

PATTERNS OF PLASMINOGEN ACTIVATOR PRODUCTION IN CULTURED NORMAL EMBRYONIC CELLS

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ABSTRACT

Cultured normal low-passage embryo fibroblasts, from a number of species, and two untransformed clones of a Balb/3T3 line elaborate increasing amounts of plasminogen activator (PA) as they approach confluence; the low-passage cells then lose this PA activity after reaching confluence, while the 3T3 cells retain it indefinitely. Even at their peaks, however, the PA activities of the low-passage cells remain well below those of the corresponding virally or spontaneously transformed cells. The PA increases in normal cells are probably a result of PA production rather than of adsorption of secreted PA to the cell surface, or of changes in cell-associated protease inhibitors. The elaboration of PA by normal cells is dependent upon their metabolic activity, such that the level of serum supplementation and the growth phase of the culture directly influence the level of cell-associated PA observed. In addition, there may be a component of serum which exerts a negative control on PA production and which is not an acid-labile protease inhibitor.

KEY WORDS plasminogen activator ·
protease · cell growth · serum effects ·
normal low-passage embryo fibroblasts ·
Balb/3T3 · transformation

The production of a high level of plasminogen activator (PA) in culture has recently come to be recognized as a distinctive property of many transformed cells. Production of this enzyme has been associated with viral, chemical, and spontaneous transformation in a number of avian and mammalian species (8, 16-19, 22, 31, 33), and with specific properties of the transformed phenotype, including rounded cell morphology (16, 18), migration into a wound (16, 17), growth in soft agar or methocel (16, 18, 19), and tumorigenicity in nude mice (19).

Some normal cells are also capable of producing this enzyme, however. Activated macrophages and cells from the lung, heart, and kidney are

generally good sources of PA (1, 3, 4, 32). Other cells produce PA only at specific, restricted times. For instance, before ovulation and during embryo implantation in the mouse the amount of PA synthesized by follicle cells and trophoblast cells, respectively, rises dramatically and later falls again in an apparently regulated manner (2, 28, 29).

Clearly, any assessment of the significance of PA in bringing about or maintaining the transformed phenotype must be made in the context of the function and regulation of PA in normal cells. In this study, we have analyzed the patterns of PA production in cultures of normal low-passage embryonic cells and of two clones of an untransformed mouse cell line. Reported here are our observations on the variations in PA level that result from differences in the seeding density, growth rate, and serum supplementation of untransformed cells.

MATERIALS AND METHODS

Cells

Primary embryo fibroblast cultures were prepared from 13-day-old C57Bl/6 mouse embryos (pregnant mice from Jackson Laboratories, Bar Harbor, Maine). Whole embryos were minced extensively, washed in 0.14 M NaCl, 0.005 M KCl, 0.024 M Tris-HCl, and 0.0004 M Na₂HPO₄, pH 7.4 (TD) and subjected to three successive 20-min trypsinizations in 0.25% trypsin. Cells released by the trypsin were then suspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FCS) and plated at 10⁷ cells per 100-mm plate (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). Confluent primary cultures were trypsinized and either stored in liquid nitrogen or passed to new plates for direct use in experiments.

Secondary or tertiary C57Bl/6 mouse embryo fibroblasts were used for most of the experiments. Cells were seeded on 100-mm plates at a density of 5 × 10⁶ cells per dish. Each day for 10 or more days, the cells on one plate were trypsinized and counted to monitor cell growth, and the cells on companion plates were scraped for PA determinations. The medium in the remaining plates of cells was changed daily, except where otherwise noted.

Plates of cells for vital staining experiments were washed with TD and stained at room temperature for 10 min with 0.4% trypan blue in 0.137 M NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1 mM KH₂PO₄, 1 mM CaCl₂, 1 mM MgCl₂, pH 7.4 (PBS). The dye solution was then removed by aspiration, the plates were washed twice with TD and the cells trypsinized with 2 ml of 0.25% trypsin at 37°C. After 5 min, 2 ml of medium with 10% FCS were added to the plate, and the cells were suspended thoroughly and counted in a hemocytometer. Both stained (blue) and total cell counts were recorded.

In addition to inbred C57Bl/6 mouse embryos, embryos from Balb/c mice (Jackson Laboratories), wild mice (collected in Bouquet Canyon, Calif., and furnished through the kindness of M. B. Gardner and V. Klement, University of Southern California School of Medicine, Los Angeles, Calif.), rats ("Fisher" rats from Charles River Breeding Laboratories, Inc., Wilmington, Mass.), hamsters (Lakeview Golden Hamster Colony, Newfield, N. J.), chickens (Spafas Inc., Norwich, Conn.), and humans (from therapeutic abortions) were also used as sources of secondary or tertiary embryo fibroblasts. Primaries were prepared from these embryos at 11 days (chicken), 13 days (mouse, rat, and hamster), or 6–8 wk (human) of gestation, using the method described above for C57Bl/6. Two A31 clones of the Balb/3T3 line, one a gift from E. Scolnick (National Institutes of Health, Bethesda, Md.) and the other a gift from R. Pollack (State University of New York at Stony Brook, N. Y.), a human melanoma, RPM1 No. M7272, and a liposarcoma, RPM1 No. 1922, both from G. Moore (Denver General Hospital, Denver, Colo.), and normal human embryonic lung and skin cells from Flow Labora-

tories (Rockville, Md.), were also used in some experiments.

All cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS except for the chicken cells, which were cultured in Eagle's minimal medium supplemented with 10% FCS, and the human cells, which were cultured in either Eagle's minimal medium or RPM1 1640 supplemented with 10% FCS. Cells were cultured in an atmosphere of 10% CO₂-90% air at 37°C.

