PRODUCTION OF PLASMINOGEN ACTIVATOR BY ESTABLISHED CELL LINES OF MOUSE ORIGIN

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ABSTRACT

The correlation between malignant transformation and increased plasminogen activator synthesis has been studied in a variety of established cell lines. In contrast to the behavior of secondary mouse embryo cultures, which always show increased fibrinolytic activity when transformed, no such correlation was found within the BALB/c 3T3 line and its transformed derivatives. Cell lines were established from tumors initiated in BALB/c mice by several transformed cell lines. These lines were generally found to contain no more plasminogen activator than the cells used for inoculation. A correlation was found between transformation and plasminogen activator synthesis within Swiss 3T3 cell lines. However, the correlation was not maintained by serum revertants of transformed Swiss 3T3 cells.

The production of plasminogen activators (PA) by primary fibroblasts transformed to malignancy and the consequent ability of these cells to promote the hydrolysis of fibrin via the activation of plasminogen to plasmin has been demonstrated to occur in a variety of cell types (3, 6, 10, 13, 14, 20). Thus, the fibroblasts of chicken, mouse, hamster, rat, and human all secrete appreciable amounts of plasminogen activators after transformation by either viruses or chemicals, while the parental cells produce rather little of this enzyme. Although the majority of cells employed in these studies had been in culture for only a few generations, a significant proportion of in vitro experiments on transformation utilize nontransformed and transformed pairs of established cell lines rather than primary cells and their transformants. Since cell lines are isolated after long periods in culture and almost always have abnormal karyotypes, it was of interest to determine whether the correlation between transformation and increased PA production also applied to cell lines. We have found that the correlation does not rigorously hold for two sets of mouse cell lines. The cells from some nonmalignant cell lines contain high levels of PA, while, conversely, certain transformed cells derived from them may contain low levels of PA.

MATERIALS AND METHODS

Cells and Virus (Table I)

Swiss 3T3 and the Simian virus 40 (SV40) transformant SV101 cell lines were originally derived by Todaro and Green (18). The F1SV11 is a flat revertant of SV101 (11). The Col 2, and Aγ7, and the LS6 revertants of SV101 were isolated by Vogel and Pollack (21), and Vogel et al. (22). The BALB 3T3 clone A31CL10 was a generous gift from Dr. E. Scolnick (National Institutes of Health, Bethesda, Md.) as were the following transformed derivatives of this clone: SVT_2, and SV40 transformant; KA31, a Kirsten sarcoma virus transformant; KA/RLV, a KA31 transformed clone also infected with Rauscher leukemia virus; B77A31, a clone transformed by B77 avian sarcoma virus; FSV/RLV, a clone transformed by feline sarcoma virus and coinfected with

TABLE I

Properties of Cells Used

Name	Species of origin	Transforming agent	Phenotype	Tumorigenicity*	
				10^4‡	10^6‡
BALB/c 3°	BALB/c mouse primary	–	Normal	ND§	0/7
4-71-1	BDIX rat	Ethylnitrosourea	Transformed	"	ND
A31CL10	BALB/c established cell line	–	Normal	0/5‖	0/5‖
SVT2	A31	SV40 virus	Transformed	1/5	ND
KA31	"	Kirsten sarcoma virus	"	3/5	2/2
Ki/RLV	"	Kirsten sarcoma virus and Rauscher leukemia virus	"	2/5	ND
B77	"	B77 avian sarcoma virus	"	2/5	"
PYA31	"	Polyoma virus	"	1/5	"
R4-4	"	Radiation	"	3/5	"
FSV/RLV	"	Feline sarcoma virus and Rauscher leukemia virus	"	3/5	"
S^+L^-	"	Moloney sarcoma virus	"	2/5	"
3T3	Swiss mouse established cell line	–	Normal	ND	0/4¶
SV101	3T3	SV40 virus	Transformed	"	5/6¶
F1SV11	SV101	–	Normal	"	0/2¶
Col 2	"	–	"	"	ND¶
Aγ7	"	–	"	"	2/4¶
LS6	"	–	"	"	0/2¶

* Animals with tumors/total animals tested.

