

## FIBRINOLYSIS ASSOCIATED WITH ONCOGENIC TRANSFORMATION

### REQUIREMENT OF PLASMINOGEN FOR CORRELATED CHANGES IN CELLULAR MORPHOLOGY, COLONY FORMATION IN AGAR, AND CELL MIGRATION\*

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We have previously confirmed the original report of Fischer (1) that fibroblast cultures from several species, transformed to malignancy by viruses or by chemicals, generate high levels of fibrinolytic activity under conditions in which cultures of normal cells do not. We have since found that a comparable fibrinolytic enzyme is produced by primary cultures of a large number of avian and mammalian tumors (2, 3) and by human tumor cell lines.<sup>1</sup> The enzymatic basis for this tumor-associated fibrinolysis has been shown to depend on two proteins: one of these (the cell factor) is a protein produced by transformed cells, and the other (the serum factor) is a protein found in all vertebrate sera tested (2, 3). The serum factor has now been purified and rigorously identified as the known zymogen plasminogen;<sup>2</sup> the cell factor is a specific serine protease that functions as a plasminogen activator.<sup>3</sup>

Because sera can be selectively freed of plasminogen by simple fractionation procedures and by affinity chromatography (4),<sup>2, 4</sup> the role of the fibrinolytic system as a determinant of established parameters of transformation can be assessed. We have therefore compared the effects of plasminogen-depleted serum with those of native and reconstituted serum on the following phenotypic properties that are considered to be associated with oncogenic transformation: (a) capacity to support growth of SV-40-transformed hamster embryo fibroblasts in soft agar; (b) capacity to mediate the de-

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<sup>2</sup> Quigley, J. P., L. Ossowski, and E. Reich. 1973. Plasminogen: the serum proenzyme activated by factors from malignant cells. Manuscript submitted to *J. Biol. Chem.*

<sup>3</sup> Unkeless, J. C., J. Danø, G. M. Kellerman, and E. Reich. 1973. Purification and properties of fibrinolytic activator produced by transformed cells. Manuscript submitted to *J. Biol. Chem.*

<sup>4</sup> Ossowski, L., J. P. Quigley, and E. Reich. 1973. Fibrinolysis associated with oncogenic transformation: morphological correlates. Manuscript submitted to *J. Biol. Chem.*

velopment of a distinct sequence of morphological changes recently described<sup>4</sup> in cultures of SV-40-transformed hamster embryo fibroblasts; and (c) capacity to support the migration of cells from a monolayer into a "wound."

The findings presented below indicate that the expression of these parameters of transformation is dependent on the presence of plasminogen in the culture medium. We have also prepared radioactive plasminogen and have studied its interaction with normal and SV-40-transformed hamster embryo fibroblasts. The results of these experiments reveal that plasminogen or possibly plasmin, the activated product of plasminogen, becomes cell associated in transformed cultures to a much greater extent than in normal cultures.

### *Materials and Methods*

Primary fibroblast cultures were prepared from hamster embryos as previously described (2, 3); the clone transformed by SV-40 virus was the same as that used in previous work (3), and the cultures were maintained and propagated according to standard procedures (2, 3). 3T3 and SV-101 cells were kindly given to us by Dr. R. Pollack.

*Plasminogen-Free Serum Preparation.*—A column of Sepharose 4B, containing covalently bound L-lysine, is prepared according to Deutsch and Mertz (4) and is equilibrated with phosphate-buffered saline (PBS)<sup>5</sup> buffer. When serum (2–5 ml/ml column bed volume) is passed through the column, the plasminogen is specifically adsorbed, and the emerging nonadsorbed peak of protein contains only 3% of the original concentration of plasminogen; this can be reduced to less than 0.5% of the original level by passing the depleted serum through a second column of lysine-Sepharose 4B. After washing with potassium phosphate buffer, 0.3 M, pH 7.4, the plasminogen that is adsorbed to the column is readily eluted by means of  $\epsilon$ -aminocaproate (4) and further purified by a second cycle of affinity chromatography.