Cells to be transformed by murine sarcoma virus (MSV) were pretreated for 40 min with 25 µg/ml DEAE-dextran (Pharmacia Fine Chemicals, Piscataway, N. J.) in TD, then infected with 1 ml of virus-containing medium per sparse 100-mm petri dish. After 40 min, medium was added and the cells were left at 37°C for 3 days or until all the cells became morphologically transformed. They were used without further passaging. Transformed chicken cells were prepared by infecting secondary cultures with Rous sarcoma virus (RSV), subgroup A, using an isolate obtained from H. Hanafusa (The Rockefeller University, New York). Hamster cells transformed with simian virus 40 (SV40) were a gift from L. Ossowski (The Rockefeller University).

Determination of Intracellular

PA Levels

Cells to be assayed for PA were washed twice with ice-cold TD, scraped off the plate with a Teflon or rubber policeman, and collected by centrifugation at 2,000 g for 3 min. The supernate was then removed by aspiration and the cell pellet was spun in 0.5–1.0 ml of ice-cold 0.1 M Tris-HCl, pH 8.1, containing 0.5% Triton X-100. This extract was centrifuged for 10 min at 500 g and the supernate retained for protein determination (12) and PA assay.

The radioactive assay of Unkeless et al. (33) was used to determine PA levels: 2.5, 5, 10, or 15 µg of extract protein were added to a 35-mm petri dish, previously coated with ¹²⁵I-fibrinogen (10 µg/cm², 50,000 cpm) and activated for 3 h, together with 2 ml of 0.1 M Tris-HCl, pH 8.1, 500 µg of bovine serum albumin (BSA) and 4 µg of purified FCS plasminogen (6). Purified chicken plasminogen, 14 µg per dish, was used instead of FCS plasminogen for assays of chicken cell extracts. The dishes were incubated at 37°C, and the supernatant fluid was assayed for solubilized ¹²⁵I-fibrin degradation products in a gamma counter at 2 and 4 h. Sometimes, linbro dishes (Linbro, New Haven, Conn.) were used rather than 35-mm dishes. In these assays, the amounts of sample protein were reduced to 0.5, 1, 2, and 3 µg per well, the amounts of Tris buffer and BSA were reduced fourfold, and the amount of plasminogen was unchanged (4 µg of FCS plasminogen per well). The value for each time-point is the average of two determinations performed on a given sample. Only values in the range of the assay linear with respect to both time and sample

concentration were used. For this purpose, each sample was assayed at a number of protein concentrations and the assay was monitored at more than one time. In general, the range in which this assay is linear will vary according to the activity of the sample, so that sample concentrations and assay time-points must be chosen carefully in accordance with the desired sensitivity. No plasminogen-independent fibrinolysis was observed. Negative activities were recorded for samples releasing fewer than the background number of counts (buffer plus BSA and plasminogen).

A relative unit (R.U.) of PA is defined as that activity which will solubilize 5% of the radioactivity in a dish, after correction for background, in 1 h at 37°C in the presence of 2 µg of plasminogen and 1 ml of Tris-HCl buffer, pH 8.1. Since the extent of fibrin cross-linking can vary among batches of assay plates, urokinase standards were run with each assay to permit quantitative comparisons among assays. One Ploug unit of Urokinase Reference Standard (Leo Pharmaceutical Products, Ballerup, Denmark) was equated to 30 R.U. in the presence of FCS plasminogen, or to 1.5 R.U. when chicken plasminogen was used. The presence of BSA in the assay increased the urokinase activity 3.33-fold. Although the PA units for mammalian and chicken systems are both defined arbitrarily here with respect to human urokinase activity, these two units are not equivalent. Species-specificity plays a marked role in the efficiency of plasminogen activation; while activation efficiencies of different mammalian plasminogens by human urokinase are similar, the activation efficiencies of bird and mammalian plasminogens by human urokinase differ by an order of magnitude. One is therefore left with two distinct activations that cannot be compared directly: the activation of bird plasminogen by bird PA and the activation of mammalian plasminogen by mammalian PA.

Measurement of PA Secretion

Cells whose PA secretion was to be measured were washed with TD and incubated in 10 ml of serum-free medium for 4 hr. The conditioned serum-free medium was collected, centrifuged for 10 min at 3,000 g, and 50 µl of the supernatant fluid were assayed in ¹²⁵I-fibrin-coated linbro wells as described above, with the medium substituted for cell extracts as the source of PA.

Extracts of Transformed Cells and Tumor Lines

Transformed cells were extracted in the same way as the normal cells, with 0.5% Triton X-100 in 0.1 M Tris-HCl, pH 8.1. Since in some cases their cell-associated PA levels were also found to vary with cell density, although not in the regular fashion reported here for normal cells (S. Rohrllich, unpublished results), we always chose to scrape them at their peak of PA produc-

tion, which generally corresponded to visual confluence of the culture.

Overlay Assays for Fibrinolysis

C57Bl/6 cells seeded at 4×10^4 (sparse) or 2×10^6 (confluent) cells per 60-mm plate were washed twice with serum-free medium a day after plating, then overlaid in the presence or absence of 50 µg of FCS plasminogen with a mixture of Eagle's minimal medium, purified agar, topical thrombin, and bovine fibrinogen, according to the method of Jones et al. (7). After incubation for 25 h at 37°C, the culture dishes were stained with 0.02% Coomassie Blue in a mixture of methanol:glacial acetic acid:water, 7:1:2 (vol:vol:vol), and examined for areas of fibrinolysis.

