Modulation of the Plasminogen Activator Activity of a Transformed Cell Line by Cell Density

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Received 15 April 1982/Accepted 2 August 1982

The effects of variations in cell density on the expression of the plasminogen activator activity of a tumorigenic rat cell line were analyzed. At low cell densities, the plasminogen activator activity per cell was high and independent of cell density. As the cell density increased, the plasminogen activator activity per cell decreased until it eventually became inversely proportional to cell density. Inhibition of the plasminogen activator activity per cell by increases in cell density was not the result of the presence of a soluble inhibitor but seemed to require cell-to-cell contact. The $V_{\max}$ per cell for the activation of plasminogen changed at high cell densities, but the K_m did not change. This change in the $V_{\max}$ per cell was in part the result of a change in the catalytic rate constant for the conversion of plasminogen to plasmin. This was inferred from studies on the kinetics of inhibition of plasminogen activator activity by diisopropyl fluorophosphate as a function of cell density. For cells growing at high densities, the rate of inhibition was constant, exhibiting a second-order rate constant of $2.6 \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$. For cells growing at low densities, the plasminogen activator activity was inhibited at two different rates, one exhibiting a second-order rate constant of $2.6 \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$ and the other exhibiting a second-order rate constant of $9.4 \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$. We discuss the importance of cell density in assays of the plasminogen activator activity of cells, the use of this cell line to study the biochemical basis of the density dependence of plasminogen activator activity, and the density-dependent role of plasminogen activator activity in tumor formation and metastasis.

Many normal and abnormal physiological processes requiring extracellular proteolysis are thought to be mediated by plasminogen activators (PAs) that cleave plasminogen to the active protease plasmin. These physiological processes can be categorized in general as involving cell migration (17), tissue remodeling (18), or fibrinolysis (30). The employment of PAs in these physiological processes may be a reflection of the ubiquitous presence of the zymogen plasminogen in all body fluids and the presence in all cells of PAs with highly regulated activity (24, 25).

Recently, much attention has been focused on the role of PAs in tumor formation and metastasis. Levels of PA activity in numerous transformed cell lines are enhanced, compared with levels in normal-cell counterparts (for review, see reference 23). Increased levels of PA activity in cells have been correlated with the ability of cells to grow in agar (16, 21), the ability of cells transformed by tumor viruses to form tumors in nude mice (22), and the ability of malignant melanoma cells to form tumors (6). Some normal cells have high levels of PA activity (1-3, 28). For some clonal isolates of tumorigenic cell

lines, no correlation between levels of PA activity and malignant transformation has been observed (8, 15, 27, 31). Studies of a human epidermal carcinoma that metastasizes with a high efficiency into the organs of chicken embryos have shown that anchorage independence and serum independence inversely correlate with metastatic ability and tumorigenicity but directly correlate with enhanced levels of PA activity (19, 20).

These discrepancies among reports on the correlation between enhanced levels of PA activity in cells in cultures and the ability of those cells to form tumors and metastasize may be the result of variables in the conditions under which PA activity of cells in cultures was assayed. One variable is the number of times a cell line has been passaged. A human epidermal carcinoma cell line has been shown to lose progressively both its PA activity and its ability to form tumors and metastasize as a function of the number of passages in vitro (20). Another variable is the medium used for growth. In serum, protease inhibitors comprise approximately 10% of the total plasma protein concentration (9). Variations in serum protease inhibitors occur among

species and, depending upon the physiological state of an individual animal, even within species. A third variable is plasminogen. There are numerous forms of plasminogen, and some are more easily activated than others (20a; H. Y. Liu, S. W. Peltz, M. L. Schmitz, and W. F. Mangel, submitted for publication). A fourth variable is cell density. PA activity has been shown to vary with cell density (5, 26). A fifth variable is the assay for PA activity. The most widely used assay for PA is the solubilization of ^{125}I -fibrin (29), which, under some conditions, has been reported not to be a quantitative assay (11).

We have begun to reexamine some of the variables in assaying the PA activity of cells in cultures by a highly quantitative assay we developed (12, 13). This assay is based upon the new active-site titrant for plasmin, 3',6'-bis(4-guanidinobenzoyloxy)-5-[N'-(4-carboxyphenyl)thioureido]spiro[isobenzofuran - 1 - (3H), 9'-[9H]xanthen]-3-one, abbreviated FDE for fluoresceindiester (14). In this study, we analyzed the effect of cell density on the PA activity of a clonal subline, RT4-71-D2, from a neurotumor, RT4, which was induced by the subcutaneous injection of ethylnitrosourea into newborn BDIX rats (10, 11). The cells are tumorigenic when injected into syngeneic BDIX rats. Our results show that cell density is indeed a major variable in assays of the PA activity of cells and thus must be taken into account in any study correlating levels of PA activity of cells with transformation parameters. Analysis of the biochemical basis for the variation in PA activity with cell density implies that one of the kinetic parameters that changes with cell density is the catalytic rate constant for the conversion of plasminogen to plasmin.

