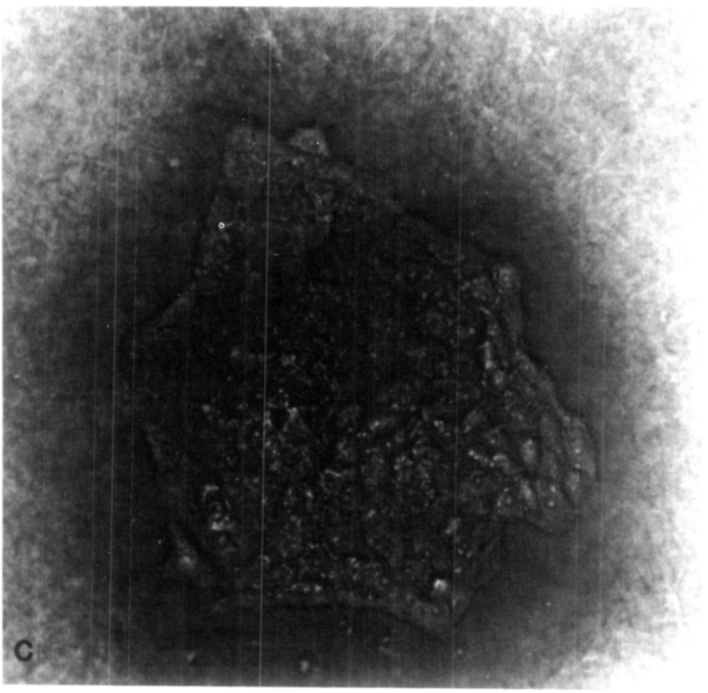


Figure 1 (continued).

c. Colony of cells formed from the same cell in b after 6 days, surrounded by an area of complete fibrinolysis.

cell death. Advantage was taken of this observation to prepare partially purified enzyme. After cells were grown in 40 to 80 - 10 cm plates, the medium was aspirated and the plates were rinsed with a small volume of serum-free medium. Three ml of serum-free medium then was added to each plate and they were incubated for 48 hrs. The medium was collected, concentrated by lyophilization, dissolved in a small volume of PBS and dialyzed for 6 hrs. against one liter of the same to eliminate excess salt. Denatured precipitated protein was eliminated by centrifugation.

Assay of fibrinolytic activity. When concentrated conditioned serum-free medium (CSFM) was assayed for fibrinolysin in fibrin-agar plates as described under methods there was a linear relationship between protein concentration and ring diameter over a wide range (Fig. 4).

