PROPERTIES OF PLASMINOGEN ACTIVATORS FORMED BY NEOPLASTIC HUMAN CELL CULTURES*

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We have previously reported that cell cultures transformed by oncogenic viruses develop a two-stage fibrinolytic system whose activity is generated by the interaction of a serum protein and a factor released by transformed cells (1, 2). The serum factor has been identified as the proenzyme plasminogen (3), and the cell factor is a specific serine protease that functions as a plasminogen activator (4). High levels of cell factor are released by avian and mammalian fibroblasts transformed in vitro, and by primary cultures of malignant tumors, such as chemically or virally induced mammary carcinomas. Normal fibroblasts, and cultures of other normal control tissues, release little or no activator.

To determine whether similar fibrinolytic activity is associated with human neoplasia, we have studied the properties of three human tumor cell lines. As shown below, all of these cultures produce high levels of fibrinolytic activity and release proteins whose properties correspond to those found previously for plasminogen activators from transformed chicken, rat, hamster, and mouse cells (1–4).

Materials and Methods

The three lines of human neoplastic cells used were derived respectively from an osteosarcoma (RPMI-41), a mesothelioma (RPMI-212), and a malignant melanoma (RPMI-8352). The cells were cultured in plastic petri dishes (Falcon Plastics, Division of BioQuest, Oxnard, Calif.) using RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% of fetal bovine serum. The cells were maintained and subcultured as previously described (5).

Human cell cultures of nonmalignant origin were purchased from GIBCO; these included cultures of embryonic skin, embryonic lung, and embryonic kidney. Alternatively, primary

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cultures of human embryonic fibroblasts were prepared from fresh surgical material. The cells were cultured using the same conditions as described for the malignant human cells.

The preparation of conditioned serum-free culture medium, the assay for fibrinolytic activity, and the preparation of the protease inhibitors were as previously described (1–3). All determinations were performed in duplicate. The assay is based on the release of [^{125}I]fibrin from the surface of petri dishes into the supernatant solution; the solubilization of radioactivity depends on partial digestion of the insoluble fibrin. For assaying the effect of diisopropylphosphofluoridate (DFP), conditioned medium was incubated with DFP (0.002 M) for 2 h, dialyzed overnight against 1,000 vol of Eagle's medium, and then assayed in the usual manner. A control sample was processed in the same way except that DFP was omitted.

To test the effects of other presumed inhibitors of fibrinolysis, assays were performed in petri dishes containing [^{125}I]fibrin (10 μg/cm^2; total radioactivity 4×10^5 cpm). The inhibitor and a standard amount of conditioned medium were mixed, and the reaction started by the addition of monkey serum to a final concentration of 2.5%. The vol was 1 ml and incubation was for 6 hr at 37°C.

Partially purified cell factor (plasminogen activator) was isolated from conditioned medium according to the procedure of Unkeless et al. (4). The crude culture fluid containing active cell factor was first clarified by centrifugation (5,000 *g*, 10 min) and then brought to pH 3.5 by the addition of 1 M HCl; solid ammonium sulfate (560 g/liter) was added slowly with constant stirring at 4°C, and the precipitate was collected by centrifugation (5,000 *g*, 10 min.) at 4°C. The precipitate was dissolved in the minimal volume of 0.005 M glycine-HCl buffer, pH 2.3, and then was adsorbed to a column of SP-Sephadex (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) as described by Unkeless et al. (4); after washing the column with 0.05 M ammonium acetate at pH 5.25 the cell factor was eluted with 0.2 M ammonium sulfate, pH 5.25. The pooled active fractions were then dialyzed against water and concentrated to a small volume. Aliquots of these partially purified preparations were incubated with [^{3}H]DFP (New England Nuclear, Boston, Mass.) (4°C, 24 h); control incubations were performed in parallel without DFP, and samples of both were analyzed by SDS-polyacrylamide gel electrophoresis as described elsewhere (4, 6); the gels were cut into 1-mm slices and these were assayed either for radioactivity ([^{3}H]DFP) or for cell factor (plasminogen activator) activity (1, 4).