MATERIALS AND METHODS

Materials. The synthesis, partial purification, and initial characterization of FDE as well as its use as an active-site titrant of plasmin have been described previously (14). Stock solutions of 2.96 mM were prepared in redistilled dimethylformamide and stored at 4°C. Further dilutions (1:1,000) were made in phosphate-buffered saline (PBS) before use. PBS contained 0.137 M NaCl, 2.68 mM KCl, 8.08 mM Na_2HPO_4 , 1.47 mM KH_2PO_4 , 0.91 mM CaCl_2 , and 0.49 mM MgCl_2 (pH 7.2). Dog plasminogen was purified from fresh plasma by a modification of the method of Castellino and Sodetz (4) described by Leytus et al. (12). Diisopropyl fluorophosphate (DFP) was purchased from Aldrich Chemical Co. Immediately before use, DFP was diluted in culture medium.

Cell culture. The RT4-71-D2 cell line was a gift from N. Sueoka, University of Colorado, Boulder (10, 11). The cells were grown in tissue culture flasks (25 cm²; Lux Scientific Corp.) in a Forma Scientific Incubator

set at 37°C and containing a 5% CO₂ atmosphere. The complete culture medium was Dulbecco modified Eagle medium with 12.5% horse serum, 2.5% fetal calf serum, 100 U of penicillin per ml, and 110 µg of streptomycin per ml (GIBCO Laboratories). The cells were passaged twice weekly at semiconfluency with trypsin-EDTA in Hanks balanced salt solution (GIBCO). The number of cells in suspension and the number of cells in each microwell after an assay for PA activity were determined with a Coulter Counter.

Assay for PA activity. Aliquots (0.10 ml) of a cell suspension in complete culture medium were placed into microwells (Costar 96-well, flat-bottom tissue culture clusters with a 0.32-cm² growth area) 16 h before an assay for PA activity was performed. After this seeding period, the medium was removed, and the cells were washed three times with PBS. To each microwell, 0.05 ml of dog plasminogen in PBS was added. After incubation at 37°C for 30 min, the 0.05-ml aliquots were withdrawn and added to 0.05-ml aliquots of 2.96 µM FDE in PBS. After 10 min at 25°C, 0.9 ml of PBS was added, and the magnitude of the fluorescence burst was determined with a MPF-44A fluorescence spectrophotometer with a universal digital readout (The Perkin-Elmer Corp.). The excitation and emission wavelengths were 491 and 514 nm, respectively (bandwidth of each, 4 nm). The change in fluorescence (ΔF) was equal to the magnitude of the fluorescence in the sample cuvette minus the magnitude of the fluorescence in a reference cuvette containing the same concentrations of FDE and plasminogen but plasminogen that had been incubated in microwells without cells. The fluorescence spectrophotometer was standardized each day with a polymethacrylate block embedded with rhodamine B so that the relative fluorescence values in different experiments were comparable and a standard curve relating ΔF units to molar concentrations of plasmin could be used.

RESULTS

Relationship between cell density and PA activity per cell. To determine the relationship between cell density and PA activity per cell, we seeded microwells (0.32-cm² growth area) with 1,500 to 150,000 RT4-71-D2 cells. The medium was removed 16 h later, and the cells were washed three times with PBS and then incubated with 11 µM dog plasminogen for 30 min. The concentration of the plasmin that had formed was then determined by active-site titration with FDE, and the number of cells in each microwell was counted. The data in Fig. 1 are presented as initial velocity per cell for the activation of plasminogen to plasmin versus area per cell.