*Preparation of [<sup>125</sup>I]Plasminogen.*—Plasminogen (2.6 mg) was isolated from dog serum by affinity chromatography on a lysine-Sepharose column (4)<sup>2</sup> and iodinated by the method of Helmkamp et al. (5) using 250  $\mu$ Ci of [<sup>125</sup>I]sodium iodide. This small quantity of radioactive iodide did not necessitate removal of hydrogen peroxide by sulfite treatment. The iodinated plasminogen was rechromatographed on a lysine-substituted Sepharose column (0.8  $\times$  6 cm) and separated from  $\epsilon$ -aminocaproate by further chromatography on Sephadex G-50 (0.8  $\times$  19 cm) equilibrated and eluted with PBS. The plasminogen obtained after iodination and subsequent purification (1.6 mg) had a specific activity of  $6 \times 10^4$  cpm/ $\mu$ g corresponding to approximately 3–4 atoms of iodine per plasminogen molecule.

Control experiments demonstrated that the fibrinolytic activity of the iodinated plasminogen was indistinguishable from that of the original plasminogen. In addition, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate- $\beta$ -mercaptoethanol (6) showed that the single plasminogen chain (mol wt 90,000) was replaced by the expected two plasmin chains (mol wt 65,000 and 25,000) after incubation with cell factor from SV-40-transformed hamster cells.

The additional methods used are given in the description of the individual experiments.

### RESULTS

*Growth of Transformed and Normal Hamster Embryo Fibroblasts in Media Depleted of Plasminogen.*—A quantity of fetal bovine serum was freed of

<sup>5</sup> Abbreviations used in this paper: PBS, phosphate-buffered saline (0.01 M phosphate buffer, pH 7.4, 0.15 M NaCl); TD, 0.024 M Tris, pH 7.4, 0.14 M NaCl, 0.05 M KCl, 0.0037 M Na<sub>2</sub>HPO<sub>4</sub>.

plasminogen by two cycles of affinity chromatography; the residual zymogen was less than 0.5% of that present in the original serum when assayed using fibrinolysis of [<sup>125</sup>I]fibrin-coated plates (2) stimulated by hamster SV-40 cell factor (3). This plasminogen-depleted serum, at a final concentration of 10%, was used to supplement the basic growth medium for normal and SV-40-transformed fibroblasts. The data in Table I show that the growth of both normal and transformed cells in liquid monolayer cultures proceeds at normal or nearly normal rates in the virtual absence of plasminogen. In contrast to this, the plating efficiency of transformed cells in soft agar is markedly depressed by the absence of plasminogen, and the restoration of plasminogen to the depleted serum partially restores colony formation in soft agar (Table II). We have previously observed that colony formation in soft agar is decreased

TABLE I  
*Growth of SV-40-Transformed and Normal Hamster Cells in Native or Plasminogen-Depleted Fetal Bovine Serum*

Fetal bovine serum (10%)	No. of cells per dish ( $\times 10^5$ )					
	SV-40 hamster			Normal hamster		
	Day 1	2	3	Day 1	2	3
Native	3.6	7.5	37.0	5.6	12.5	17.5
Plasminogen depleted	3.1	6.7	38.0	2.9	7.9	14.5

Normal hamster embryo fibroblasts and SV-40 hamster embryo fibroblasts were plated ( $2.5 \times 10^5$ /plate) in 60 mm Petri dishes in Dulbecco's medium supplemented with either 10% native fetal bovine serum or 10% plasminogen-depleted fetal bovine serum. The cells were allowed to settle for 16–18 h and were washed with TD, and fresh Dulbecco's medium with the appropriate serum was added. A set of normal and SV-40-transformed cultures were trypsinized daily and the number of cells in each culture was determined. The preparation of plasminogen-depleted fetal bovine serum has been described in Materials and Methods.

by a trypsin inhibitor known to block the action of plasmin (3), and the present finding is, therefore, a second and more direct line of evidence indicating that plasminogen participates in the expression of this parameter of transformation. Colony counts were performed as long as 14–16 days after plating, and since macroscopic colonies are ordinarily visible after 6–7 days of growth, it is apparent that the absence of plasminogen must at least greatly retard, if not completely block, the growth of most colonies in agar. The small number of colonies that form in plasminogen-free soft agar require further study.