Trypsin and Plasmin

Inhibitor Assays

An adaptation of the PA assay was used to determine levels of inhibitors of proteases such as trypsin or plasmin. The sample to be assayed was preincubated for 30 min at room temperature with 1 ml of 0.1 M Tris-HCl, pH 8.1, containing 0.25 µg of trypsin and 0.25 µg of BSA, then 500 µl of the mixture were transferred to each of two linbro wells containing ¹²⁵I-fibrin as described for the PA assay. The assay was incubated at 37°C for 1 h, and 250 µl of the supernatant fluid were withdrawn for determination of counts, as in the PA assay. Again, a range of concentrations of sample was assayed, and the percent of inhibition was calculated by subtracting the per cent of solubilized radioactivity, after correction for background, from the total solubilizable counts in the well, or 100 per cent. Some, but not all, of the samples were also tested for plasmin inhibition by the substitution of plasmin for trypsin in this assay. The plasmin was generated from plasminogen by preincubating 25 Ploug units of urokinase with each 1 µg of FCS plasminogen for 30 min at 37°C in activation buffer (25% glycerol in 0.04 M Tris-HCl, pH 9.0, containing 0.01 M lysine and 0.08 M NaCl). The equivalent of 0.25 µg of plasminogen was used for each sample to be assayed. Assay time-points were taken at 1 and 2 h.

Acid Treatment to Destroy

Acid-Labile Inhibitors

Cell extracts and FCS were acid-treated in some experiments to remove acid-labile inhibitors of fibrinolysis. Samples were brought to pH 2 by adding 1 N HCl, held at room temperature for 2½ h, then reneutralized with 1 N NaOH.

Perchloric Acid Extraction of Acid-Stable Protease Inhibitors

Cell extracts, made as described earlier, were dialyzed against two changes of 10 mM sodium acetate, pH 5.0 at

4°C. The dialyzed samples were then acidified with $1/10$ vol of concentrated perchloric acid (70–72%), and the mixture was centrifuged at 12,000 g for 10 min at 4°C. The supernate was collected and again dialyzed against two changes of 10 mM sodium acetate, pH 5.0, then assayed for protein and for acid-stable inhibitors in the trypsin and plasmin inhibitor assays described above.

Drug Experiments

Concentrated stock solutions of drugs were diluted in serum-free medium to the following final concentrations: 10 $\mu\text{g/ml}$ cytosine arabinoside, 4 $\mu\text{g/ml}$ actinomycin D, 5 $\mu\text{g/ml}$ cycloheximide, 10 $\mu\text{g/ml}$ colchicine, 7.6 $\mu\text{g/ml}$ dibucaine hydrochloride, and 1 mM NaN_3 + 10 mM NaF. 10 ml of one of these drug-supplemented media or a nondrug control medium were added to each plate of TD-washed cells and left on the cells for the duration of the experiment. Sample plates of cells were washed and scraped at 2, 4, and 24 h of incubation, and were extracted and assayed as described above.

Reagents

Cytosine arabinoside, cycloheximide, and colchicine were purchased from Sigma Chemical Co. (St. Louis, Mo.) and dibucaine hydrochloride from the Amend Drug and Chemical Co., Inc. (New York). Actinomycin was a gift from Merck Sharp and Dohme (Rahway, N. J.). Crude fibrinogen, obtained from Pentex Biochemical (Kankakee, Ill.) was purified and freed of plasminogen by ammonium sulfate precipitation and two ethanol precipitations, according to the methods of Laki (10) and Mosesson (14). Plasminogen was purified from chicken serum or from FCS by the method of Deutsch and Mertz (6). Crystalline bovine albumin was purchased from Pentex Biochemical.

Powdered media were purchased from Grand Island Biological Co. (Grand Island, N. Y.). They were supplemented with 60 $\mu\text{g/ml}$ tylosin (Associated Biomedic Systems, Buffalo, N. Y.), 200 $\mu\text{g/ml}$ streptomycin and 500 U/ml penicillin (both from Pfizer Chemicals Div., New York). The Eagle's minimal medium was further supplemented with $4 \times$ glucose. Sera were purchased from Grand Island Biological Co. and from Reheis Chemical Co. (Phoenix, Ariz.), Hanks' saline from Grand Island Biological Co., and trypsin from ICN Pharmaceuticals Inc., Life Sciences Group-NBC Division (Cleveland, Ohio). All other reagents were of the highest commercial grade available.

RESULTS

Early Passage Embryonic Cells

We have compared the cell-associated PA levels of normal embryonic cells from a number of species with their viral or spontaneous transformants. Figs. 1–5 show representative PA profiles as a function of cell density from early passage embryo

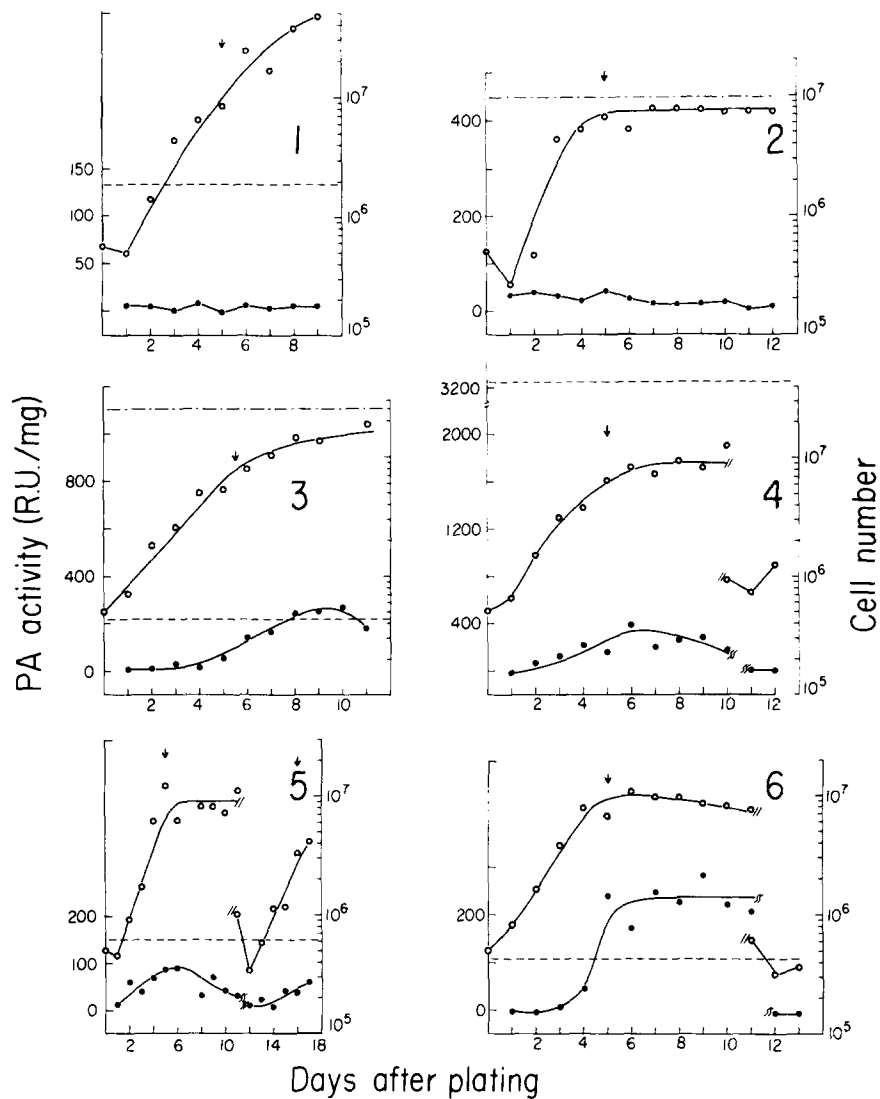
fibroblasts. All of the normal embryonic cells have barely detectable intracellular PA levels at low density. All but the chicken and hamster cells develop increasingly higher PA levels as they approach and attain confluence, and gradually lose some or all of the activity again after confluence.