RESULTS

Fibrinolytic Activity Produced by Human Tumor Cell Lines.—Previous work with nonhuman material has shown that transformed cultures incubated in serum-free medium release a plasminogen activator that promotes fibrinolysis when mixed with serum (1, 2). We have examined similar conditioned media from human osteosarcoma, melanoma, and mesothelioma cell lines for their content of activator. The data in Table I show that conditioned medium for each of the three cell lines produced fibrinolysis in the presence of appropriate sera. Two elements were required for fibrinolysis; one of these was contributed by the conditioned media and the other by the serum. Thus, neither medium alone, nor medium plus serum, nor conditioned medium alone produced fibrinolysis; however, conditioned medium supplemented with the appropriate serum effectively hydrolyzed the radioactive fibrin. The properties of conditioned media from human tumor cell lines therefore resembled those previously reported for comparable materials from nonhuman cultures.

Serum Specificity of Fibrinolysis: Activating and Inhibitory Sera.—One important characteristic of the fibrinolytic activity produced by other transformed

TABLE I

Serum Specificity of Fibrinolytic Activity of Conditioned Medium from Human Tumor Cells

Serum supplement	Conditioned medium tested: Radioactivity released		
	Osteosarcoma*	Melanoma*	Mesothelioma‡
		% of total	
Dog	3.8	5.8	2.3
Monkey	44.5	73.8	14.3
Mouse	51.0	35.4	29.6
Human (DBR)	57.3	78.0	12.4
Fetal bovine	8.6	9.9	2.3
Horse	24.3	50.1	5.3
Fetal pig	2.6	1.17	1.8
Bovine	20.7	39.4	NT§
Lamb	3.8	15.9	1.4
Rat	10.0	7.9	5.6
None	2.5	4.5	2.3
	RPMI	RPMI	RPMI
None	2.5	1.9	1.5
Mouse	1.9	2.3	1.4

Petri dishes (35 mm) containing [^{125}I]fibrin (10 μg/cm^2) were prepared as described (1). Fresh or conditioned serum-free RPMI-1640 (2 ml) was mixed with the indicated serum supplement to yield a final serum concentration of 2.5% (vol/vol), added to the [^{125}I]fibrin-coated plates, and incubated for 6 h at 37°C. The radioactivity in the incubation medium was then measured. The melanoma-conditioned medium was diluted 1:10 with RPMI-1640 before assay.

* Total radioactivity 22,000 cpm/plate.

‡ Total radioactivity 43,000 cpm/plate.

§ NT, not tested.

cultures was the apparent serum specificity of the reaction; for each type of cell, there was a species-specific spectrum of activating and nonactivating sera (1–3). As also seen in Table I, the same was true for conditioned media from the human tumor cell lines: monkey, mouse, human, bovine, and horse sera were activating, whereas sera from rat, fetal bovine, dog, and fetal pig were nonactivating. With all of the transformed cultures previously investigated the spectrum of activating sera was species specific, and was not influenced either by the nature of the transforming agent or by the tissue of origin. One apparent inconsistency with previous results, shown by conditioned medium from one of the human tumor cell lines, was the failure of horse serum to be activated by mesothelioma-conditioned medium. This resulted from the fact that the mesothelioma cultures produced significantly less activator than the other two cell lines, as reflected in the generally lower fibrinolysis obtained with all sera; the ability of this conditioned medium to activate horse serum

was therefore detectable only after somewhat longer periods of incubation (12 h).