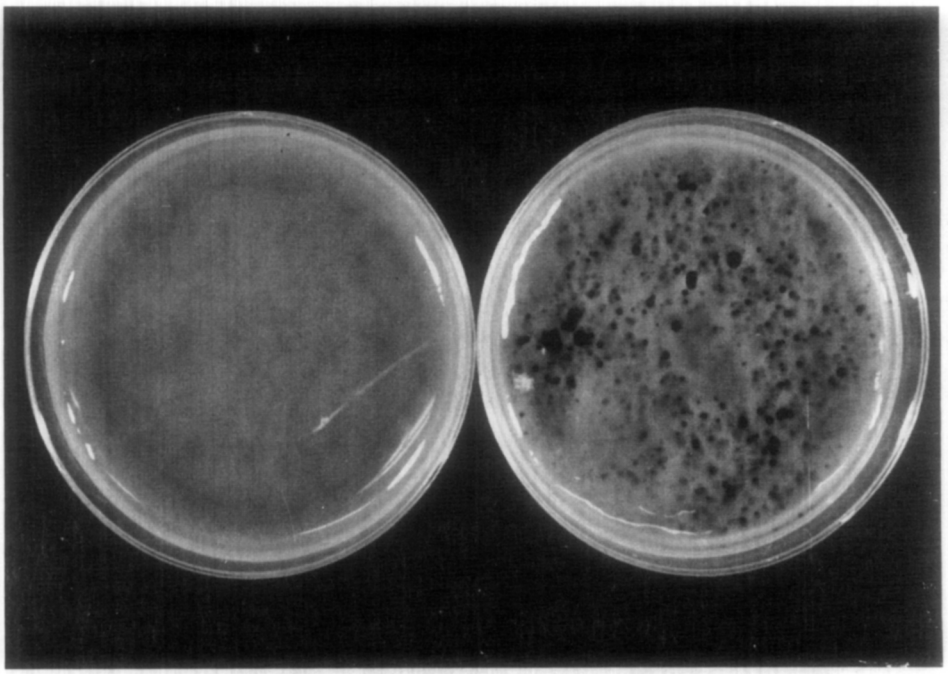


Figure 2 Seven-day old fibrin plate (right) in which 2×10^4 cells were plated on day 0 (plate on left was the control without cells).

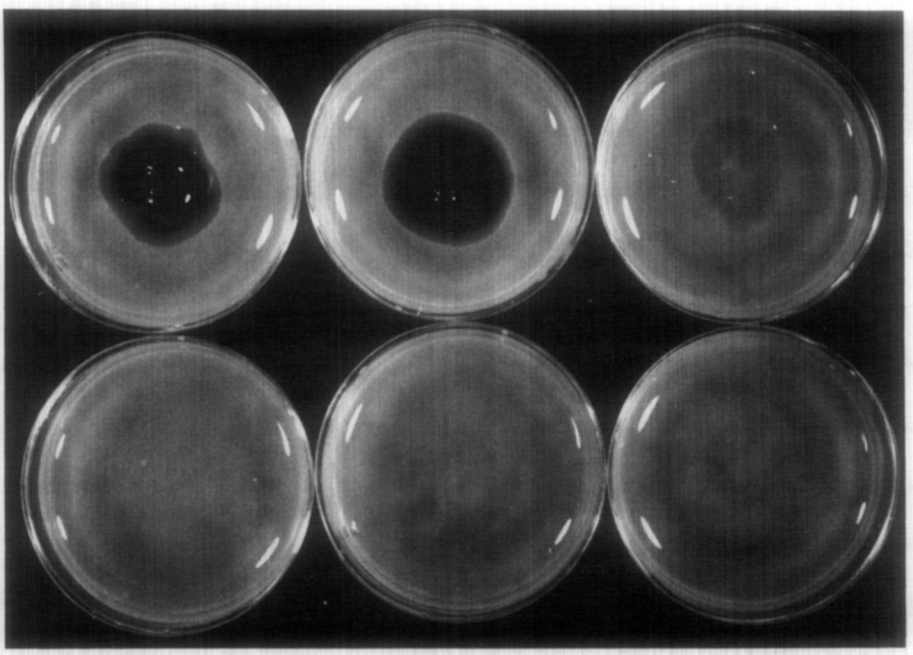
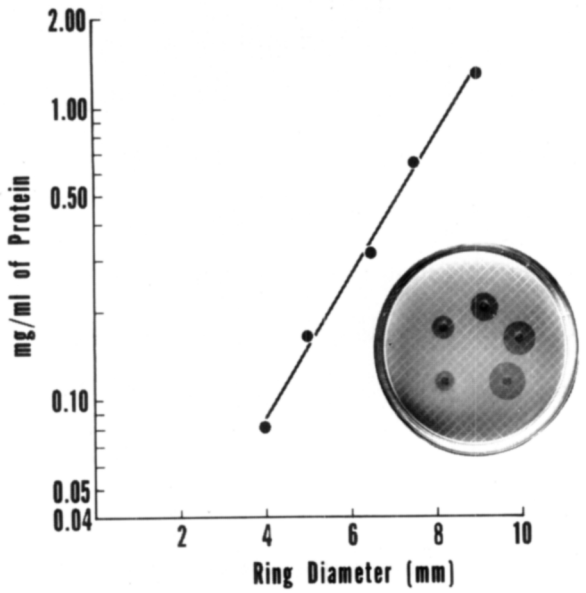
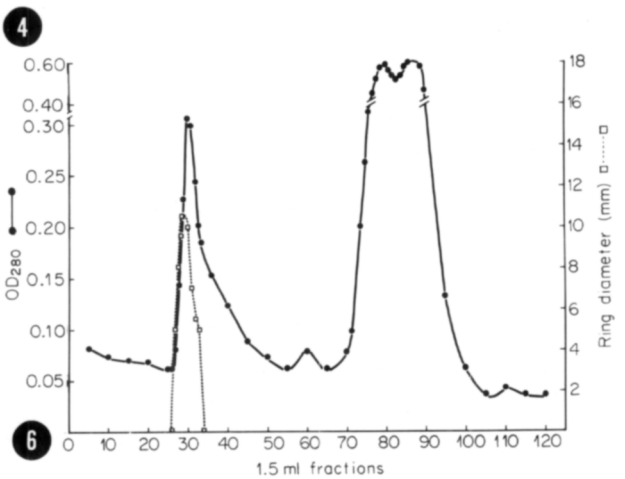