The peak level of PA varies greatly from species to species. Chicken embryo fibroblasts (CEF) never develop any measurable PA activity (Fig. 1) although RSV-A-transformed CEF are relatively active (133 R.U./mg). This lack of detectable PA activity in normal CEF cells maintained in 10% FCS also holds true when the cells are maintained in medium supplemented with 5, 1, or 0.1% FCS (data not shown). In the 0.1% serum supplement, the cells go through only one doubling in 19 days despite daily changes of medium. Clearly, then, the PA level in CEF cells does not change as a function of their growth rate.

Normal hamster embryo fibroblasts (Fig. 2) have the lowest measurable activity, 41 R.U./mg, compared with 448 R.U./mg for an SV40 transformant of the same cells, and this low PA level remains fairly constant throughout their growth cycle. At the opposite end of the spectrum are human embryo fibroblasts (HEF), which start with very low PA activities at low densities but develop much higher PA activities as they approach confluence. Although the intracellular PA level of sparse HEF (Fig. 3) is only 6 R.U./mg, at confluence their PA activity rises to 142 R.U./mg and continues to rise for several days after the cells have become confluent, reaching 268 R.U./mg at its peak. Still, even this high level of PA activity is fourfold lower than the PA activity of an active human melanoma line (1,100 R.U./mg); it is comparable to the activity generated by a relatively inactive human liposarcoma line (220 R.U./mg).

Human embryonic skin (HES) and human embryonic lung (HEL) cultures are different from the HEF cultures obtained from whole embryos and may represent more specialized, homogeneous cell populations. HES cells show patterns of cell-associated PA activities qualitatively similar to those seen for HEF cells, but HES cells have a much lower peak value, 20 R.U./mg. HEL cells have considerably more activity than HEF cells; their PA level, 1,065 R.U./mg, remains fairly uniform throughout their life in culture and is about equivalent to that of the human melanoma line (data not shown).

The patterns of PA production for rat embryo fibroblasts (REF) and C57Bl/6 (mouse) embryo



FIGURES 1-6 Cell-associated PA activity (●) as a function of cell density in cultures of normal low-passage CEF (Fig. 1), hamster embryo fibroblasts (Fig. 2), HEF (Fig. 3), REF (Fig. 4), C57Bl/6 embryo fibroblasts (Fig. 5), and the Scolnick A31 clone of Balb/3T3 (Fig. 6). Daily cell counts on 100-mm dishes (○), visual confluence of the cells (arrows), and passaging of the cells to new plates (double slash marks) are all indicated. On days of cell passaging, the samples assayed for PA came from confluent plates before trypsinization. Also shown for comparison are the cell-associated PA levels of corresponding transformed cells or tumor-derived lines: RSV-A-transformed CEF (---), Fig. 1; SV40-transformed hamster embryo fibroblasts (---), Fig. 2; a human melanoma (---), and liposarcoma (---), Fig. 3; MSV-transformed REF (---), Fig. 4; MSV-transformed C57Bl/6 embryo fibroblasts (---), Fig. 5; and a fresh MSV transformant of the Scolnick Balb/3T3 clone (---), Fig. 6.

fibroblasts, which develop intermediate PA levels, are shown in Figs. 4 and 5, respectively, along with their MSV-transformed counterparts. Wild mouse and Balb/c cells behave identically with C57Bl/6. Furthermore, no differences in activity

are observed between C57Bl/6 cells derived from eviscerated embryos rather than whole embryos, nor in cells from 15-day embryos as compared with cells from 13-day embryos (data not shown). In each of these examples, the normal fibroblasts

also have peak PA levels below those of their transformants.

Balb/3T3

As shown in Fig. 6, the Balb 3T3 clone A31 obtained from Scolnick also elaborates PA in a cell density-dependent manner. As with low-passage normal embryo fibroblasts, A31 cells go from no cell-associated PA when they are sparse, to a peak level of 248 R.U./mg at confluence. There are two major differences, however, between the PA pattern of A31 cells, which are an established cell line, and that of low-passage mouse embryo fibroblasts. The peak PA level of A31 is almost three times higher than the peak PA level of the low-passage mouse cells. Furthermore, it remains high indefinitely beyond confluence, instead of falling again. (The Balb/3T3 clone A31 from Pollock has qualitatively the same PA profile as that from Scolnick, but the peak level is only 170 R.U./mg.) In addition, the fresh MSV transformant of the Scolnick A31 clone has a lower cell-associated PA level, 107 R.U./mg, than does its untransformed counterpart. This transformed PA level is close to the level of PA in MSV-transformed low-passage mouse embryo fibroblasts, which is 148 R.U./mg.