‡ D. B. Rifkin, unpublished observations and this study.

§ ND is not determined.

‖ Under certain conditions these cells may form tumors (see reference 2).

¶ Determined in the nude mouse (17).

Rauscher leukemia virus; RLV, a clone infected with Rauscher leukemia virus; R4-4, a clone transformed by radiation; S^+L^-, a clone of cells transformed by the Moloney sarcoma virus. The polyoma virus transformant of A31, PYA31, was generously given to us by Dr. T. Benjamin, (Harvard Medical School, Cambridge, Mass.). The KA/RLV, FSV/RLV, and RLV cell lines continuously produced infectious virus. All other cell lines did not produce virus. A31, Swiss 3T3, SV101, and all revertants were doubly recloned approx. 3 mo before these experiments.

Tumor-derived sublines of the cell lines described above were isolated after injecting 10^3, 10^4, or 10^5 transformed cells into 3-wk-old BALB/c mice. The mice were checked for tumors by palpation every 3–4 days. When a palpable mass was detected, it was surgically removed, minced, trypsinized in 0.25% trypsin, 0.001 M EDTA, and the cells were plated in Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, N. Y.) containing 10% fetal bovine serum (Reheis Chemical Co., Chicago, Ill.). Each tumor-derived subline is denoted by a T after the abbreviation used for the parental cell line.

Primary BALB/c cells were obtained after trypsinization of decapitated 16-day-old mouse embryos. At confluence, the cells were trypsinized, frozen, and stored in liquid nitrogen until further use.

The 4-71-1 cells were obtained from a rat neurotumor induced by ethylnitrosourea in a BDIX rat and were a gift from Dr. N. Sueoka (University of Colorado, Boulder, Colo.). These cells have been found to produce exceptionally high levels of PA and are used as a standard reference line for high PA production. Rauscher leukemia virus (RLV195) was a gift of Dr. E. Scolnick.

All cells were grown in Dulbecco's modified medium containing 10% fetal bovine serum (DBFC10) and tylosin (60 μg/ml) (Associated Biomedic Systems, Inc., Buffalo, N. Y.). The medium was changed twice a week. The cells were passaged once each week at a dilution of 1:20. All cells were grown at 37°C in an atmosphere of 10% CO_2, 90% air with a relative humidity of 100%.

Secondary BALB/c cells were infected with virus after they were first incubated with DEAE-Dextran, 20 μg/ml in 2 ml of TD buffer (0.14 M NaCl, 0.005 M KCl, 0.024 Tris-HCl, and 0.0004 M Na_2HPO_4, pH 7.4), for 45 min at 37°C. The DEAE-Dextran was then removed, and 2 ml of culture medium obtained from virus-producing cells was placed in each 100-mm dish. The cells were incubated for an additional 1 h at 37°C. At the end of this period, 10 ml of DBFC10 was added to each plate.

Fibrinolysis Assays

The preparation of ^{125}I-fibrin plates was performed as described by Unkeless et al. (20) and Beers et al. (1). Secretion of plasminogen activator by living cells was assayed as follows: cells were dispersed by mild trypsin treatment, suspended in Eagle's minimal medium containing 10% fetal bovine serum (EFC10), centrifuged at 500 *g* for 5 min, resuspended in EFC10, and plates on 17 mm ^{125}I-fibrin-coated dishes (Linbro Chemical Co., New Haven, Conn.). The cells 5×10^4 were allowed to attach for 6 h, the medium was removed, the cells were washed once with TD buffer, and 1 ml of Eagle's minimal medium containing the appropriate serum was added. Duplicate assays were performed in the presence of 2.5% dog serum, 10% fetal bovine serum, or medium alone. The plates were then incubated at 37°C and, at the indicated items, 0.5-ml aliquots of the medium were removed. The amount of soluble ^{125}I-fibrin liberated by a given cell line was determined by counting each aliquot in a Packard γ-counter (Packard Instrument Co., Inc., Downers Grove, Ill.).

