Proc. Natl. Acad. Sci. USA
Vol. 75, No. 10, pp. 4967–4971, October 1978
Cell Biology

Lack of correlation between tumorigenicity and level of plasminogen activator in fibroblasts transformed by Rous sarcoma virus

(protease/caseinolysis/fibrinolysis/soft agar colony formation/altered morphology)

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Communicated by Igor Tamm, July 10, 1978

ABSTRACT **We have previously isolated, from agar suspension culture, clones of chicken embryo fibroblasts transformed by B77 and Prague strains of Rous sarcoma virus (RSV) that varied in the expression of plasminogen activator activity [Wolf, B. A. & Goldberg, A. (1976) *Proc. Natl. Acad. Sci. USA* 73, 3613–3617]. All of the clones exhibited an altered cellular morphology, an increased rate of sugar transport, and a high efficiency of colony formation in agar suspension regardless of the level of plasminogen activator. Because B77 and Prague strains of RSV replicate as well as cause sarcomas in chickens, the tumorigenicity of the transformed cells could not be evaluated with clones of these cells. In order to determine the oncogenicity of clones with various levels of plasminogen activator, it was necessary to isolate cells transformed by the replication-defective Bryan strain of RSV, which release noninfectious virus. All of the agar suspension clones of transformed cells, derived by infection of chicken embryo cells with replication-defective Bryan RSV, fell within the continuum observed for B77- and Prague-transformed clones with respect to altered morphology, increased rate of sugar transport, efficiency of colony formation in agar suspension, and variations in plasminogen activator activity. All of the clones, regardless of the level of plasminogen activator, produced tumors when as few as 5×10^2 cells were injected into the wing web of 1-day-old chicks. The latency period for tumor formation after injection of cells was similar regardless of the level of plasminogen activator of the injected cell. Primary explants of tumors resulting from inoculation of clones having low, intermediate, or high activator activity displayed a spectrum of activator activity.**

After infection of tissue culture cells with Rous sarcoma virus (RSV), cellular behavior is modified (1, 2). Genetic and biochemical analyses of the RSV genome have indicated that a viral gene, called *src*, located near the 3′ terminus of the linear map, is responsible for the modifications in the behavior of fibroblasts (1–7). It is conceivable that one viral gene can pleiotropically influence several cellular properties. One mechanism that will be considered in this paper involves perturbation of the integrity of the cell by means of proteolysis after viral transformation. Three lines of evidence support this hypothesis. First, proteases have been found to induce some of the properties of transformed cells. Protease treatment of fibroblasts has resulted in cell division (8, 9), increased cellular agglutinability with plant lectins (10), altered cytoskeleton structure of the cell (11), and the removal of a transformation-sensitive protein from the surface (12, 13). The second line of evidence arises from experiments involving the addition of inhibitors of serine proteases to intact cells. These inhibitors have been shown to reduce cellular growth rate (14–16), alter cellular morphology and adhesion (14, 17–20), and reduce agglutination by plant lectins (20). Third, elevated levels of a plasminogen activator that can cleave the serum zymogen plasminogen were shown to be associated with transformation of embryonic fibroblasts (14, 21–28). The kinetics of elevation of plasminogen activator activity parallels transformation of fibroblasts during high-multiplicity infection by RSV (28), during production of focal areas of transformed cells due to infection and replication of a single infectious virion (14), and during expression of the *src* gene at permissive temperature in cells transformed by temperature-sensitive transformation mutants of RSV (refs. 21 and 27; B. A. Wolf, unpublished observations).

We have investigated the correlation between increased plasminogen activator activity and the pleiotropic alterations of the cellular properties of fibroblasts. We reported the isolation of RSV-transformed embryonic fibroblasts with low levels of plasminogen activator (29). Although these clones showed levels of activator similar to those of uninfected cells, they exhibited several properties of transformed cells, including increased rate of sugar transport, altered cellular morphology, and high efficiency of growth in suspension culture. We have extended these studies to an examination of tumorigenicity of clones with low levels of plasminogen activator. Because cells transformed by nondefective strains of RSV release infectious virus that is sarcomagenic in chickens, it was necessary to isolate cells transformed by the replication-defective Bryan strain of RSV, which releases noninfectious virus (30, 31), to be able to study the tumorigenicity of the cell itself. Jones *et al.* (32) have reported that clones of a human fibrosarcoma cell line that exhibited low levels of plasminogen activator activity were tumorigenic in immunosuppressed hamsters. They also observed increased plasminogen activator activity in explants of these tumors.