The level of fibrinolysis was dependent on the concentration of both conditioned medium and serum in the assay mixture. When the conditioned medium was diluted (e.g., with RPMI-1640 medium) corresponding decreases in enzyme activity were produced (Fig. 1). The effect of changing serum concentrations

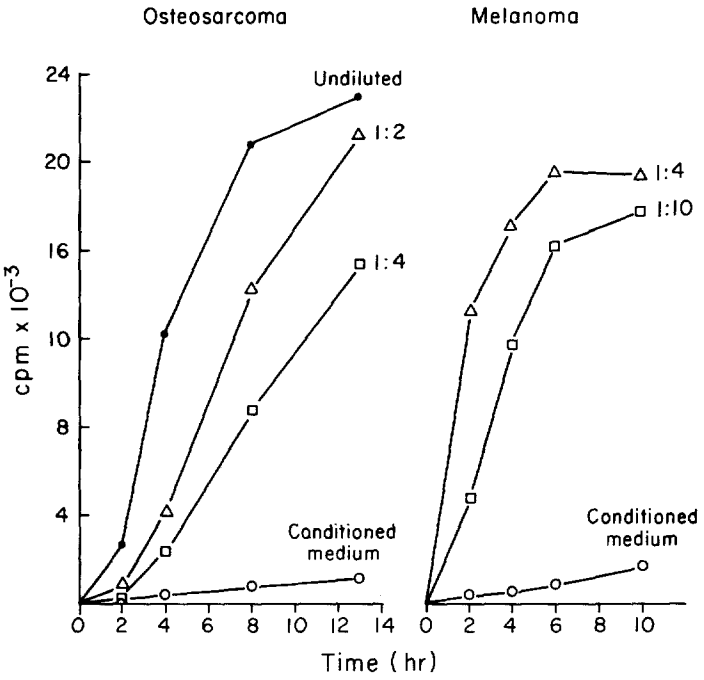

FIG. 1. Conditioned serum-free media from osteosarcoma and melanoma cell cultures were diluted as shown with RPMI and incubated in petri dishes (35 mm) containing [^{125}I]fibrin (10 μg/cm; total radioactivity 6 $\times$ 10^5 cpm). Final vol was 3 ml, the serum concentration was 2.5%, and incubation was in a CO_2 incubator at 37°C. At the indicated times aliquots of the medium were removed and assayed for soluble radioactivity. The curves labeled "conditioned medium" are controls incubated in absence of serum.

was more complex (Table II). As the serum concentration was progressively increased the rate of fibrinolysis first increased, then reached a plateau, and finally decreased. We have analyzed this result and have found that at low concentrations of serum, the rate of fibrinolysis was limited by the amount of serum factor, plasminogen. At the higher levels of serum, the rate of enzyme action was limited by the increasing concentration of serum protease inhibitors; this was shown by the effect of briefly exposing serum to mild acid (pH 3 for

120 min), a treatment which inactivated the serum inhibitors.[1] This treatment abolished the inhibition of fibrinolysis previously seen at high serum concentrations; the rate of fibrinolysis then became proportional to both the cell and serum factors over a wide concentration range for each, and permitted the fibrinolytic assay to be used for purification of the human cell factor.

It appeared likely that the low rates of fibrinolysis observed with nonactivat-

TABLE II

Effect of Serum Concentration on Fibrinolysis by Conditioned Medium from Malignant Human Cell Cultures

Monkey serum	Conditioned medium tested	Radioactivity released
% vol/vol		*% of total*
10	Osteosarcoma	3.2
5	"	6.8
2	"	31.6
1	"	37.2
0.5	"	36.4
0.1	"	9.4
None	"	2.6
10	Melanoma	10.0
5	"	34.9
2	"	61.9
1	"	66.0
0.5	"	41.6
0.1	"	8.0
None	"	1.6
10	Mesothelioma	5.2
5	"	8.3
2	"	17.0
1	"	22.6
0.5	"	17.0
0.1	"	10.0
None	"	1.0

Petri dishes (35 mm) containing [^{125}I]fibrin (10 $\mu g/cm^2$) were prepared as described. Conditioned medium (2 ml) was mixed with serum to yield the indicated final serum concentration and then incubated for 8 h at 37°C. The radioactivity in the incubation medium was determined at the end of this period. Total radioactivity in each dish was 46,000 cpm.

ing sera might be due to high concentrations of inhibitors, and therefore assays were performed with different serum mixtures to test for this (Table III). In all cases the addition of small amounts of nonactivating sera (e.g., dog serum) reduced the level of fibrinolysis obtained in reactions with activating sera, such as monkey serum. For example, the addition of dog serum (1%) to an assay of osteosarcoma-conditioned medium containing monkey serum (2.5%) re-

[1] D. Loskutoff, unpublished observations.