Total cell-associated levels of PA were assayed in the following manner (13). Initially, 5×10^5 cells were plated in 60-mm petri dishes and allowed to grow for 24 h. The medium was then removed, and the cells were washed twice with TD buffer and incubated at 37°C in serum-free Eagle's minimal medium for 3 h. The serum-free medium was removed, and the cells were washed twice with ice-cold TD buffer and scraped with a rubber policeman. The scraped cells were suspended in approx. 10 ml of ice-cold TD buffer, centrifuged at 2,000 *g* for 5 min, and the supernate was discarded. The cell pellet was dissolved in 0.1 M Tris-HCl, 0.5% Triton X-100, pH 8.1, centrifuged at 500 *g* for 10 min, and the supernate, which contained greater than 90% of the total PA, was removed for PA assays. The protein concentration was determined by the method of Lowry et al. (8). Measured aliquots of the cell extract were added to 17 ^{125}I-fibrin-coated plates containing 0.5 ml of 0.1 M Tris-HCl buffer, pH 8.1, and 4 μg/ml of purified fetal bovine plasminogen. Plasminogen was purified according to the method of Deutsch and Mertz (5) and was at least 95% pure as judged by SDS-polyacrylamide gel electrophoresis. The assays were performed at 37°C. At the appropriate times, aliquots of the assay reaction were removed and the ^{125}I-content was determined in a Packard γ-counter. The background levels of fibrinolysis with controls of plasminogen alone or Triton X-100 extract alone were never more than 3% of the total counts removed by trypsin (25 μg/ml). The values for the plasminogen control were subtracted from each sample value.

The secretion of soluble plasminogen activator was measured in cell cultures prepared as described for the assay of intracellular plasminogen activator except that the incubation in serum-free minimal Eagle's medium was continued for 24 h. During this period, aliquots of medium were periodically removed from the cultures and frozen. The assays for PA were then performed in the same way as those described for intracellular plasminogen activator except that 0.1-ml samples of the serum-free, conditioned Eagle's medium were used in place of cell lysates.

A unit of plasminogen activator activity is the amount of enzyme which will catalyze the release of 5% of the substrate radioactivity in 1 h at 37°C, using 4 μg/ml of fetal bovine plasminogen in 1.0 ml of 0.1 M Tris-HCl, pH 8.1 in a 17-mm petri dish. The total substrate radioactivity was determined by measuring the amount of ^{125}I-fibrin solubilized by 1 ml of 0.25% trypsin at pH 7.6 in 1 h.

The different levels of fibrinolysis associated with various cell lines tested were not merely the result of differential cell plating or growth. Cell counts at the initiation and conclusion of each experiment varied by no more than 20%. Moreover, as has been described before (10), cells which produce high levels of PA over long periods of time tend to detach from the surface of the petri dish. Consequently, those assay plates containing cell lines having the highest fibrinolytic activity always contained fewer cells at the termination of an experiment than cell lines producing low levels of PA.

It should be noted that the figures and tables present only the level of fibrinolysis generated by cells in dog serum. Each cell line was also tested for its ability to hydrolyze fibrin in the absence of serum as well as in the presence of fetal bovine serum. As has been described elsewhere (10, 14, 20), fibrinolysis was strictly dependent upon the presence of plasminogen. No more than 3% of the substrate was hydrolyzed in the absence of plasminogen. In addition, the amount of proteolysis when fetal bovine serum was employed was about 10% of that found with dog serum. This result is also in agreement with earlier results with other rodent cells (3, 10, 13).