In summary, it is clear that the PA profile of embryonic cells from a number of different species varies with cell density and growth rate, though the peak level of cell-associated PA activity, at least for early-passage embryonic cells, always remains well below the PA level of corresponding transformed cells. The absolute value of the PA activity at its peak differs considerably from species to species.

Mouse Embryo Fibroblasts

In an attempt to determine the factors that might be responsible for the observed PA variations, we examined low-passage C57Bl/6 embryo fibroblasts in some detail.

The serum supplement with which C57Bl/6 cells are provided affects their growth rate and has a small quantitative effect on their cell-associated PA levels, although it has no qualitative effect on the pattern of PA elaboration with time. Thus, in 10% serum, which supports $4^{1/2}$ cell doublings in 10 days, the peak of cell-associated PA activity is 91 R.U./mg (Fig. 5), whereas in 2% serum ($2^{1/2}$ doublings in 10 days) it is 68 R.U./mg, and in 1% serum ($1^{1/2}$ doubling in 10 days) it is 61 R.U./mg (data not shown). If C57Bl/6 cells are maintained

in a 2% serum supplement without any medium change in the course of a 10-day experiment ($1^{1/2}$ doublings), the PA activity formed is still lower, reaching a peak of only 30 R.U./mg (data not shown).

Fig. 7 shows the overall PA pattern for C57Bl/6 cells maintained for four passages. In the course of each passage, the cell-associated PA level rises as the culture becomes confluent and falls again with each trypsinization and sparse replating. These PA changes are observed with trypsinization and sparse replating of other cell types also, for example, REF and Balb/3T3 clone A31, shown in Figs. 4 and 6, respectively. The only progressive change in the PA pattern that occurs as the C57Bl/6 cells go through their finite, characteristic number of generations in culture is that the peak of the PA activity is successively lower at each passage. Thus, the PA level of confluent primary cells is 136 R.U./mg whereas that of confluent fourth-passage cells, which will not undergo further divisions in culture, is only 52 R.U./mg. The decline in peak PA levels accompanies a gradual decline of the cell growth rate in the aging cultures and may be related to it.

At the same time that the cell-associated PA levels are changing in growing cultures of C57Bl/6 cells, similar density-dependent changes occur in the amount of PA that the cells secrete. As shown in Fig. 8, the amount of PA secreted (recovered in the serum-free conditioned medium) per 10^6 cells is not measurable when the cells are sparse but

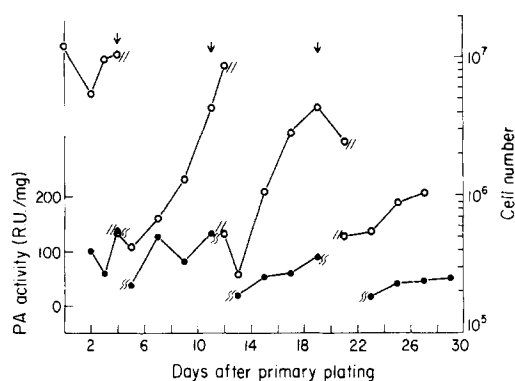


FIGURE 7 Cell-associated PA activity (●) as a function of cell density and passage number in normal C57Bl/6 embryo fibroblast cultures. Cell counts on 100-mm dishes (○), visual confluence (arrows), and passaging of the cells to new plates (double slash marks) are all indicated. On days of cell passaging, the samples for PA determination came from confluent plates before trypsinization.

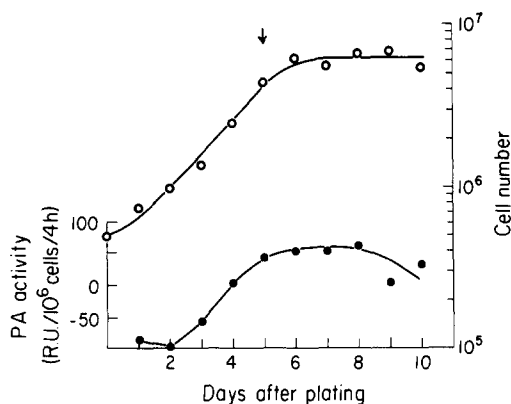


FIGURE 8 Secreted PA activity (●) collected in 10 ml of serum-free medium per 100-mm plate as a function of cell density, in cultures of normal low-passage C57Bl/6 embryo fibroblasts. Daily cell counts (○) and visual confluence (arrow) are also indicated.

increases gradually to 55 R.U./10⁶ cells per 4 h as the cells become confluent. A broad plateau of active PA secretion is followed by a gradual decrease as the cells progress into the stationary phase of growth. Thus, both the cell-associated and the secreted PA levels of normal mouse embryo cells in culture vary as a function of cell density and reach their peak at confluence.

Overlay fibrinolysis assays of sparse and confluent C57Bl/6 cultures were performed to determine whether the peak of cell-associated PA activity resulted from a few particularly active cells in confluent cultures or from the development of moderately high PA activity in many cells as they became confluent. The large, multi-focal area of fibrinolytic digestion in confluent plasminogen-containing plates, contrasted with the lack of plaques in similar sparse plates, indicates that a significant fraction of the cells in a given culture undergo the same changes in PA production and release as are observed grossly for the culture as a whole, though the cells of a given culture are not uniformly active (data not shown). No fibrinolytic plaques are present in confluent plates lacking plasminogen, demonstrating that the observed activity is entirely plasminogen-dependent.