TABLE III

Effect of Inhibitory Sera on Fibrinolytic Activity of Conditioned Medium

Concentration of serum		
Monkey serum	Fetal bovine serum	Radioactivity released into solution
% vol/vol	*% vol/vol*	*% of total*
2.5	0	83.0
2.5	1	65.0
2.5	5	4.9
2.5	10	3.2
0	5	3.7
Monkey serum	Dog serum	Radioactivity released into solution
% vol/vol	*% vol/vol*	*% of total*
2.5	0	82.0
2.5	1	15.8
2.5	5	2.4
2.5	10	1.5
0	5	1.6
Concentration of serum		
Monkey serum	Fetal pig serum	Radioactivity released into solution
% vol/vol	*% vol/vol*	*% of total*
2.5	0	100
2.5	1	78
2.5	5	4
2.5	10	1.2
0	2.5	1.5

Petri dishes (35-mm diameter) containing [^{125}I]fibrin (10 μg/cm^2) were prepared as described previously. Serum supplements were added to conditioned medium from tumor cell lines (2 ml) and the dishes incubated for 8 h at 37°C. The radioactivity released into solution was then assayed. Total radioactivity: A, 72,000 cpm; B, 40,000 cpm.

duced the [^{125}I]fibrin release from 82% to 15.8%. This inhibitory effect of nonactivating sera was abolished by mild acid treatment, as was previously found for the inhibition occurring at high concentrations of activating sera (vide supra). Thus, the difference between activating and nonactivating sera was only quantitative and reflected differences in the effective concentrations of protease inhibitors. The inhibitory effects of nonactivating sera could also be overcome by increasing concentrations of conditioned medium, i.e., plasminogen activator.

Inhibitors of Fibrinolytic Activity.—Fibrinolysis stimulated by conditioned medium from human cultures was inhibited by the same compounds (1, 2) that had previously been found to inhibit the activity from other animal tumor cells. The concentrations required for 95% inhibition of fibrinolysis by the combination of osteosarcoma-conditioned medium and monkey serum (2.5%

final concentration; conditions as in Table I) were as follows: nitrophenyl-*p*-guanidobenzoate, 0.1 μg/ml; soybean trypsin inhibitor, 20 μg/ml; ε-amino caproic acid, 50 μg/ml; and sodium chloride, 0.5 M. Similar results were obtained with melanoma-conditioned medium, and the activity of all of the conditioned media was abolished by 2×10^{-3} M DFP.

Molecular Size and Activity of Human Cell Factor.—When conditioned media or partially purified cell factor were exposed to DFP, the ability to stimulate fibrinolysis by activating sera was irreversibly lost. This suggested that the cell factor in conditioned media probably contained an active serine residue that could be labeled with [^{3}H]DFP and thereby identified after SDS-polyacrylamide gel electrophoresis. Furthermore, since Christman and Acs (7) found that the cell factor from transformed hamster cells was not inactivated by exposure to SDS, polyacrylamide gels could also be analyzed for catalytically active cell factor. As seen in Fig. 2, partially purified cell factor preparations contained two distinct components that stimulated fibrinolysis by monkey serum. The mobility of the major component corresponded to a mol wt of 48,000, while that of the minor component was approximately 60,000. In electrophoretic mobility and size, the cell factors in melanoma-conditioned medium therefore resembled those produced by transformed mouse (4) and hamster (7) cultures (mol wt $\sim$ 50,000), rather than that released by transformed chick (4) cells (mol wt $\sim$ 39,000).