No attempt has been made to analyze the kinetics of the reaction as they are obviously complex and would not be expected to follow simple enzyme kinetics. The substrate employed is insoluble, and the assay is a coupled one employing two proteases; in some cases, there is continuous elaboration of plasminogen activator, and the end product of the reaction, plasmin, will destroy plasminogen activator. Repeated assays of the same cell line may vary in absolute activity, as can be seen by comparing the activity of B77 in Figs. 1 and 2. Such variation may be due, in part, to the state of the fibrin substrate. However, each assay is performed with standard cells, and, relative to these standard lines, the differences between different cell lines have always been constant. This is true for some cell lines which have been compared several times over a three-year period. While the activities measured may have been the result of the release of proteases other than plasminogen activator, dependence of fibrinolysis upon the presence of plasminogen would indicate that any plasminogen-independent hydrolysis of the substrate must be a minor activity.

RESULTS

Fibrinolysis by BALB/c 3T3 cells and BALB/c 3T3 transformants

One of the cell lines widely used in tissue culture is the BALB/c 3T3 line and its transformed derivatives. When cells of the A31 clone of the BALB/c 3T3 line were plated on ^{125}I-fibrin-coated plates, it was found that the untransformed cells (A31CL10) generated significantly more fibrinolysis than third passage BALB/c embryo cells (BALB/c 3°) (Fig. 1 A), but considerably less than malignant 4-71-1 cells. In the absence of serum, there was no fibrinolysis (Fig. 1 A).

SVT2, an SV40 virus transformant of the A31 clone, had a higher fibrinolytic activity than the parental A31 cells as did the Kirsten sarcoma virus transformant KA31 (Fig. 1 B). This is consistent with previous observations with other normal and transformed cells. However, a number of other lines displaying transformed characteristics derived from A31 by irradiation (R4-4) or by infection with different tumor viruses produced levels of fibrinolysis either equivalent to or lower than that associated with the parental A31CL10 cells (Fig. 1 A and B).

The variation in PA production evident when lines were plated on ^{125}I-fibrin was also apparent when the total cell-associated plasminogen activator or the secretion of soluble PA (Table II) was assayed. Attempts to detect plasminogen activator or plasmin inhibitors associated with those cell lines containing low levels of plasminogen activator have thus far been unsuccessful. Thus, the differences in the fibrinolytic activities illustrated in Fig. 1 and Table II are indicative of differences in the levels of synthesis and secretion of PA. Since some of the cell lines tested failed to produce or secrete PA but were indeed tumorigenic (see below), the correlation between tumorigenic transformation and increased levels of PA synthesis does not hold for this set of transformed cell lines.