To assess the importance of cell death in the decline of PA activity after cell confluence, we carried out vital staining experiments on cultures of different densities. The number of trypan blue-stained, presumably nonviable cells, is uniformly small throughout the 10-day experiment, never reaching even 2% of the total cell number. By the criterion of dye exclusion, then, stationary C57Bl/

6 cultures do not amass dead cells, and cell death probably cannot account for the loss of cell-associated PA activity after confluence.

Cell-Associated Inhibitors of Fibrinolysis

Inhibitors of fibrinolysis are found associated with cell extracts from normal embryo fibroblasts of many species cultured in the presence of serum, as determined by the depression of the PA assay when these extracts are mixed with similar extracts from transformed cells (S. Rohrllich, unpublished results); whether this inhibitory activity derives from the cells or from the serum in which they are cultured has not yet been determined. Whatever their origin, however, cell-associated inhibitors of fibrinolysis could mask true cell-associated PA levels. We therefore attempted to use acid treatment (pH 2, at room temperature, for 2½ h, then reneutralization), which destroys the inhibitory capacity of several serum inhibitors (11), to remove as much of the cell-associated inhibitory capacity of cell extracts as possible. Such a strategy cannot be used with C57Bl/6 cell extracts; the mouse PA activity cannot be recovered from acid-treated extracts. The PA in HEF extracts, however, is still recoverable after acid treatment, and the results of acid treatment of HEF extracts are shown in Fig. 9. The difference between the curve for acid-treated extracts and that for untreated extracts indicates that changes in the amount of cell-associated acid-labile inhibitors take place, with an increase in such inhibitors after 3 days. These changes in the level of cell-associated acid-labile

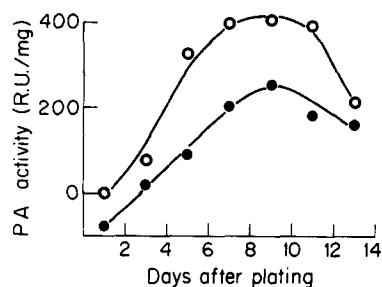


FIGURE 9 Cell-associated PA activity of normal low-passage HEF cells extracted and assayed without further treatment (●) or acid-treated at pH 2 for 2½ h at room temperature, then reneutralized before assay (○). The difference between the two curves represents the amount of cell-associated acid-labile inhibitors of fibrinolysis as a function of days in culture. Refer to Fig. 3 for growth curve.

inhibitors occur in the same direction as the PA changes in these cells, however, increasing as the PA level increases and decreasing as it decreases, and so they cannot account for the rise and subsequent fall of apparent cell-associated PA as a function of cell density.

Since acid-stable protease inhibitors (stable to 7% perchloric acid) have been found in several tissues (9, 25, 26) and in FCS (D. Rifkin, unpublished results), the level of these inhibitors in the cell extracts also seemed of interest. As shown in Table I A, we found that protease inhibitors stable to 7% perchloric acid are present at similar specific activities in sparse and confluent HEF extracts, so that these also cannot account for the PA changes seen.

Perturbations of Mouse

PA Production

To determine how serum affects cell-associated PA levels, we performed a number of experiments in which serum was first removed from the cells and later added back. After an initial drop in cell-associated PA resulting from TD washing and the addition of new medium, C57Bl/6 cells maintained in serum-free medium develop increased cell-associated PA levels (Fig. 10). Over a 16-h period, the PA level may increase as much as 20-fold. By 20–24 h in serum-free medium, the peak of PA has passed and the cell-associated PA be-

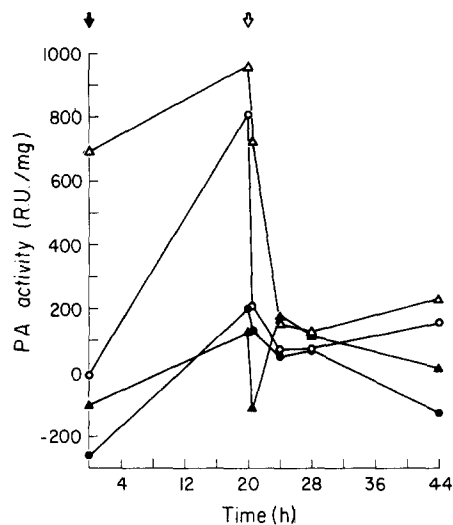


FIGURE 10 Variations in cell-associated PA activity related to the presence or absence of serum, as a function of the plating density of C57Bl/6 cells on 100-mm dishes. Cells seeded at 5×10^5 (●), 1×10^6 (▲), 5×10^6 (○), and 1×10^7 (△) cells/plate in medium containing 10% FCS were washed with TD the next day and placed in 10 ml/plate of serum-free medium. Sample plates were scraped just before the change to serum-free medium (solid arrow), after 20 h in serum-free medium, and at the indicated times after the re-addition of medium with 10% FCS (empty arrow).

gins to decline again (not illustrated). The timing of this PA rise and decline can vary by a few hours. If medium containing serum (10% FCS) is added to the cells at any point, the cell-associated PA drops immediately and remains at a very low level or declines further for a few hours; only gradually over the course of the next 24 h does it approach its original baseline level. Fig. 10 shows this pattern of PA increase, decrease, and return to baseline level for cells plated at four different densities. As illustrated, the cells plated at different densities start with relative PA levels characteristic of their seeding densities, and they regain this same relative pattern at the end of the experiment. The actual values of the initial PA levels for a given plating density, however, are somewhat variable, and are not quantitatively identical to the PA levels found for cells at corresponding densities in long-term culture, either in these C57Bl/6 experiments or in those with HEF cells (see below). We cannot explain this observed initial variability. Not illustrated are qualitatively similar results from an equivalent experiment performed with HEF cells. In this experiment, the control PA