Both of the peaks found in the assays of catalytic activity were also visible in the radioactivity profile after reaction with [^{3}H]DFP, and both were therefore serine enzymes; in addition, there were several lesser peaks of incorporated [^{3}H]DFP that might indicate the presence of other serine enzymes in the conditioned medium. When the gel was assayed for fibrinolysis using purified human plasminogen the resulting activity profile was identical with that obtained using monkey serum. Thus, the human cell factor, like those found in transformed cultures from other species, is a serine protease that functions as a plasminogen activator.

Fibrinolytic Activity of Nonmalignant Human Cells.—Several types of human cells derived from nonmalignant tissues have been tested for fibrinolytic activity. Of the cultures listed in Table IV only the embryonic kidney cells were an established cell line, and the remainder were from early subcultures of primary cultures. Neither fibroblasts obtained from trypsinized embryos nor embryonic skin cells showed significant fibrinolytic activity. Because cultures of normal kidney tissue have been reported to form the fibrinolytic activator urokinase (8), the failure of the embryonic kidney cell line to show activity was unexpected.[2] In contrast, cultures of normal embryonic lung cells produced very

[2] Several other independently isolated kidney cell lines have recently been tested and some of these do show serum-dependent fibrinolysis. The latter include BHK, MDBK, and primary monkey kidney cells as well as primary human embryonic kidney cells purchased from Flow Laboratories, Inc., Rockville, Md. and from Microbiological Associates, Bethesda, Md.

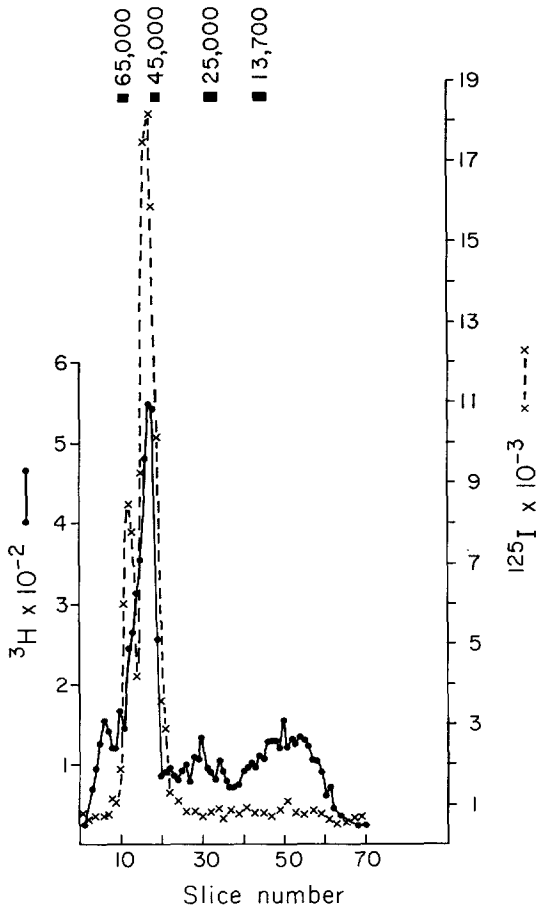

FIG. 2. SDS-polyacrylamide gel electrophoresis of human melanoma cell factor. Partially purified cell factor was inactivated with [^{3}H]DFP as described in the text. The [^{3}H]DFP-inactivated material and a control were dissolved in buffer containing 5% glycerol, 1% SDS, and 5% ethylene glycol and applied to a slab gel (10% acrylamide, 0.1% SDS) along with standard protein markers. The gel was cut into separate lanes and these were sliced and assayed either for radioactivity or for cell factor activity.

high levels of fibrinolysis, but some of this was independent of serum and might therefore have been due to the action of other proteases. Further work will be required to characterize the nature of this activity.[3]

Properties of Different Individual Human Sera.—Because the fibrinolysis observed in culture might be related to cell function in the body, it was of interest to explore for possible differences in the ability of individual sera to generate

[3] Several additional independent cultures of cells derived from human embryonic lung, including WI38, have been assayed and all produce significant levels of fibrinolysis. Several of these release plasminogen activator in culture (J. O'Malley and D. Rifkin, unpublished observations).