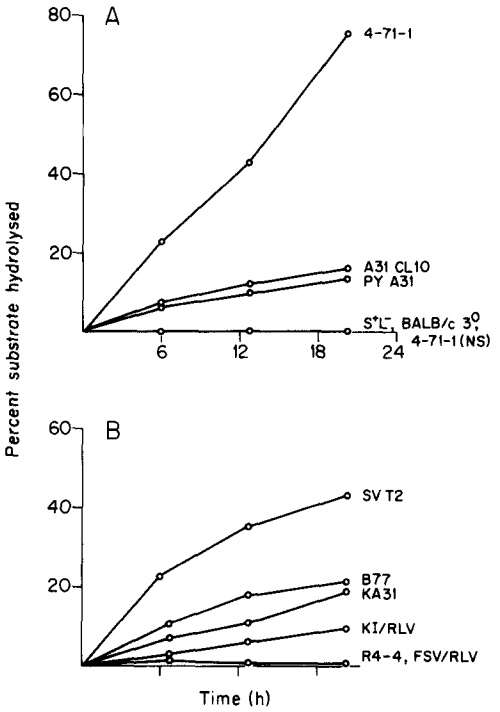

FIGURE 1 Fibrinolytic activity of the BALB/c 3T3 (A31CL10) cell line and transformed derivatives of this cell line. The fibrinolytic activity of living cells plated on ^{125}I-fibrin coated surfaces was determined as described in Materials and Methods. The data presented represent those values found for cells grown in medium containing 2.5% dog serum. The level of fibrinolysis seen when the cells were incubated on ^{125}I-fibrin in the absence of serum is illustrated (4-71-1 NS) for the 4-71-1 cell line. A31CL10 is a nontransformed line of BALB/c 3° cells, Ki/RLV is a KA31 clone also infected with Rauscher leukemia virus, B77 is a B77 avian sarcoma virus transformant of A31CL10 cells, PYA31 is a polyoma virus transformant of A31CL10 cells, R4-4 is a radiation-induced transformant of A31CL10 cells, FSV/RLV is a clone of A31CL10 cells coinfected with feline sarcoma virus and Rauscher leukemia virus, and S+L$^-$ is a clone of A31CL10 cells transformed by Moloney sarcoma virus.

Since the transformed cell lines used above were all selected by various in vitro criteria of transformation (15), it was important to establish which of these A31CL10-derived lines were tumorigenic and to determine the fibrinolytic activity of cell lines derived from these tumors. Cells from most of the cell lines rapidly formed tumors when injected into 2-wk-old BALB/c mice. (While all of the transformed cell lines caused tumors in vitro, we were not able to culture in vitro all of these tumors.) These included the KA31, KI/RLV, FSV/RLV, S$^+$L$^-$, and B77 cell lines.

When cell lines derived from the tumors were compared to the parental cell lines (Fig. 2, Table III), it was apparent that there was no specific selection for cells containing either increased or decreased levels of PA. In most instances, the amount of PA in the tumor-derived cell lines was virtually identical to that found in the parental cell lines. Nor was there an obvious correlation be-

TABLE II

Plasminogen Activator Levels of BALB/c 3T3 Cell Lines

Cell type	Release of soluble plasminogen activator	Total cell-associated plasminogen activator
	U/ml/10 h	*U/mg*
A31CL10	7	178
4-71-1	52	1,480
BALB/c 3°	1	38
SVT2	28	130
KA31	27	240
Ki/RLV	11	136
B77	11	148
PYA31	9	140
R4-4	0	0
FSV/RLV	0	0
S^+L^-	0	0

The release of soluble plasminogen activator and total cell-associated plasminogen activator were assayed as described in Materials and Methods. For the determination of soluble plasminogen activator, 100 μl of conditioned medium were assayed after 10-h incubation of the cells in serum-free Eagle's medium. For the determination of total cell-associated plasminogen activator, 5 μg or 100 μl of each sample was assayed. The data illustrated were taken from a 4-h assay. A31CL10 is a nontransformed line of BALB/c° cells, Ki/RLV is a KA31 clone also infected with Rauscher leukemia virus, B77 is a B77 avian sarcoma virus transformant of A31CL10 cells, PYA31 is a polyoma virus transformant of A31CL10 cells, R4-4 is a radiation induced transformant of A31CL10 cells, FSV/RLV is a clone of A31CL10 cells coinfected with feline sarcoma virus and Rauscher leukemia virus, and S^+L^- is a clone of A31CL10 cells transformed by Moloney sarcoma virus.

tween plasminogen activator levels and tumorigenic potential as determined by the number of cells required to form a tumor or the frequency of tumors (data not shown).

It was important to eliminate the possibility that cells producing high levels of PA were lost during the in vitro establishment of tumor sublines since experiments, in which MTV-, DMBA-, or RSV-induced tumors and cells derived from these tumors were assayed, have uniformly yielded high levels of fibrinolysis in well over 200 tumors each tested individually (reference 20; D. Rifkin, unpublished observations; and A. Piperno, unpublished observations). Therefore, extracts were made from several of the tumors upon excision, and the level of PA in each extract was compared to that found when cells from the same tumor were grown in culture. The amount of PA associated with these tumors was low and was comparable to the activities associated with the tumor cell lines after culture (results not shown). Thus, not only were some of the original cell lines with low PA levels tumorigenic, but also no selection for cells producing high levels of PA occurred during tumorigenesis.