TABLE I
Inhibitors of Trypsin and Plasmin Associated with
HEF Cell Extracts

Sample	Protein assayed	Trypsin inhibition	Trypsin inhibition/ μ g	Plasmin inhibition	Plasmin inhibition/ μ g
			%		
A.* HEF sparse	28	38.92	1.39	26.05	0.93
HEF confluent	28	44.51	1.59	44.39	1.59
B.‡ Control HEF					
Sample a	90	22.26	0.25		
Sample b	100	20.36	0.20		
17-h HEF					
Sample a	102	17.81	0.17		
Sample b	103	22.70	0.22		
4-h HEF					
Sample a	102	26.39	0.26		
Sample b	96	20.76	0.22		

* Cells extracted with 0.1 M Tris-HCl, pH 8.1, containing 0.5% Triton X-100, and assayed in the trypsin and plasmin inhibitor assays after perchloric acid treatment and dialysis as described in Materials and Methods.

‡ HEF cells, plated at 1×10^6 cells/100-mm plate in Eagle's minimal medium supplemented with 10% FCS (control HEF), were deprived of serum for 17 h (17-h HEF), then resupplied with 10% FCS for 4 h (4-h HEF). Duplicate samples were scraped, extracted, and assayed in the trypsin inhibitor assay at each time, giving the values recorded here.

level for HEF cultures, seeded at 1×10^6 cells/100-mm plate, was 600 R.U./mg, rose to 2,975 R.U./mg after 17 h in serum-free medium, and fell again to 1,175 R.U./mg after 4 h in serum-supplemented medium.

Analysis of the mouse cells' response to serum-free medium (Fig. 11) shows that the induced increase in PA activity requires that RNA and protein synthesis be sustained, if not newly induced, and that it is energy-dependent. No increase in cell-associated PA is seen in the presence of 5 μ g/ml cycloheximide or 4 μ g/ml actinomycin D (although the depression of PA production in response to this drug is delayed), nor in the presence of a mixture of 1 mM sodium azide and 10 mM sodium fluoride which together block 80-90% of the cells' aerobic and anaerobic respiration (27). By contrast, neither 10 μ g/ml cytosine arabinoside, which inhibits DNA synthesis, nor 2×10^{-5} M dibucaine or 10 μ g/ml colchicine, both of which have been reported to affect secretion and cytoskeletal integrity (13, 20, 24, 30), has any effect.

The PA increase in the absence of serum probably results from increased PA production rather

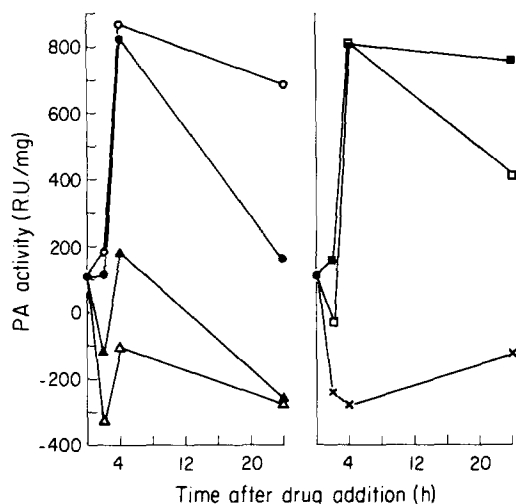


FIGURE 11 The effect of drugs on the rise in cell-associated PA in the absence of serum. C57Bl/6 cells were seeded on 100-mm plates at 2×10^6 cells/plate. The next day, at 0 h, sample plates were scraped and the other plates were washed with TD and put into 10 ml/plate of serum-free medium without drugs (●), or with 10 μ g/ml cytosine arabinoside (○), 4 μ g/ml actinomycin D (▲), 5 μ g/ml cycloheximide (△), 10 μ g/ml colchicine (■), 2×10^{-5} M dibucaine (□), or 1 mM sodium azide and 10 mM sodium fluoride (×). Sample plates were scraped at the indicated times.

than from adsorption of secreted PA. BSA included in the serum-free medium does not reduce the cell-associated PA; the cell-associated PA level is as high or higher in the presence of 0.1% BSA than in its absence (data not shown). Still, adsorption of secreted PA could be specific, and cannot be rigorously ruled out at present. Attempts to remove all cell-adsorbed material by trypsinization of the cells before extraction failed, since, even after extensive washing, trace amounts of residual trypsin interfered with the fibrinolytic assay. Direct measurements of the ability of these extracts to activate plasminogen in the presence of trasylol (an inhibitor of plasmin and trypsin, but not of PA), as determined by the appearance of the plasmin heavy and light chains on gels under reducing conditions, also could not be done; the assay was not sensitive enough to measure the small amounts of activator involved, even when 125 I-plasminogen with a specific activity as high as 1.3 μ Ci/ μ g was used.

Finally, as shown in Table I B, protease inhibitors associated with control HEF cell extracts are present with nearly the same specific activities in the extracts from the serum-deprived cells and from the cells after they have been in serum-supplemented medium again for 4 h and therefore cannot account for the apparent serum-induced PA changes observed.

Thus the increase in cell-associated PA levels induced by the removal of serum from C57Bl/6 or HEF cells is energy-dependent, requires RNA and protein synthesis, and presumably results from increased PA production, rather than from PA accumulation or adsorption or from changes in cell-associated protease inhibitors. Furthermore, it cannot be accounted for by a shift from secretion of PA to intracellular accumulation of PA, since secretion is active and measurable in the absence of serum. Fig. 8 shows PA secreted by cells deprived of serum for 4 h. By 16 h (not shown) the amount of secreted PA has increased on all days except days 8-10, where it had already reached saturation levels in the medium by 4 h. Thus, PA levels in the serum-free medium increase rather than diminish with time, indicating that removal of serum does not block PA secretion.