The amount of PA activity per cell could be dramatically influenced by cell density (Fig. 1). As the area per cell increased, there was an almost linear increase in the amount of PA activity per cell. The slope of the line then decreased until eventually the amount of PA activity per cell became independent of the area per cell. The PA activity per cell became independent of the area per cell at approximately the

FIG. 1. Relationship between PA activity per cell and cell density. Microwells (0.32-cm^2 growth area) were seeded with various numbers of cells, the cells were incubated with $11\text{ }\mu\text{M}$ dog plasminogen for 30 min, and the resulting plasmin was titrated with FDE, as described in the text. The initial velocities (atto-moles of plasmin per second) for the activation of plasminogen were normalized per cell and plotted against the growth area divided by the cell number.

same point that the cells ceased to become confluent. The range in PA activity per cell as a function of the area per cell was quite dramatic. The PA activity per cell of 10,000 cells was sevenfold greater than that for 150,000 cells growing in an area of the same dimensions.

Inhibition of PA activity at high cell density. The progressive decrease in PA activity per cell at high cell densities as the cell density increased could be mediated by the release of a soluble inhibitor. To determine whether a soluble inhibitor with an activity that was concentration dependent was released, we incubated 62,500 cells growing in microwells of the same size (0.32 cm^2) with samples of plasminogen ranging from 0.05 to 0.20 ml. The plasminogen concentration was also varied. After 30 min, the amount of plasmin formed was titrated with FDE. From the data, the Michaelis-Menten parameters were calculated and plotted as a function of the volume per cell (Fig. 2). The results show that neither the $V_{\max}$ nor the K_m changed as the volume per cell was varied.

The data in Fig. 2 imply that inhibition of PA activity per cell at high cell densities did not decrease as the cell density increased because a soluble inhibitor was released. If an inhibitor of PA or plasmin were released, the $V_{\max}$ would increase as the volume in which the assay was performed was increased because the concentration of the inhibitor would be decreased. To show that a soluble inhibitor of PA was not released from the cells during the 16-h seeding period, we took conditioned media from cells seeded at high density and used it to seed cells at low density. The initial velocities per cell for the activation of plasminogen were independent of the use of fresh or conditioned medium (data not shown). The experiment described in Fig. 2 also

eliminated the possibility that the decrease in initial velocity per cell at high cell densities as the cell density increased (Fig. 1) was the result of a decreasing plasminogen concentration. If a significant fraction of plasminogen bound to the cells, the K_m values (Fig. 2) would have increased as the volume per cell decreased. All of these data imply that density-dependent inhibition of PA activity is mediated not by the release of a soluble factor but rather by cell-to-cell contact.

Determination of the Michaelis-Menten parameters for the activation of plasminogen as a function of cell density. The variation at high cell densities in the initial velocity per cell for the activation of plasminogen as a function of cell density must be the result of changes in $V_{\max}$, K_m , or both. To determine which of these macroscopic kinetic constants varied with cell density, we seeded microwells of the same surface area (0.32-cm^2) with 5,700 to 62,500 cells. After attachment, the cells were incubated with various concentrations of plasminogen, and the initial velocities for activation were determined. The Michaelis-Menten parameters were calculated and are presented in Fig. 3 as a function of the area per cell. The results indicate that the $V_{\max}$ per cell varied at high cell densities as a function of cell density and that the K_m was independent of cell density.

Inhibition of PA activity by DFP. The decrease

FIG. 2. Michaelis-Menten parameters for the activation of plasminogen as a function of the assay volume per cell. Microwells seeded with 62,500 cells were incubated with 0.05, 0.10, 0.15, and 0.20 ml of dog plasminogen at concentrations of 2.2, 4.4, and $8.8\text{ }\mu\text{M}$. After 30 min, the resulting plasmin was titrated with FDE. The initial velocities (atto-moles of plasmin per second) were plotted against the plasminogen concentrations, and the Michaelis-Menten parameters were calculated with a computer program described by Cleland (7). The $V_{\max}$ values were normalized per cell. The lines for $V_{\max}$ and K_m indicate the average of the four data points.

FIG. 3. Michaelis-Menten parameters for the activation of plasminogen as a function of cell density. Microwells seeded with various numbers of cells were incubated with 0.20 ml of dog plasminogen at concentrations of 2.2, 4.4, and 8.8 μM . After 30 min, the resulting plasmin was titrated with FDE. The initial velocities (attomoles of plasmin per second) were plotted against the plasminogen concentrations, and the Michaelis-Menten parameters were calculated with a computer program described by Cleland (7). The V_{max} values were normalized per cell. The line for K_m indicates the average of the four data points.

in the V_{max} per cell at high cell densities as the cell density increased could be the result of a change in the PA concentration ($[E]_0$), a change in the catalytic rate constant for the conversion of plasminogen to plasmin (k_{cat}), or both:

$$V_{\text{max}} = k_{\text{cat}}[E]_0 \quad (1)$$

To determine which kinetic parameters were altered, we needed to directly assay the PA activity as a function of cell density. One approach to measuring PA activity directly is to assay its inhibition by DFP. DFP interacts irreversibly with the active-site serine residue in serine proteases to form a diisopropylphosphoryl-enzyme complex. To determine whether the PA activity of the RT4-71-D2 cell line could be inhibited by DFP, we incubated 30,000 cells for 30 min with various concentrations of DFP. After removing the DFP, the amount of residual PA activity was determined. All of the RT4-71-D2 cell PA activity could be inhibited by DFP (Fig. 4).