Infection of Secondary Mouse Cells with Virus Recovered from Transformed Cell Lines

To establish that the low fibrinolytic activity associated with certain cell lines was not the result of transformation by a virus genetically incapable of inducing the synthesis of PA, BALB/c secondary cells were infected with virus collected from

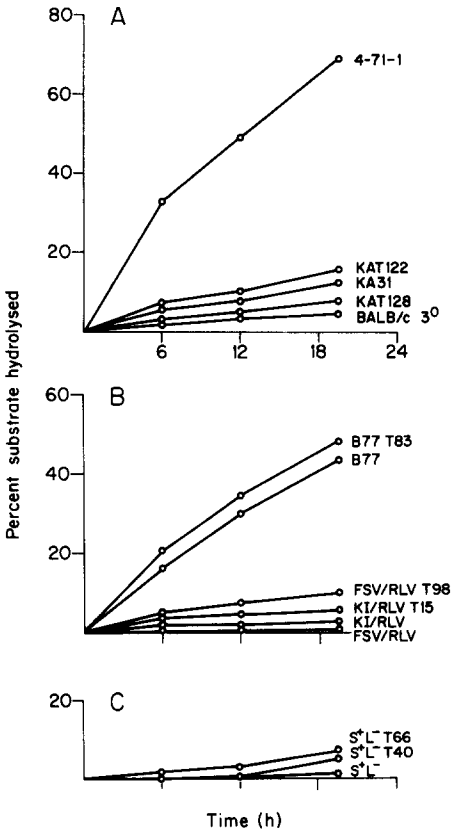

FIGURE 2 Fibrinolytic activity of the BALB/c 3T3 cell line A31CL10, transformed derivatives of this cell line, and in vitro derived sublines of these malignant cells. The in vitro derived sublines are denoted by a T after the cell line abbreviation. The fibrinolytic activity of living cells plated on ^{125}I-fibrin coated surfaces was determined as described in Materials and Methods and the legend to Fig. 1.

TABLE III

Plasminogen Activator Levels in Transformed BALB/c 3T3 lines and Tumor-Derived Sublines

Cell type	Release of soluble plasminogen activator	Total cell-associated plasminogen activator
	U/ml/10 h	*U/mg*
4-71-1	50	1,132
BALB/c 3°	0	36
Ki/RLV	17	100
Ki/RLV T15	13	58
B77	15	258
B77 T83	17	286
KA31	9	216
KA31 T122	4	78
KA31 T128	3	162
S^+L^-	0	64
S^+L^-T66	0	72
S^+L^-T40	0	74
FSV/RLV	0	28
FSV/RLV T98	0	28

The release of soluble plasminogen activator and total cell-associated plasminogen activator were assayed as described in Materials and Methods and the legend to Table I.

cultures of the Ki/RLV, FSV/RLV, and RLV cell lines.

The infected cells were passaged once and assayed for the production of PA (Table IV). Consistent with the finding in other systems that only sarcoma viruses are capable of inducing the production of PA in fibroblasts (10, 20), there was no increase in the production of PA by cells infected with RLV. After infection with either a stock of Ki/RLV virus grown on primary BALB/c cells or culture fluid obtained from the Ki/RLV cells, or FSV/RLV, the morphology of the cells was altered and the amount of PA increased dramatically (Table IV). Thus, although the Ki/RLV and FSV/RLV cell lines do not produce significant amounts of PA, the virus produced by these cells retains the capability of causing an increased synthesis of PA upon transformation of secondary BALB/c mouse embryo cells.