MATERIALS AND METHODS

Cells and Viruses. Primary chicken embryo fibroblasts were prepared from 10-day-old gs-antigen-negative SPAFAS eggs as described previously (23, 33). The replication-defective Bryan high-titer RSV(RAV-1) strain of RSV was the kind gift of H. Hanafusa. [(RAV-1) indicates the viral coat is that of Rous-associated virus 1 (RAV-1).]

Isolation of Soft Agar Clones. Twelve hours after plating, secondary cultures of chicken embryo fibroblasts were infected in monolayer culture with defective Bryan high-titer RSV-A [BH-RSV(RAV-1)] at a multiplicity of infection of 10^{-4} focus-forming units per cell in the presence of DEAE-dextran at 5 μg/ml. After adsorption for 2 hr at 37°C with occasional rocking, cells were treated with trypsin and suspended in medium containing 5% calf serum, 10% tryptose phosphate broth, 0.3% agar, and 1% anti-RAV-1 antiserum as described by Friis *et al.* (34). Two weeks later, visible colonies were removed from the soft agar overlay with a syringe fitted with a 23-gauge needle. Cell clumps were broken by shear force by vigorous passage through the syringe needle. Cells were plated in Linbro

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Abbreviations: RSV, Rous sarcoma virus; RAV, Rous-associated virus.

tissue culture multi-dishes (FB-16-24-TC) in medium containing 1% anti-RAV-1 antiserum. All clones were maintained in Eagle's minimal essential medium containing 5% calf serum and transferred to Scherer's maintenance medium as indicated.

Casein/Agar Overlay Assay. Clones were overlayed with Scherer's medium containing 0.75% agar, 1.4% non-fat milk, and chicken plasminogen at 0–5 μg/ml as described previously (23). The degree of digestion of the casein was estimated as indicated in the legend to Table 2. Chicken plasminogen was purified by affinity chromatography on lysine-Sepharose 4B as described by Deutsch and Mertz (35). The level of plasminogen activator activity of different cell clones did not depend on whether they had been grown in Scherer's or Eagle's growth medium, nor did it vary with the number of passages *in vitro*.

Transformed Cell Properties. Cells were assayed for uptake of 2-deoxy-D-[*G*-^{3}H]glucose, 10.0 Ci/mmol, as described by Hatanaka and Hanafusa (36). Efficiency of growth in soft agar was tested as described previously (29).

Characterization of Virus Released from Bryan Clones. Undiluted 12-hr harvests of supernatant medium from each clone were used to infect secondary chicken fibroblasts in the presence of DEAE-dextran at 10 μg/ml. Cells were overlaid with 0.45% agar containing 5% calf serum, 10% tryptose phosphate broth, and 1% beef embryo extract (33). After 1 week, cultures were examined under phase contrast optics for transformed cells. Negative cultures were subcultured and overlaid with agar again. The cultures were incubated 1 week and examined for transformed cells. Negative cultures were subcultured twice more. A culture was considered negative for infectious transforming virus (i.e., releasing only noninfectious transforming RSV) if no transformed cells were observed during the three subcultures (over a 4-week period). Only the clones producing noninfectious transforming Bryan RSV were used in the tumorigenicity studies.

Tumorigenicity in Newborn Chicks. Only clones of replication-defective Bryan-transformed cells that were releasing noninfectious transforming virus were injected into chicks. One-day-old gs-antigen-negative SPAFAS chicks were injected in each wing web with 0.05 ml of a clone of replication-defective Bryan virus-transformed cells diluted to either 10^4 or 10^5 cells per ml in Scherer's medium containing 5% calf serum and 10% tryptose phosphate broth. Chicks were incubated in a 37°C incubator for 1 week and then transferred to brooder cages. The wing webs were examined periodically for tumor formation. Lesions ≥5 mm in diameter were considered to be positive. The latency period for development of a tumor 5 mm in diameter was noted.