Kinetics of inhibition of PA activity by DFP as a function of cell density. Since RT4-71-D2 cell PA activity could be inhibited by DFP, a kinetic analysis of the inhibition by DFP as a function of cell density allowed us to determine whether $[E]_0$ or k_{cat} changed as a function of cell density. The inhibition of PA by DFP can be described as follows:

where E is PA, I is DFP, and EI' is the irreversibly inhibited diisopropylphosphoryl-PA complex. The rate of inhibition by DFP is derived from the following:

$$\frac{d[E]}{dt} = -k_2[E][I] \quad (3)$$

where $[E]$ is the concentration of PA, $[I]$ is the concentration of DFP, and k_2 is the second-order rate constant for inhibition. When the concentration of DFP does not change during the assay,

$$k_{\text{obs}} = k_2[I] \quad (4)$$

where k_{obs} is the apparent first-order rate constant for inhibition at a specific DFP concentration. Under those conditions, equation 3 can be integrated to yield:

$$\ln \frac{[E]_t}{[E]_0} = -k_{\text{obs}}t \quad (5)$$

where $[E]_t$ is the concentration of PA at a given time (t).

To determine the kinetics of inhibition by DFP of PA activities of cells at high and low densities, we seeded microwells with 60,000 and 10,000 cells. After attachment, the cells were incubated with fresh medium with or without 2 mM DFP. After various intervals, the medium was removed, and the cells were incubated with 11 μM

FIG. 4. Inhibition of PA activity by DFP. Microwells were seeded with 30,000 cells. The medium was removed 16 h later, and the cells were incubated at 25°C with 0.10 ml of fresh medium containing the indicated concentrations of DFP. After 30 min, the medium with DFP was removed, and the cells were washed with PBS and then incubated with 11 μM dog plasminogen. The resulting plasmin was titrated with FDE 30 min later. Control experiments were performed in an identical manner but with fresh medium without DFP.

FIG. 5. Kinetics of inhibition by DFP of the PA activity of cells at different densities. Microwells (0.32-cm² growth area) were seeded with 10,000 (○), or 60,000 (●) cells. The medium was removed 16 h later, and the cells were incubated with fresh medium containing 2 mM DFP. After the indicated intervals at 25°C, the medium with DFP was removed, and the cells were washed three times with PBS and then incubated with 11 μ M dog plasminogen. The resulting plasmin was titrated with FDE 30 min later. Control experiments were performed in an identical manner but with fresh medium without DFP.

dog plasminogen for 30 min. The amount of plasmin formed was then titrated with FDE. The results are shown in Fig. 5, in which the percent PA activity is plotted against the time of incubation with DFP.

The rate at which DFP inhibited PA activity of cells growing at high density differed from the rate for cells growing at low density. For cells growing at high density, the rate of inhibition was constant, with a k_{obs} of $6.08 \times 10^{-5} \text{ s}^{-1}$ ($t_{1/2} = 173 \text{ min}$). For cells growing at low density, the PA activity seemed to be inhibited by DFP at two rates, one exhibiting a k_{obs} of $19.9 \times 10^{-5} \text{ s}^{-1}$ ($t_{1/2} = 58 \text{ min}$) and the other exhibiting a k_{obs} of $6.08 \times 10^{-5} \text{ s}^{-1}$. By repeating the experiments described in Fig. 5 at different DFP concentrations, we obtained the second-order rate constants for inhibition by DFP. These data are shown in Fig. 6, in which the apparent first-order rate constants are plotted against the DFP concentrations. The slopes of these lines can be used with equation 4 to calculate values for k_2 of 9.4×10^{-2} and $2.6 \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$. Thus, the catalytic rate constant of the PA activity of the RT4-71-D2 cell line changed as a function of cell density.