The decrease of PA upon serum-readdition seems to represent repression of PA. As discussed above and shown in Table I B, cell-associated protease inhibitors are not important in the change. The use of medium supplemented with 10, 1, or 0.1% of either native or acid-treated

FCS (Fig. 12) to replace serum-free medium after 16 h shows that acid-treated serum is just as effective as native serum in bringing about the deinduction within 4 h after serum readdition, although it is depleted of about 90% of its protease inhibitors. We do not understand the transient increase in apparent PA immediately after the addition of acid-treated serum.

As shown in Fig. 12, the final depression of PA activity (after 4 h) is less in cells with the 1 and 0.1% serum supplements as compared to cells with the 10% serum supplement, for both the native and the acid-treated sera. Thus, the extent of PA depression is dependent upon the concentration of serum added back, but not upon its content of protease inhibitors. These results suggest the presence in serum of some other regulatory effectors, such as hormones, which modulate cellular PA synthesis.

DISCUSSION

Our results show that the cell-associated PA activity of low-passage normal embryo fibroblasts is not uniformly low throughout their life in culture. On the contrary, fibroblasts from a number of different species develop variously elevated levels of intracellular PA as they approach and attain confluence, and gradually lose this activity as they pass confluence. In all cases, however, even the

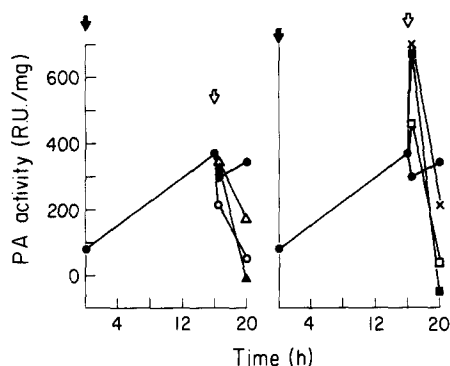


FIGURE 12 Serum effect on cells deprived of serum for 16 h. C57Bl/6 cells plated at 2×10^6 cells/plate were TD-washed the next day and put into 10 ml/plate of serum-free medium. Sample plates were scraped before the addition of serum-free medium (solid arrow), after 16 h in the absence of serum (empty arrow), and at 15 min and 4 h after a medium change to new serum-free medium (●), medium with 10% FCS (▲), 1% FCS (○) or 0.1% FCS (△), or medium with 10% acid-treated FCS (■), 1% acid-treated FCS (□), or 0.1% acid-treated FCS (×).

peak PA activities of normal low-passage fibroblasts remain well below those of their virally or spontaneously transformed counterparts.

A different situation prevails for the two Balb/3T3 clones studied. Although the Balb/3T3 line is generally regarded as "normal", the cells of both clones tested attain much higher peaks of PA activity at confluence than any of the low-passage embryonic mouse cells tested, and they maintain this peak activity indefinitely beyond confluence. In this regard, our findings with Balb/3T3 do not agree with those reported by Chou et al. (5) who found that the PA of Swiss 3T3 declined after the cells reached confluence. Furthermore, fresh MSV transformants of the Balb/3T3 clones have lower cell-associated PA activities than the untransformed cells. We cannot provide an explanation for these observations, but we think that this anomalous behavior of cell lines, as compared to low-passage cells, is worth noting. Differences in the behavior of cell lines and primary cultures with respect to PA production have been investigated extensively by Rifkin and Pollack (23), and were also noted by Mott et al. (15) and by Jones et al. (8).

Our main interest has been in analyzing the regulation of PA in normal low-passage embryo fibroblasts. We have considered the interplay of cellular metabolism, activator production and secretion, activator adsorption, and cell-produced or adsorbed inhibitors of fibrinolysis. The increase of PA levels in cells maintained in serum appears to represent changes in PA synthesis. It requires active cell metabolism, and, so, is directly influenced by the level of serum supplementation of the culture medium and by the growth rate of the culture. Other possible explanations for the PA increase, such as changes in intracellular PA storage, cell-surface bound secreted PA, and cell-associated inhibitors of fibrinolysis, were also considered. At least in the C57Bl/6 cells, secreted PA patterns are similar to cell-associated PA patterns over a 10-day period, an observation which agrees with previous findings that PA is rapidly secreted from many cells as it is made (21). It is, therefore, unlikely that the observed differences in cell-associated PA levels can be attributed to changes in PA storage patterns in the cells. They are also unlikely to result simply from an accumulation of secreted material adhering to the cell surface. Since the cell-associated PA level falls during the cells' stationary phase, some mechanism other than trypsinization and passaging would have to

be invoked for the loss of adsorbed PA after confluence, if such adsorption occurred. PA adsorption cannot be rigorously ruled out at present, however, because the appropriate experiments are prevented by technical difficulties. Changes in the levels of cell-associated inhibitors of fibrinolysis also cannot account for the observed changes in cell-associated PA levels of growing cells.

In cells deprived of serum, PA synthesis appears to go unchecked or even be stimulated for 16–20 h. The observed PA increase is energy-dependent and requires ongoing RNA and protein synthesis; it is not clear whether an increased level of synthesis must be induced. The increase in cell-associated PA in serum-deprived cells results from changes in PA production rather than from changes in PA secretion, since secreted PA levels increase over the same 16-h period during which the cell-associated levels increase. Over-production of PA stops by 20–24 h, and a gradual decline in PA occurs at this time. If serum is resupplied to the cells at 20 h, the apparent PA level of the cells falls precipitously to zero, and only over the next 24 h do the cells begin to recover their baseline PA levels. The extent of the decline in PA is dependent upon the concentration of serum added but not upon its content of acid-labile protease inhibitors. This would suggest that some as yet unidentified acid-stable serum component exerts a negative regulatory effect on PA production.

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This article is dedicated to Professor Keith R. Porter, on the occasion of his 65th birthday. Professor Porter, we salute you as scientist, teacher, and friend, and we wish you many happy returns of the day.

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