TABLE IV

Effects of Different Sera on Fibrinolytic Activity of Nonmalignant Human Cell Cultures

Cell Type	Period of Incubation	Serum supplement: No serum	Monkey	Dog	Human	Fetal bovine
		Radioactivity Released				
	h	*% of total*				
Human embryonic fibroblasts	4	0.7	2.3	1.02	1.9	1.5
	16	2.3	5.3	2.8	5.2	2.8
Human embryonic lung	4	21.2	75.4	56.5	81.0	46.2
	16	69.4	85.1	86.7	95.1	88.9
Human embryonic skin	4	1.4	6.1	2.1	3.3	1.3
	16	4.6	12.3	7.2	9.9	3.8
Human embryonic kidney	4	0.7	1.2	1.3	1.7	.9
	16	2.0	3.0	2.9	4.1	3.7
Medium alone	4	0.5	0.5	0.8	0.4	0.5
	16	1.3	1.2	1.9	1.2	1.4

Cells were plated in Eagle's medium containing 10% of fetal bovine serum in 60-mm petri dishes coated with [^{125}I]fibrin (10 μg/cm^2) and allowed to settle for 4 h. The medium was then removed, the cells washed twice with buffer, and 3 ml of Eagle's medium containing the indicated serum supplement was added. After a 4 h incubation at 37°C, the medium was removed for measurement of radioactivity. Fresh medium of identical composition was added (3 ml), incubation was continued for a further period of 12 h, after which the radioactivity released was again measured. The final concentration of serum was 2.5%, and the total radioactivity in each dish was 20,000 cpm.

enzyme activity. As an initial approach to such a survey we have examined sera from more than 100 individuals. A representative sampling is presented in Table V; there was a wide range of variation, the activity of the most active serum being approximately 30-fold higher than that of the least active. The fibrinolytic activity of serum from a single donor (under standard conditions of cell factor concentration)appeared to be reproducible when blood was drawn at intervals over a period of months, and no distinctive pattern of activity has so far been correlated with any category of disease. The range of fibrinolytic activities seen with the different sera probably reflected the concentration of serum inhibitors, since the differences were eliminated, and the level of fibrinolysis was increased, by treatments that inactivated serum protease inhibitors (e.g., brief exposure to pH 3).

DISCUSSION

Previous work with avian and mammalian fibroblasts has established that primary cultures, transformed by either DNA or RNA viruses, produce levels of fibrinolytic activity much higher than those of the normal, control cultures. Since the publication of our initial reports (1, 2), comparably elevated levels of fibrinolysis have been observed in primary cultures of chemically induced rat hepatomas and mammary carcinomas, and in mouse mammary carcinomas

TABLE V

Effect of Individual Human Sera on Fibrinolytic Activity of Osteosarcoma-Conditioned Medium

Test Fluid	Serum Supplement	Radioactivity Released	
		cpm	*% of total*
Fresh RPMI medium	D.L.	767	1.3
Osteosarcoma conditioned medium	—	2,442	4.1
"	D.L.	1,367	2.3
"	D.E.	1,875	3.1
"	L.O.	11,347	18.8
"	Gz	29,342	48.8
"	968	22,370	37.2
"	440	25,954	43.2
"	Gd	17,698	29.4
"	Jhn	3,498	5.8
"	2191	16,442	27.4
"	D.R.	18,000	30.0
"	CAL	862	1.4
"	MEL	1,460	2.4

Petri dishes (35 mm) containing [^{125}I]fibrin (10 μg/cm; 6 $\times$ 10^5 cpm) were prepared as described previously. Conditioned or fresh medium was mixed with the individual sera at a final concentration of 2.5%, incubated for 7.5 h, and then assayed for radioactivity.