*Morphological Changes Occurring in SV-40-Transformed Hamster Fibroblast Cultures.*—We have reported<sup>4</sup> that SV-40-transformed hamster fibroblast cultures undergo a sequence of morphological changes that are correlated with high levels of fibrinolytic activity in the growth medium. The sequence of changes—rounding and increasing refractility of cells, followed by retraction and extensive formation of cell cords that finally separate into large aggregates

TABLE II  
*Colony Formation in Agar in Native, Plasminogen-Depleted, or Reconstituted Fetal Bovine Serum*

Fetal bovine serum (10%)	No. of colonies per dish*	Plating efficiency
		%
Native	109.0 (107-111)	10.9
Plasminogen depleted	33.1 (21-41)	3.3
Reconstituted†	63.0 (48-78)	6.3

SV-40-transformed hamster cells were plated at  $3 \times 10^5$  cells/60 mm Petri dish in Dulbecco's medium supplemented with 5% of native, plasminogen-depleted, or reconstituted fetal bovine serum. The cells were trypsinized after 4 days of incubation and 1,000 cells were plated in soft agar according to the method of MacPherson (7). The bottom and top layer each contained 10% of the appropriate serum. Each sample was plated in quadruplicate and incubated for 14-16 days. The colonies formed in agar were counted in a light microscope with a  $\times 10$  eyepiece and  $\times 0.7$  objective.

\* The values represent the average of three experiments, and the range is given in parentheses.

† The reconstitution: plasminogen is isolated by affinity chromatography and added to the depleted serum at the level of 100  $\mu\text{g}/\text{ml}$  of serum.

and detach from the surface of the Petri dish—ordinarily requires 2-3 days for full expression; however, this process can be compressed into a 16 h period by plating the cells on a fibrin layer. As seen in Fig. 1, transformed cells plated on fibrin in medium containing whole serum show the characteristic progression of morphological changes; in contrast, no comparable changes occur in plasminogen-depleted medium even after a much longer interval (2 days). The restoration of plasminogen to the medium restores the morphological changes, and plasminogen is therefore a necessary intermediate in the development of these changes.

*Cell Migration: Effect of Plasminogen.*—The work of several laboratories (8, 9) has suggested that, under certain conditions, the locomotion of normal and transformed cells may differ. Moreover, it has been reported that media from cultures of transformed cells contain a factor that can induce nontransformed cells to migrate when they ordinarily do not. We have tested normal and transformed cells for migratory activity using the method described by Burk (9). In this procedure a wound is produced in cell monolayers by means of a razor blade, and the migration of cells across the sharply defined edge of the wound is easily monitored by counting the migrating cells after fixation and staining.

The influence of plasminogen on cell migration was tested by incubating cultures in native, plasminogen-depleted, or reconstituted serum before and after wounding. The data in Table III show the following: (a) The presence of serum is required for maximal migration by both transformed and control cells; however, the number of cells migrating into the wound is much greater in transformed cultures both in the presence and absence of serum. (b) The migra-