DISCUSSION

The PA activity per cell of the tumorigenic cell line RT4-71-D2 can be severely dependent upon

cell density. At low cell densities, the PA activity per cell was high and independent of cell density. As the cell density increased, the PA activity per cell decreased until it eventually became inversely proportional to cell density. Cell-to-cell contact seemed to be required for density-dependent inhibition, as no diffusible inhibitor whose activity was concentration dependent could be detected. The density-dependent variations in the PA activity per cell of cells at high densities were the result of changes in the V_{max} and not the K_m for the activation of plasminogen.

Our experiments on the inhibition of PA by DFP imply that the catalytic rate constant for the activation of plasminogen by the PA activity of the RT4-71-D2 cell line can change as a function of cell density. This is based on the observation that the PA activity of cells growing at high densities was inhibited by DFP at two rates, one with a k_2 of $9.4 \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$ and the other with a k_2 of $2.6 \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$. Thus, the changes in the V_{max} for the activation of plasminogen by the PA activity of cells at high

FIG. 6. Determination of second-order rate constants for the inhibition of PA activities of cells growing at low and high density. Microwells were seeded with 10,000 or 60,000 cells. The medium was removed 16 h later, and the cells were incubated with 0.10 ml of fresh medium containing the indicated concentrations of DFP. At five successive 6-min intervals, the medium containing DFP was removed, and the cells were washed with PBS and then incubated with 11 μ M dog plasminogen. The resulting plasmin was titrated with FDE 30 min later. Control experiments were performed in an identical manner but with fresh medium without DFP. The data were plotted as logarithm (percent PA activity) against time. The slopes of those lines are plotted here against the DFP concentration. Data from the initial rate of inhibition of the PA activity of cells growing at low density (○) and data from the rate of inhibition of the PA activity of cells growing at high density (●) are shown.

densities can, in part, be attributed to changes in k_{cat} . These data cannot be used to deduce whether the PA concentration of cells at high densities also changes or whether at low densities there are two different PAs. We now know, however, the conditions under which the PA activity of cells at low densities is inhibited by DFP at two different rates, and thus, we should be able to inhibit and radiolabel either activity individually for further studies.

Our observations on the density dependence of PA activity are consistent with models for tumor formation and metastasis. For a tumor to grow, many processes must occur simultaneously. Outward growth of a tumor requires tissue remodeling at the tumor surface and thus presumably extracellular proteolysis. Cells at the surface of a tumor are at low density and therefore have high levels of PA activity. For a tumor to grow, it must obtain an internal blood supply. Internally, high levels of extracellular proteolytic activity would be deleterious, and cells are at high density where their PA activity is low. Metastatic cells may leave a tumor at the surface, where they are at low density and thus have high levels of PA activity. Penetration of physical barriers such as blood vessels may occur at low cell density and be mediated by high levels of PA activity.

Variations in PA activity as a function of cell density have been reported by others. Chou et al. (5) reported that actively growing 3T3 mouse cells seeded at low density exhibit a high level of PA activity which progressively decreases as the cells become confluent. This diminution in PA activity was shown to be the result of a decrease in PA secretion, because intracellular levels of PA are independent of cell density. In contrast, the secretion of PA by simian virus-transformed 3T3 mouse cells is high and independent of cell density. Rohrlrich and Rifkin (26) reported that the amount of cell-associated PA activity of normal, low-passage, embryonic fibroblasts from a number of species and from two transformed clones of a BALB/3T3 mouse cell line increases as the cells approach confluence. The low-passage cells lose PA activity after reaching confluence, whereas the 3T3 cells retain it indefinitely. Because the assay conditions for these studies were different from ours, our results are not directly comparable to theirs.

The experiments on cell density that we present in this study define a major variable in assays of the PA activity of cells growing in cultures. The discrepancies among reports on the correlation between the PA activity of cells in cultures and the ability of those cells to form tumors and to metastasize may solely reflect differences of cell densities in the assays of the PA activity of cells in cultures. For example, the

PA activity per RT4-71-D2 cell of 10,000 cells was sevenfold greater than that of 150,000 cells (Fig. 1). If those tumorigenic cells were assayed at high densities, there would appear to be a lack of correlation between enhanced levels of PA activity and tumorigenicity. Additional experiments are now being performed to determine if the density dependence that we observed is characteristic of other cells and, if so, whether the degree of dependence is a unique characteristic of a cell line.

ACKNOWLEDGMENTS

We thank Pin Pin Yang for technical assistance.

This research was supported by Public Health Service grant CA 25633 from the National Institutes of Health.

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