and skin tumors induced, respectively, by viruses and by chemical carcinogens.[4] In these cases also, the enzyme activity produced by normal tissues was at, or close to background levels. The data presented here show that the fibrinolytic activity of human tumor cell lines resembles that obtained with transformed cells and primary cultures of malignant tissue from other species. The similarities include the following properties of the fibrinolytic system: (*a*) The fibrinolysis produced by human cells requires two factors: one is released by the cells, and the other, found in serum, is plasminogen. (*b*) High levels of fibrinolysis are not observed with all sera. The activating sera for the human system are monkey, human, mouse, bovine, and horse. Dog serum, which activates fibrinolysis with transformed hamster, mouse, and rat cells, permits only minimal levels of fibrinolysis with human cells. Fetal bovine serum also is inhibitory, just as it is in all other systems studies so far. (*c*) The activity of the human cell enzyme is inhibited by all of the trypsin-specific inhibitors that block the fibrinolytic activity of other cell cultures. (*d*) The human cell factor, like those of transformed chick and hamster cells (4, 7), is a DFP-sensitive serine protease that functions as a potent plasminogen activator. (*e*) The concentration of activator produced by the three human tumor cell lines is in the same range as that found in transformed chick fibroblast cultures. Based on measurements of [^{3}H]DFP labeling, the level in melanoma-conditioned medium (20–200 pM) is somewhat higher, and that in mesothe-

[4] Rifkin, D. B., A. Piperno, A. Tobia, and L. Ossowski. Unpublished observations.

lioma-conditioned medium is somewhat lower (2–10 pM) than in transformed chick cultures (10–30 pM).

All of the above characteristics of the human fibrinolytic enzyme suggest that it is homologous with those previously observed in neoplastic and transformed cultures from other species. These similarities, and the widespread occurrence of fibrinolysis and disorders of coagulation as complications of human metastatic disease (9–11), suggest that a fibrinolytic activity is likely to be associated with at least some human malignancies.

In spite of these correlations the formation of high levels of plasminogen activator cannot be taken as diagnostic of neoplasia. Although the concentration in most normal tissue is generally low (1, 2, 4), a number of nonmalignant cells and cultures contain plasminogen activators at levels comparable to those found in neoplastic cells;[5] these include granulocytes, activated macrophages, spermatozoa, endometrium, and vampire bat saliva, in addition to the cell lines of nonmalignant origin reported by other laboratories (12–14) and in this paper.

There is one reservation that applies specifically to the interpretation of the findings in this paper since they are based on the use of established cell lines, some of which had been maintained in culture for several years. It seems possible that the process of establishing cell lines in culture might favor the selection of variants that produce a fibrinolytic activator. Therefore these data while fully consistent with the results given by the animal cell models, are not by themselves as persuasive as those obtained either with primary cultures of embryo fibroblasts transformed in vitro, or with primary cultures of neoplastic tissues. In the latter two instances, the transformed cells can be compared with the normal parental cell type, or with adjacent normal tissues, respectively. Since the in vitro transformation of human cells proceeds with significantly lower efficiency than that of other mammalian cells, a firm correlation between fibrinolysis and human neoplasia will require the examination of larger numbers of cell lines and transformants, as well as primary cultures derived from biopsies and other surgical specimens. Because plasminogen activator has already been detected at high frequency in fresh samples of human tumors (15), it is reasonable to expect that a firm correlation of this activity with human malignancy may soon be forthcoming.

SUMMARY

A series of human cell lines has been examined for fibrinolysis in culture. The sera that are activating for fibrinolysis by human cells are mouse, monkey, human, horse, and bovine. Individual human sera show considerable variation in the ability to activate fibrinolysis. In common with other neoplastic or transformed mammalian and avian cell cultures, human cell lines of neoplastic

[5] Rifkin, D. B., J. C. Unkeless, W. Beers, and D. Luskotoff. Unpublished observations.

origin produce substantial amounts of plasminogen activator. Several cultures of nonmalignant origin also produce plasminogen activator, whereas cultures obtained from trypsinized human embryos, or from human embryonic skin do not. The human melanoma plasminogen activators are of two kinds: a major component with a mol wt of 50,000, and a minor species with a mol wt of approximately 60,000. Both are DFP sensitive, serine proteases.

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