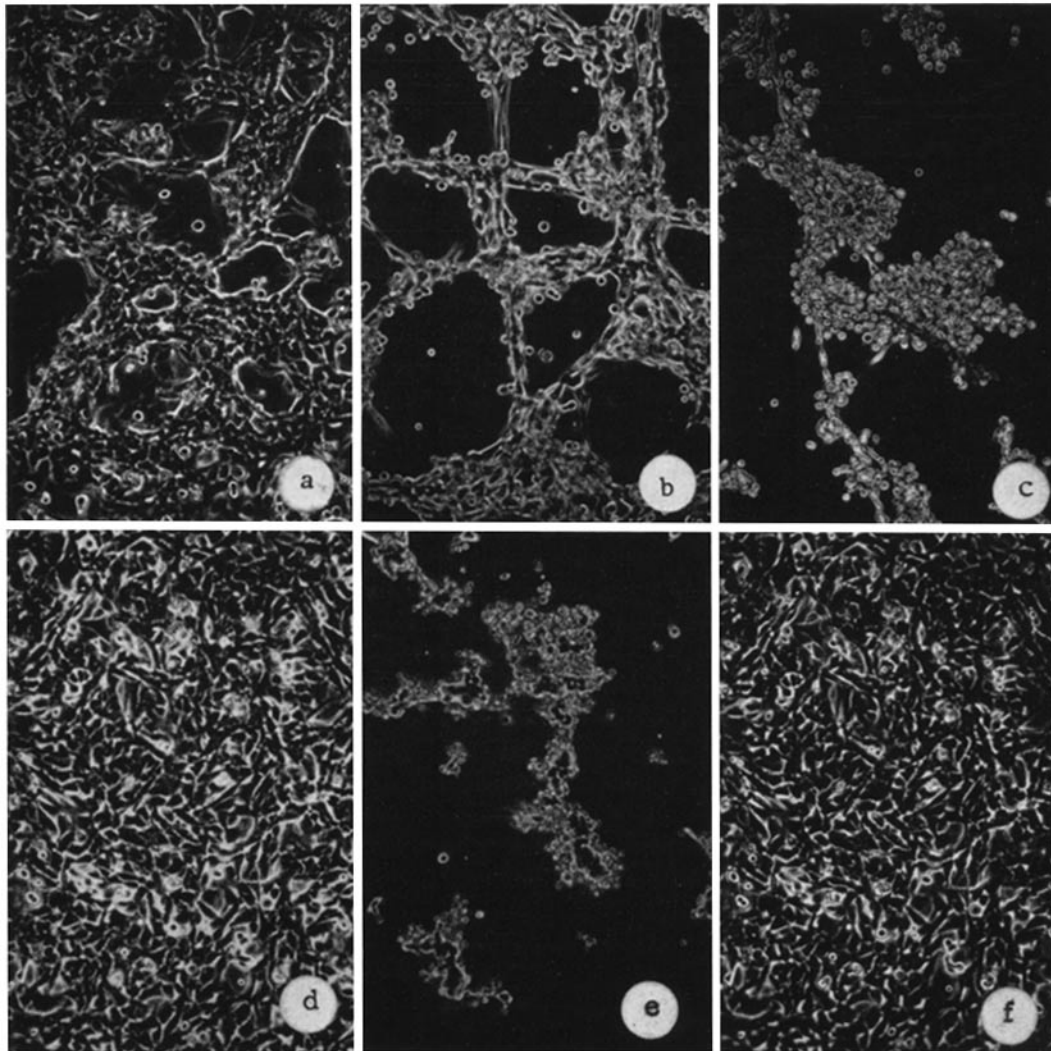


FIG. 1. SV-40-transformed hamster embryo fibroblasts were plated at  $10^6$  cells/60 mm Petri dish coated with  $2 \mu\text{g}/\text{cm}^2$  of fibrin (2) in Dulbecco's medium supplemented with 10% fetal bovine serum. The following day, the cultures were washed once, and fresh medium containing 10% of native or plasminogen-depleted dog serum or native fetal bovine serum was added. Pictures were taken at the indicated time intervals. SV-40 hamster cells in: (a, b, c) native dog serum (5, 8, and 16 h of incubation); (d) plasminogen-depleted dog serum (48 h of incubation); (e) plasminogen-depleted dog serum reconstituted with 0.5 mg of plasminogen/ml of serum (16 h incubation); (f) native fetal bovine serum (48 h of incubation).  $\times 66$ .