Figure 3 Fibrinolysis by cell free conditioned medium from 8-day old plates. One drop of medium (ca. 0.05 ml) was placed on fibrin and the plates were examined 12 hours later. From left to right - Top: 53 I, 31 A clone 1 and 31 A clone 2. Bottom: 31 A clone 3, clone 5 and clone 12.





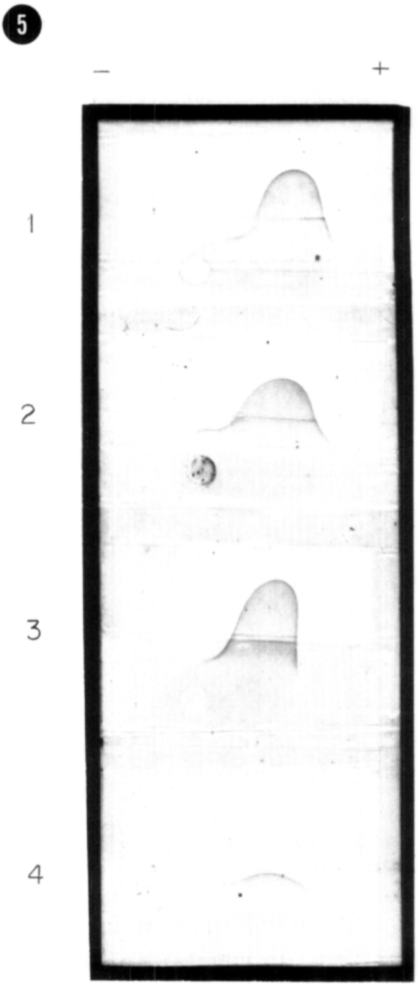


Figure 4 Assay of fibrinolysis in fibrin-agar plates. Serial dilutions of CSFM (1.8 mg/ml) were assayed and activity plotted as shown. Relationship of diameter of fibrinolytic area to protein concentration was linear.

Figure 5 Degradation of human fibrinogen by the fibrinolytic enzyme. PBS or enzyme were added to the fibrinogen (final conc. 1 mg/ml), incubated for 4 to 24 hrs. at ca. 25°C, and samples of each mixture were run in antigen-antibody crossed electrophoresis: Nos. 1 and 3 are buffer + fibrinogen 4 and 24 hrs. 2 and 4 enzyme + fibrinogen 4 and 24 hrs.

Figure 6 Filtration of concentrated serum-free conditioned medium on Sephadex G-100. (See text for details). The large bifurcated absorbance peak that eluted last consisted mainly of phenol red and other small molecules not eliminated by the brief dialysis prior to filtration; absorbance was largely eliminated by dialyzing the pooled fractions.