Tumor Explants. Primary cultures of tumors were prepared in the same manner as primaries from chicken embryos.

RESULTS

We had previously observed a spectrum of plasminogen activator activity in the agar suspension clones isolated after infection of chicken embryo cells with B77 or Prague RSV (20). The frequencies of B77 or Prague clones with low caseinolytic activities were 13% (3/23) and 33% (8/25), respectively. All of the clones derived from infection with B77 or Prague strain of RSV released infectious transforming virus (37). Because infectious transforming RSV is sarcomagenic in chickens, it would be difficult to ascertain the tumorigenicity of the cells themselves. Therefore, clones derived from infection with the replication-defective Bryan strain were isolated. Clones derived from a cell transformed by one focus-forming unit of the replication-defective Bryan high titer strain of RSV do not release infectious transforming virus (30, 31). Thus, by eliminating the production of transforming virus, the tumorigenic potential of the transformed cells could be ascertained. The frequency of Bryan RSV clones exhibiting a low level of caseinolytic activity was 34% (5/17). The fluctuations in the observed frequency of clones with low levels of plasminogen activator among the different strains of RSV may be due to the limited sampling. Of the 65 clones derived from infection with B77 or Prague or Bryan strain RSV, 25% exhibited low plasminogen activator activity, 43% had an intermediate level, and 32% showed high activity. The distribution of low, intermediate, and high activator clones appeared similar with the three strains of RSV studied.

A summary of the properties exhibited by uninfected cells and by the agar clones is shown in Table 1. The range of properties exhibited by the Bryan strain of RSV-derived clones fell within the continuum observed for the B77 and Prague clones (29). We have listed the range of values for individual clones as well as the mean to show the extent of the variation of these properties within our categories. By definition, the low activator clones possessed the same or only slightly more proteolytic activity than uninfected cells. Yet all clones exhibited three properties of RSV-transformed cells, namely an altered (round) morphology, a 2- to 5-fold greater rate of sugar transport, and a 10^5- to 10^6-fold higher efficiency of growth in agar suspension.

Of particular interest was the degree of tumorigenicity in chickens, the natural host, of the three types of clones. Five clones, transformed by the Bryan strain of RSV and showing low, intermediate, or high plasminogen activator activity, were tested for tumorigenicity in newborn chicks. The caseinolytic activities of the five clones are shown in Table 2. The three clones with low activity, 2119, 211-17, and 211-20, exhibited little caseinolysis relative to the high-activity clone, 211-14, even though 3–4 times more cells were assayed. Clone 211-11 exhibited an intermediate level of caseinolysis. Tumorigenicity of the five clones was determined as described in *Materials and Methods*. All of the clones induced tumors at the two sites of

Table 1. Properties of Bryan RSV-transformed clones

Cells	Caseinolysis	Morphology	Sugar transport, cpm/mg cell protein × 10^{-3}		Efficiency of growth in agar suspension	
			Mean	Range	Mean	Range
Uninfected	Low	Flat	8.4 ± 1.0 (4)	7.2–10.4	$< 10^{-6}$	
RSV Clones	Low	Round	51 ± 12 (5)	25–87	0.90 ± 0.04 (7)	0.8–1.0
RSV Clones	Intermediate	Round	44 ± 19 (5)	23–121	0.87 ± 0.04 (9)	0.8–1.0
RSV Clones	High	Round	23 ± 5.2 (4)	20–37	0.84 ± 0.04 (5)	0.8–1.0

Plasminogen activator was assayed by casein/agar overlay. Morphology under phase contrast optics and efficiency of growth in agar suspension were determined as described previously (29). Sugar transport was assayed as rate of uptake of 2-deoxy-D-[*G*-^{3}H]glucose in 10 min as described by Hatanaka and Hanafusa (36). The standard error of the mean is given for each set of determinations. The numbers in parentheses are the number of clones that were tested in each instance.