TABLE III  
*Migration of 3T3-SV-40-Transformed (SV-101) and 3T3 Cells into a Wound*

Preincubated in fetal bovine serum (3%)	Fetal bovine serum added after wounding (2%)	No. of cells migrating into a wound in 24 h/1.7 mm length unit		
		Transformed		Normal
		Exp. I	Exp. II	
Native	Native	195	147	24
	None	45	23	<1
Plasminogen depleted	Plasminogen depleted	95	66	7
	None	40	18	<1
Reconstituted*	Reconstituted	178	170	NT†
	None	53	35	NT

$4 \times 10^5$  3T3 or 3T3-SV-101 cells were plated in 5 ml of Dulbecco's medium supplemented with 3% of native or plasminogen-depleted fetal bovine serum. After 3 days the monolayer was wounded with a razor blade as described by Burk (9). After wounding, the medium and debris were aspirated, the cells were washed once, and fresh medium with either native or plasminogen-depleted fetal bovine serum (2%) or medium without serum was added. The cultures were fixed and stained after 24 h and the number of cells migrating into the wound in  $5-16 \times 1.7$  mm length units (depending on the cell number) was counted.

\* The reconstitution: plasminogen is isolated by affinity chromatography and added to the depleted serum at the level of 130  $\mu\text{g}/\text{ml}$  of serum.

† NT, not tested.

tion of both transformed and control cells is markedly reduced by incubation in plasminogen-depleted media. (c) The migration of transformed cells is fully restored when depleted serum is reconstituted by the addition of purified plasminogen.

Thus, plasminogen, or, more likely plasmin, is required for optimal migration by both normal and transformed cultures. It appears likely that the reduced migration observed in cultures exposed to plasminogen-depleted medium is due to residual traces of the zymogen because the addition of pure plasminogen alone restores cell migration when transformed cultures are incubated in serum-free medium.

*Uptake of [ $^{125}\text{I}$ ]Plasminogen by SV-40-Transformed and Normal Hamster Fibroblasts.*—The preceding results, which implicate plasminogen in cell migration, are, to some extent, in conflict with previous reports that transformed cells migrate into wounds in serum-free media. It appeared that these differences might be reconciled if plasminogen became associated with transformed cells for appreciable lengths of time; such cell-associated plasminogen might be sufficient for cell migration under the conditions of the assay. We have therefore studied the interaction of  $^{125}\text{I}$ -labeled plasminogen with transformed and normal fibroblasts. As seen in Table IV, both normal and transformed cells accumulate radioactivity when exposed to [ $^{125}\text{I}$ ]plasminogen, although the quantity bound

TABLE IV  
*Adsorption of [<sup>125</sup>I]Dog Plasminogen to SV-40-Transformed and Normal Hamster Embryo Fibroblasts*

Cell type	Radioactivity bound			
	Hours	0	4	24
		<i>cpm/10<sup>6</sup> cells</i>		
Hamster SV-40		4,500	4,500	38,604
Hamster normal		1,917	1,830	2,564

SV-40-transformed or normal hamster embryo fibroblasts ( $4 \times 10^5/60$  mm Petri dish) were plated in Dulbecco's medium supplemented with 10% fetal bovine serum. After 1 day the medium was removed, the cultures were washed twice, and fresh Dulbecco's medium supplemented with 2% of plasminogen-depleted dog serum was added. <sup>125</sup>I-labeled dog plasminogen ( $4 \times 10^4$  cpm/ $\mu$ g at 8  $\mu$ g/ml of medium) was added to all the cultures at zero time. After the indicated time intervals the medium containing iodinated plasminogen was removed and the cultures were washed four times with fresh medium containing 2% of plasminogen-depleted dog serum. (The radioactivity of the individual washes was measured separately and the radioactivity present in the final wash did not exceed the background level.) The cells were detached by trypsinization and the radioactivity was measured in a gamma spectrometer. Both transformed and normal cells approximately doubled in the 24 h period.