Fibrinolysis by Swiss 3T3 Cells and Derivatives of Swiss 3T3 Cells

We also examined the fibrinolytic activities of another group of mouse cell lines: the Swiss 3T3 cell line, an SV40 complete transformant of these cells, SV101, and partial revertants of SV101 selected by three different procedures. In Table V and Fig. 3 are shown the results of experiments to determine the total cell-associated, secreted, and intracellular plasminogen activator levels in these cells. The SV40 transformed cells had more activity than the parental 3T3 cells. When the revertants of SV101 were examined for PA production, a complex pattern was observed. Thus, F1SV11, selected by its inability to grow at high density (11), and Col 2, selected by its resistance to colchicine (21), both had lost the high level of PA production associated with the SV101 parental

TABLE IV

Fibrinolytic Activity of BALB/c Cells Infected with RNA Tumor Viruses

Cells	Substrate removed
	%
–	1.4
BALB/c tertiary	3.1
BALB/c tertiary infected with Ki/RLV from primary mouse cells	46.4
Ki/RLV	3.5
BALB/c tertiary infected with virus from Ki/RLV cells	19.6
FSV/RLV	1.2
BALB/c tertiary infected with virus from FSV/RLV cells	17.5
BALB/c tertiary infected with RLV	2.3

The fibrinolytic activity of control and virus-infected cells was determined as described in Materials and Methods. The data represent the amount of substrate hydrolyzed by intact cells after 16 h incubation in medium containing 2.5% dog serum. The total radioactivity in each sample well was 47,000 cpm. The RLV was prepared after infecting BALB/c 3T3 cells with RLV (195).

TABLE V

Plasminogen Activator Levels in Transformed Swiss 3T3 Cell Lines

Cell type	Release of soluble plasminogen activator	Total cell-associated plasminogen activator
	U/ml/10 h	*U/mg*
4-71-1	50	1,338
BALB/c 3°	0	30
3T3	2	0
SV101	8	208
F1SV11	1	10
Col 2	1	0
Aγ7	16	34
LS6	19	438

The release of soluble plasminogen activator and total cell-associated plasminogen activator were assayed as described in Materials and Methods and the legend to Table I.

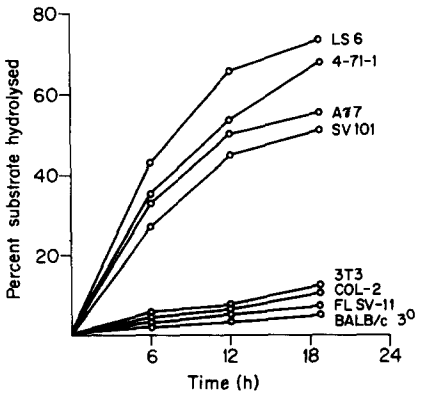

FIGURE 3 Fibrinolytic activity of the Swiss 3T3 cell line, an SV40 transformant (SV101) of 3T3, and partial revertants of SV101. The fibrinolytic activity of living cells plated on ^{125}I-fibrin coated surfaces was determined as described in Materials and Methods and the legend to Fig. 1.

line. However, two other revertants, A γ7 and LS (21), selected by their inability to grow in either γ-globulin-depleted serum or low concentrations of serum, both secreted as much or more PA than the parental SV101 line. Thus, while the SV40 transformant of the Swiss 3T3 cell line did show an increased fibrinolysis, revertants of this transformant did not uniformly produce less PA. The level of PA production by these four revertants correlated with the anchorage requirement of the cells. F1SV11 and Col 2 are unable to grow suspended in Methocel (The Dow Chemical Co., Midland, Mich.), while the revertant clones A γ7 and LS studied here retain the ability to grow in Methocel, a property associated with the original transformant.

The low fibrinolytic activities associated with 3T3 and the anchorage revertant F1SV11 may have been due to the production of an extracellular inhibitor of fibrinolysis. This was tested by plating together pairs of cell lines with different levels of PA and assaying the fibrinolytic activity. The fibrinolytic activities of various mixtures of nontransformed, revertant, and transformed cells were equivalent to the sum of activities associated with each cell type (results not shown). Thus, no soluble inhibitor of fibrinolysis was found.