Table 2. Caseinolysis by isolated replication-defective Bryan RSV clones

Clone	Caseinolysis	Cells/35-mm plate × 10^{-6}	Caseinolysis with chick plasminogen at 1 μg/ml	2 μg/ml	5 μg/ml
2119	Low	0.9	0	±	±
211-11	Intermediate	0.9	±	1	2
211-14	High	0.3	1	2	4
211-17	Low	1.2	0	0	0
211-20	Low	0.9	0	±	±

Cells were washed twice with phosphate-buffered saline and overlayed with Scherer's medium containing 0.75% agar, 1.4% nonfat milk, and chicken plasminogen at 1–5 μg/ml as indicated. After the agar hardened, plates were incubated in a humidified 5% CO_2 atmosphere for 22 hr. The degree of casein hydrolysis was estimated visually and is represented by the following symbols: 0, no clearing; ±, less than 10% of area cleared; 1, 25% of area cleared; 2, 50% of area cleared; 3, 75% of area cleared; 4, 100% of area cleared. The number of cells per dish was determined by trypsin treating equivalent plates that had not been incubated with casein/agar overlay.

injection at both cell concentrations that were tested (Table 3). Similar latency periods were observed regardless of the activator level of the clone injected. All lesions were infiltrated by blood vessels and grew progressively until the animals died or were sacrificed. Some lesions reached a diameter of 5 cm, which was the size of the brachium. No tumors regressed during the observation period (4 weeks).

It has been suggested that the process of tumor formation by low plasminogen activator clones of a human fibrosarcoma line is associated with an increase in plasminogen activator activity (32). Therefore, it was of interest to determine the caseinolytic activity of the tumors induced by the RSV-transformed chick embryo fibroblasts. Primary explants of several tumors that developed after injection of both cell concentrations were made and assayed for caseinolytic activity. The results are shown in Table 4. Every injected clone, regardless of its activator activity prior to injection, induced tumors exhibiting a spectrum of plasminogen activator activity. No correlations could be made with regard to the level of plasminogen activator, size of excised tumor, number of cells surviving explantation, or morphology of explanted tumors. Although the tumors 964T, 966T, and 984T appear to have higher caseinolytic activity than the clones that were originally injected, several times more tumor cells were assayed than cloned cells. The reason why more tumor cells were assayed for caseinolytic activity than the parental clone is related to the finite lifetime *in vitro* of chicken cells. Chicken embryo fibroblasts, even when transformed by RSV,

Table 3. Tumorigenicity of replication-defective Bryan RSV clones

Clone	Caseinolysis	Cells injected	Chicks with tumors	Latency, days
2119	Low	500	1/1*	10
		5000	1/1*	10
211-11	Intermediate	500	1/1*	16
		5000	4/4	10
211-14	High	500	4/4	11
		5000	4/4	11
211-17	Low	500	5/5	8–12
		5000	4/4	8–12
211-20	Low	500	5/5	8–14
		5000	5/5	8–14

* The rest of the chickens in this group died due to incubator malfunction.

age and die after several (~50) generations *in vitro*. Because many Bryan clones had already succumbed to crisis before these clones were injected into chicks and because these clones had undergone an undetermined number of cell divisions by the time of assay, expeditious assay of the tumors was necessary. To ensure successful plating, cells were seeded at high density. Thus, Tables 2 and 4 are not absolutely comparable. The rate of casein digestion depends not only on the activator activity of a given cell but also on the number of cells assayed. Therefore, the slight increases in activity above that of the injected cells probably reflect the increased number of cells assayed rather than a significant increase in cellular activity. Clones with low proteolytic activity generally produced tumors with higher levels of activity (60%). However, clones with intermediate or high caseinolytic activity resulted in tumors at a similar frequency (56%) with lower levels of activity. Thus, all possibilities were observed (low to low, low to high, high to high, and high to low), suggesting random selection. We have no data that indicate a mechanism for the loss of plasminogen activator activity during tumor formation. If the presence of high proteolytic activity is irrelevant or not mandatory for tumor formation, then this property could be lost. Perhaps a high level of proteolytic activity would be self-destructive in confined tumors and would mitigate against its retention. The spectrum of plasminogen activator activity of the cell-induced tumors contrasts with the uniformly high level of plasminogen activator of tumors induced by virus (ref. 21; B. A. Wolf, unpublished observations) and argues against the requirement of high proteolytic activity for tumor formation.