Fibrinogenolysis shown by antigen-antibody crossed electrophoresis. One volume of human fibrinogen (2 mg/ml) was incubated with either one volume of PBS or one volume of CSFM for 4 or 24 hrs. at room temp. (ca. 25° C) with end-to-end rotation. The samples were then run in electrophoresis and the height of each peak was measured and compared. Figure 5 shows that although the 4 hr. -peak of the fibrinogen-enzyme mixture (No. 2) compared with the fibrinogen-buffer control (No. 1) was only slightly reduced (12%), the 24 hr. peak was reduced ca. 60% (No. 4) compared to the buffer control (No. 3).

Gel filtration and partial characterization. An aliquot (1.2 ml) of CSFM was applied to a 1.3 x 105 cm Sephadex G-100 column (Fig. 6) previously equilibrated with PBS. All the activity emerged in the ascending portion of the first peak. Fractions with the highest specific activities were pooled, concentrated by dialysis against a saturated sucrose solution, and dialyzed against PBS. The final preparation when subjected to electrophoresis in polyacrylamide gel exhibited two bands, a major (70%) and a minor one (30%). (Not illustrated). The specific activities, expressed as mm lysis/μg protein, in regular conditioned medium, serum-free medium, and Sephadex filtrate were 0.04, 0.6 and 2.5 respectively.

Upon sucrose gradient ultracentrifugation, partially purified enzyme sedimented in two activity peaks, a major peak with a MW of 615,000 and a minor peak with a MW of 175,000 suggesting a tetramer-monomer relationship. (MW was determined according to Martin and Ames (6) using bovine liver catalase (MW 250,000) as a marker).

Inhibitors. Fibrinolytic activity was inhibited by soybean trypsin inhibitor and by 0.3 M ε-aminocaproic acid.

Discussion

The rat ovarian cells used in these experiments were originally derived from tumors produced by implanting luteal phase ovaries of young adult Fischer rats into spleens of young adult female castrates of the same strain (2, 3). Three of the 6 clones secreted fibrinolytic activity in culture. The others neither contained nor secreted any detectable activity. Whether the secretion of fibrinolysin is a property specific to tumor cells remains to be determined. Although these ovarian cells were originally derived from tumors, they do not produce tumors when injected into rats. Therefore, correlation of fibrinolytic activity with malignancy cannot be made at the present.

Recently Unkeless et al (7) and Ossowski et al (8) described the development of fibrinolytic activity in several lines of fibroblasts after transformation by Rous sarcoma virus. Activity was not present in normal cultures. Like the fibrinolysin described here, their enzyme was inhibited by soybean trypsin inhibitor and by ε-aminocaproic acid suggesting trypsin-like specificity.

The nature of the fibrinolytic factor is uncertain at this time. It may be the fibrinolytic enzyme itself or an activator of plasminogen which is a contaminant of the fibrinogen. Our results using highly purified preparations of enzyme and human fibrinogen favor the former possibility but further studies on the purified protein, its substrate specificity and its relationship to plasmin are necessary. The relative ease of cultivating cells in large numbers and the secretion of the enzyme into serum-free medium should allow the large scale preparation of highly purified fibrinolysin for study.

Acknowledgments

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References

1. Sato, G. H., in Molecular Genetics and Develop. Biol., M. Sussman Editor. Prentice Hall, N.J. pp. 475 (1971).
2. Clark, J. L., Jones, K. K., Gospodarowicz, D., and Sato, G. H., Nature New Biol. 236, 180, (1972).
3. Fischer, A., Arch. Entwicklungsmech. Org. 104, 210, (1925).
4. Nilsson, U. R., Tomar, R. H., and Taylor, F. B. Jr., Immunochem. 9, 709, (1972).
5. Laurell, C. B., Analyt. Biochem. 10, 358, (1965).
6. Martin, R. G., and Ames, B. N., J. Biol. Chem. 236, 1372, (1960).
7. Unkeless, J. C., Tobia, A., Ossowski, L., Quigley, J. P., Rifkin, D. B., and Reich, E., J. Exp. Med. 137, 85, (1973).
8. Ossowski, L., Unkeless, J. C., Tobia, A., Quingley, J. P., Rifkin, D.B., and Reich, E., J. Exp. Med. 137, 112, (1973).