DISCUSSION

Tumorigenicity and Plasminogen Activator

The experiments described in this paper demonstrate that some established cell lines and their transformants do not necessarily conserve the correlation between the production of PA and neoplastic transformation, a consistent finding when fibroblasts and primary tumors are examined (13, 16). The lack of uniform correlation between transformation and the production of PA is most apparent within cell lines derived from BALB/c 3T3. Most of the transformed lines examined synthesized no more PA than the parental nontransformed BALB/c 3T3 A31CL10 cells. Several of these cell lines, such as KA31, were highly tumorigenic even though they synthesized low levels of PA, demonstrating that the increased synthesis of PA after transformation is not a prerequisite for tumor growth. Moreover, cell lines derived from these tumors also produced levels of PA essentially equivalent to the parental cells, indicating that there was no selection during tumorigenesis for rare cells capable of synthesizing high levels of PA.

Although all transformed cell lines derived from the BALB/c 3T3 line do not appear to produce more PA than the parental cell line, a tumorigenic transformant of the Swiss 3T3 cell line, SV101, did show a much higher level of PA than the parental cell. Two phenotypic partial revertants selected by different mechanisms but still anchorage transformed, also still produced large amounts of PA, again demonstrating the variability of different cell lines with respect to PA synthesis. Tumorigenicity of the Swiss 3T3 cell lines has been determined in nude mice (17). Here, the correlation between tumorigenicity and PA production is good. Two cell lines that synthesized large amounts of PA (A γ7, and SV101) produced tumors upon injection into nude mice, while two lines that synthesized little PA (3T3, (Col 2), and F1SVII) were not tumorigenic (12). However, LS 6, a revertant of SV101 which synthesized large amounts of PA, was not tumorigenic.

Properties of Cell Lines

We have no explanation for the discrepancy in PA production between cell lines and primary transformants. The lack of correlation between transformation and PA production in established cell lines has also been described by Mott et al. (9). Since a large proportion of tumors do produce PA (10, 20, 4), we conclude that when a lack of correlation between transformation and PA production is observed (Tables II and III), it may be

an expression of the abnormal regulatory behavior intrinsic to established cell lines.

In the establishment of cell lines from primary cultures of normal cells, genetic alterations of unknown nature must take place since established cell lines differ from primary cells in many respects. For example, primary cultures of diploid cells have a finite life in culture while cell lines are capable of infinite growth, a property usually associated with transformation and/or malignancy. Moreover, the many different assays used to determine in vitro transformation detect separable phenotypic alterations (15). A striking example of this can be seen with the SV101 revertants LS and Aγ7, which have lost the phenotypic properties of transformation that were selected against but have retained other transformed characteristics such as tumorigenicity and anchorage-independence (17). Thus, it is not surprising that cell lines ordinarily classified as nontransformed, such as BALB 3T3 cells, may in fact be malignant (2). Alterations in the genotype may also accumulate during prolonged culturing of cell lines and thus obscure certain fundamental phenotypic changes that occur after transformation (7). Properties such as the synthesis of high levels of proteases may well be selected against in view of the reduced adherence of cells to the surface of the petri dish (7).

These considerations as well as other observations on the variable nature of both phenotypic and biochemical correlations associated with transformation (4, 19, 20, 21) give reason for caution in the use of cell lines for studies of malignant transformation.

The authors would like to acknowledge the expert technical assistance of Susan Conlon and Lucy Palmer, and to thank E. Reich for valuable comments on the manuscript.

This study was supported by grants from the National Science Foundation, the National Institutes of Health, and the Damon Runyon-Walter Winchell Cancer Fund, and by a Faculty research grant to D. B. Rifkin from the American Cancer Society.

This work was initiated at Cold Spring Harbor Laboratory and at The Rockefeller University and completed at State University of New York at Stony Brook and at The Rockefeller University.

Received for publication 12 May 1976, and in revised form 29 September 1976.

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