DISCUSSION

We have shown that clones of replication-defective Bryan strain RSV-infected chicken embryo fibroblasts vary in the level of plasminogen activator activity in the same way as clones infected by B77 or Prague strain of RSV (29). The Bryan clones displayed the same spectrum of properties—namely, altered morphology, increased rate of sugar transport, high efficiency of growth in suspension, and low-to-high levels of plasminogen activator. The low proteolytic activity exhibited by the RSV-transformed clones is not due to the presence of inhibitors. Mixing of harvests from equal numbers of cells from low- or high-activator clones or from RSV-transformed cells and uninfected cells results in a decrease in proteolytic activity due only to dilution. Furthermore, we have been unable to detect any macromolecular inhibitors of plasminogen activator.

We did not observe any correlation between the level of plasminogen activator and ability of cells to grow as a wing web tumor in chicks. In order to ascertain the tumorigenic potential of a given clone, only replication-defective Bryan clones that did not release infectious transforming RSV were injected into chicks. All of the replication-defective Bryan clones produced tumors with approximately the same latency period. The cells in the clones are highly oncogenic, because injection of only 5 × 10^2 cells resulted in 100% incidence of tumor formation. Because the clones with low proteolytic activity were highly oncogenic without passage *in vivo*, a second *in vivo* passage was not investigated. Although noninfectious transforming Bryan RSV (the virus itself) has been shown to cause wing web sarcomas when injected at high concentration (10^7 focus-forming unit equivalents/ml) (38),* it is improbable that the tumors that

* As defined by Hanafusa *et al.* (38), one focus-forming unit equivalent is the amount of radiolabeled RSV (−) (noninfectious transforming virus) equivalent to the amount of radiolabel in one focus-forming unit of RSV (f) (infectious transforming virus) when one plate of RSV (f) and one plate of RSV (−) producing transformed cell cultures are incubated for 12 hr in medium containing [^{3}H]uridine.

Table 4. Caseinolysis by isolated tumors

Injected clone		Explanted tumor	Cells/ 35-mm plate	Caseinolysis with chick plasminogen at		
No.	Activator level	cells	$\times 10^{-6}$	1 μg/ml	2 μg/ml	5 μg/ml
2119	Low	928T	4.4	0	±	2
		932T	5.5	0	±	±
211-11	Intermediate	942T	5.0	2	3	4
		946T	1.8	2	3	4
		948T	4.3	0	±	±
		949T	2.6	0	±	±
211-14	High	955T	4.5	±	1	2
		957T	3.5	0	0	1
		958T	3.1	0	0	±
211-17	Low	960T	5.3	1	2	3
		964T	2.6	0	±	1
		966T	4.1	0	0	±
		968T	3.5	3	4	4
211-20	Low	975T	3.6	±	±	2
		977T	5.3	0	2	3
		979T	1.6	1	2	4
		984T	5.6	0	0	1

Tumors were explanted and assayed for caseinolysis as described in the legend to Table 2. Incubation time was 22 hr.