by transformed cells is greater by a factor of more than ten. It may be significant that the uptake of plasminogen by transformed cells does not begin to exceed background levels until approximately 4 h of incubation. This implies that all of the available sites may be saturated when the radioactive protein is first added to the medium, and the time lag required for uptake may reflect the rate of formation of new potential binding sites. It is also significant that the addition of soybean trypsin inhibitor blocks the uptake of [<sup>125</sup>I]plasminogen by transformed cells (unpublished observations). Since this inhibitor is known to block the catalytic action of plasmin, it seems likely that it is the plasmin that must remain catalytically active in order to become associated with the transformed cells. When cultures exposed to [<sup>125</sup>I]plasminogen for 24 h are washed and incubated with fresh medium, as much as 20% of the cell-associated radioactivity does not exchange with free, nonradioactive plasminogen for at least 48 h.

#### DISCUSSION

The obvious question raised by the association of a two-step fibrinolytic system with transformed and malignant cells is whether the system is merely an artifact of the methodology, a correlated phenomenon, or an essential part of the overall malignant process. In the present experiments those features of cell growth usually correlated with transformation; growth in soft agar, morphological changes in monolayer culture, and migration in the "absence" of serum have been shown to depend largely on the activity of the fibrinolytic system, in that removal of plasminogen leads to suppression of the phenomena. In the

case of cell migration, it appears in addition, that association of plasminogen or plasmin with the cells may be responsible for maintenance of ability to migrate in the absence of serum. Some features of the overall system appear to respond to very small amounts of plasminogen; thus residual traces of plasminogen in plasminogen-depleted serum, cell-associated traces of plasminogen or plasmin, or small traces of plasminogen-activating factors associated with normal cells may be responsible respectively for the residual growth of transformed cells in soft agar, for the maintained migration of transformed cells after serum removal, and for the migration of normal cells in presence of serum.

The precise mode of action of plasmin, presumably on the cell surface, that leads to changes in mobility, morphology, and growth in agar is unknown. Despite this, a reasonable tentative conclusion is that the observed differences between normal and transformed cultures arise from the formation and release of a plasminogen activator by transformed cells. Moreover, the fact that normal cells cocultivated with transformed cells show some of the morphological changes of transformation<sup>4</sup> suggests that the continued activation of plasminogen is a necessary factor in these alterations. Further studies of the effect of the fibrinolytic system on growth characteristics of cells in various phases of the cell cycle are needed to explore the generality and normal control of this phenomenon, and these are in progress.

In view of the numerous reports describing cell surface changes associated with density-dependent growth of cells in monolayer cultures, it is tempting to suggest that the generated plasmin may continuously remove some surface components needed for this response, with consequent delay in the attainment of "stationary" phase to higher cell densities. This possibility is also under investigation.

#### SUMMARY

Fetal bovine and dog serum were selectively freed of plasminogen by affinity chromatography. The resulting serum as well as native and reconstituted serum (obtained by the addition of purified plasminogen to the plasminogen-depleted serum) were used to examine the role of plasminogen in (a) growth of normal and SV-40-transformed hamster embryo fibroblasts in liquid medium, (b) growth of SV-40-transformed hamster embryo fibroblasts in soft agar, (c) aggregation — a characteristic morphological change of SV-40-transformed hamster cells, and (d) migration of SV-40-transformed and control 3T3 cells from a monolayer into a "wound."

The results demonstrated that exponential growth of both normal and transformed cells in liquid medium proceeded at the same rate in the presence or absence of plasminogen. In contrast, removal of plasminogen markedly depressed the plating efficiency of transformed cells in soft agar, eliminated their characteristic aggregation, and substantially reduced the extent of migration. The role of plasminogen and its activation in oncogenic transformation is discussed.



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