we observed after injection of cells were due to virus for two reasons. First, in our hands, only injection of undiluted cell-free supernatants induced tumors (data not shown). Because cell suspensions of the clones involved dilutions of 1:100–1:10,000, the presence of sufficient noninfectious transforming Bryan RSV seems unlikely. Second, the tumors induced by injection of the Bryan clones did not resemble tumors resulting from injection of Bryan RSV. Virus-induced wing web tumors have an ill-defined periphery and a mucous consistency. Cell suspensions from them are difficult to dissociate from this mucous material by using trypsinization or mechanical force. The tumors observed after injection of cells were surrounded by a tough, fibrous capsule but were easily trypsinized to single cell suspensions. These spongy tumors were similar to tumors resulting from subcutaneous injection of fibroblasts into animals and quite distinct from the mucous tumors induced by RSV. However, it would be difficult to exclude the possibility that some recruitment of host cells occurred due to a local concentration of cells releasing high titers of noninfectious sarcoma virus or due to cell-to-cell spread. Nevertheless, the observation that all RSV virus-induced tumors studied to date have resulted in tumors with high levels of plasminogen activator (ref. 21; B. A. Wolf, unpublished observations) argues against recruitment being a major factor in tumor formation. Our results with soft agar clones of Bryan-transformed cells are in agreement with earlier studies by Hanafusa *et al.* (30, 31), which demonstrated that noninfectious virus-producing cells, derived from a single focus of Bryan RSV-infected cells, induced wing web sarcomas when injected into 2-week-old chicks. The slightly longer latency period we observed is probably the result of using larger lesions to determine latency times. Also, the absence of tumor regression in our experiments was probably the result of injecting younger chicks.

Our results confirm those of Jones *et al.* (32, 39) that tumorigenicity of transformed cells does not appear to correlate with the level of plasminogen activator. In our hands, tumorigenicity seems to correlate with an enhanced ability of cells to grow in suspension culture, because all of the Bryan clones grew well in agar suspension and were tumorigenic. These observations are in agreement with those of Pollack *et al.* (26) and Freedman and Shin (40). However, we have not attempted in this study to quantitate the correlation between these two properties as Pollack *et al.* and Freedman and Shin have done. Because we failed to observe a correlation between tumor induction and plasminogen activator activity, we suggest that the level of plasminogen activator of a transformed cell is not the primary factor in the tumorigenic potential of that cell at the site of injection in its natural host. Furthermore, the spectrum of caseinolysis observed in the induced tumors suggests that the level of plasminogen activator is not important in the selection process that gives rise to a tumor mass. This observation is in contrast to the results obtained by Jones *et al.* (32), who observed that all the explants of tumors induced by clones with low plasminogen activator activity exhibited an increased level of proteolytic activity. The reason for this difference is not clear but may reflect either the different hosts (immunocompetent chicks vs. immunosuppressed hamsters) or the different cell type injected (embryonic chicken cell vs. established transformed human line). In support of the latter possibility are the observations that in the cell line studied by Jones *et al.* there is a spontaneous increase in the level of plasminogen activator during *in vitro* passage (32), while uninfected and transformed chicken fibroblasts maintain a stable level during growth in tissue culture (29).

We are not able to determine if the very small increases in plasminogen activator activity in some of the low-activity clones may be sufficient to account for the tumorigenic potential of these cells. The level of plasminogen activator may nonetheless be important in the process of metastasis, although apparently not in tumor formation at the site of injection of transformed cells. It may be of interest to note that some normal cells possess very high levels of proteolytic activity (14, 41) but are not oncogenic.

It is not clear why transformation is often associated with an elevation of the plasminogen activator level, even though transformed cells with low levels of plasminogen activator can be isolated. Our results suggest that the level of plasminogen activator in RSV-transformed cells is both virally and cellularly determined (37). In many studies of the effects of transformation, the plasminogen activator activity of the cells en masse is assayed and the presence of some transformed cells with low activator acitivity would probably be masked by the presence of cells with high activator levels. The production of focal areas of transformed cells due to infection with a single infectious

virus particle would recognize transformed cells with low activator activity if the foci were well separated. In fact, foci with low plasminogen activator activity, which appear to be cell variants and not viral mutants, have been reported (14, 42). The frequency of these foci is lower than the frequency of clones with low activator activity, which may be the result of recruitment of cells exhibiting increased levels of activator during the spread of virus.

We thank Drs. I. Tamm and H. Hanafusa for many stimulating discussions during the course of these experiments. We are grateful to Miss Janet Helms and Miss Maria Victoria Docal for excellent technical assistance. This work was supported by U.S. Public Health Service Grant CA-13362. B.A.W. was a fellow of the National Cancer Institute, 5F22 CA-02607-12. A.R.G. is a Research Career Development Awardee of the National Institutes of Health and a Richard King Mellon Foundation Fellow in The Rockefeller University.

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