Effect of Cell Density on Growth Rate and Amino Acid Transport in Simian Virus 40-transformed 3T3 Cells

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ABSTRACT

Rate of proliferation and amino acid transport were assessed in simian virus 40 (SV40)-transformed 3T3 cells by measurements of growth rate quotient and L-serine uptake via System ASC, respectively. Growth rate and cell density of the cultures were varied by modifying: (a) the number of cells initially plated; and (b) the period spent by the cells in culture. The growth rate quotient of SV40 3T3 cells was not correlated with cell density. Sparse cultures exhibited marked fluctuations in their growth rate as a function of time, whereas, under comparable conditions, crowded cultures retained some form of growth control by density. Transport activity by System ASC decreased as a function of increased cell density following a complex trend described by a double-exponential equation. The density-dependent decrease in amino acid transport was not accompanied by a parallel change in the rate of cell proliferation. These results indicate that alterations in amino acid transport are not linked with cell growth and suggest that an increase in transport activity is not a prerequisite for an optimal rate of proliferation in SV40-transformed 3T3 cells.

INTRODUCTION

Cell density is known to control the rate of proliferation of cells in culture (7, 28, 30). The transport of small nutrients is also dependent on cell density. It decreases when cell density increases and vice versa (9, 15, 18, 19, 20, 34). The relationship between nutrient transport rate and cell growth is less clear. An increased membrane permeability for critical nutrients has been proposed as an essential event for optimal cell proliferation (13, 17). Indeed, increases in the uptake of low-molecular-weight nutrients are among the early events associated with the initiation of growth (1, 5, 6, 14, 23, 25, 26, 31), although some of these transport changes have been shown not to be causally linked with shifts between quiescence and proliferation (1, 4, 11, 24, 32).

Recent data from our laboratory provided evidence that the Na+-dependent transport systems devoted to translocate neutral amino acids, Systems A and ASC, exhibit a marked modulation of their activity by cell density in 3T3 cells and that this regulation is maintained in SV40-transformed 3T3 cells (2, 21, 22). These latter cells lose growth control by density, do not enter a stationary phase, and maintain high metabolic rates in crowded cultures (27). The persistence of a density-mediated regulation of nutrient transport accompanied by the loss of density-dependent control of cell growth offered the opportunity to investigate the relationship between cell proliferation and amino acid transport in a biological model whose proliferation rate was expected to be uncoupled from cell density. As a probe for evaluating changes in nutrient (amino acid) transport, we selected System ASC, a Na+-dependent agency which responds strikingly to cell density (2), being poorly affected by other factors or conditions (adaptive and hormonal controls) known to regulate nutrient transport in animal cells (12).

The results to be presented indicate that, in SV40 3T3 cells, the density-dependent changes in neutral amino acid transport (as assessed by measurements of activity of the Na+-dependent System ASC) are not accompanied by parallel changes in the rate of growth.

MATERIALS AND METHODS

Cell Cultivation. The source of SV40-transformed 3T3 cells was described previously (2). The cells were maintained in Dulbecco's modified Eagle's medium containing penicillin (100 units/ml) and streptomycin (100 μg/ml) and supplemented with 5% fetal cell serum. All cultures were kept in incubators at 37°C in a water-saturated 5% CO2 atmosphere in air and passaged twice a week. All measurements of cell counting and amino acid transport were made on subcultures resulting from SV40 3T3 cells plated onto 9-sq cm wells of disposable multiwell trays (Costar) and incubated for variable periods of time in 3 ml of growth medium. Initial subculture plating with different cell inocula required special care to ensure a uniform and even spreading of the cells before their attachment to the substrate (thus obtaining homogeneous population densities).

Transport Assay. Cell monolayers were washed with EBSSG and depleted of the cellular amino acid pool by incubating them in EBSSG for 30 min at 37°C (2). L-Serine uptake was measured under conditions approaching initial entry rates by System ASC as follows. After the depletion period, the cells were washed and incubated for 1 min at 37°C in 0.5 ml of EBSSG containing the 14C-labeled amino acid (final concentration, 0.1 μM) and 10 mM 2-methylamino-isobutyric acid to minimize L-serine uptake by System A (2, 31). Incubation was terminated by rapidly rinsing the cells 4 times with ice-cold EBSSG. Acid-soluble pools were extracted with 10% trichloroacetic acid and counted in a liquid scintillation spectrometer. The cells were dissolved in 0.5 N NaOH and assayed for protein by the method of Lowry et al. (16).

Cell Counting. Cells were detached by trypsinization from each 9-sq cm well, and the resulting suspension was counted in a Bürker hemocytometer after proper dilution. Duplicate cell counts on each suspension from 3 culture wells (partners of the 3 culture wells on which amino acid transport was measured) were performed for each experimental condition leading to different densities. No fewer than 400 to 500 cells were scored for each counting. Counts from triplicate seedings differed by less than 10% among replications throughout the experiments. Proliferation rates were estimated by the growth rate quotient Q (10) as calculated by the equation:

\[ Q = (N_n - N_0)/\Delta t 1/N_{n-1}, \]

where \( N_n \) is cell number at the final counting, and \( N_{n-1} \) is cell number at...
the penultimate counting, and \( \Delta t \) is the time elapsed between the 2 countings (in hr). This equation does not contain log terms, thus avoiding the flattening of the data which occurs along the log axis in the graphical presentation of log-containing expressions. The use of \(^{3}H\)thymidine incorporation into DNA as an index for cell growth was hindered by the large error introduced by the transport step in cell populations of largely different densities in pulse experiments (20).

RESULTS

Proliferation Rate versus Density in SV40 3T3 Cell Cultures. The experimental design adopted to alter the rate of cell proliferation and to attain the desired cell density of the cultures consisted of a combination of 2 procedures whose variable parameters were: (a) the number of cells at the initial plating; and (b) the period spent by the cells in the same medium after plating. The initial plating corresponds to the number of cells attached to the substratum at 5 hr after their inoculation into the well. The measured plating efficiency was over 90%. An example of the results obtained with this approach is presented in Chart 1. With an initial plating of a high number of cells (3.6 \( \times 10^6 \) cells/sq cm, Chart 1a), the growth rate quotient of the population did not change up to about 45 hr after seeding and then decreased to very low values. Approximately 70 hr after the seeding time, when the cells were crowded (culture density, \( 3.5 \times 10^5 \) cells/sq cm), the growth stopped (Q = 0) and the cells started to detach from the substratum. With lower plating densities (1.7 \( \times 10^4 \) cells/sq cm, Chart 1b), the growth rate quotient increased up to a maximum around 45 hr after seeding and decreased slowly thereafter. At about 70 hr after cell inoculation, the culture density was around \( 2 \times 10^5 \) cells/sq cm. With cultures started by a very low cell plating (2 \( \times 10^3 \) cells/sq cm, Chart 1c), the growth rate quotient remained low for about 45 hr when a critical point was reached, after which pronounced alterations in the growth rate were recorded. A maximum was attained after 53 hr (culture density, 0.9 \( \times 10^4 \) cells/sq cm) followed by a rapid decrease leading to a low Q value at about 70 hr (culture density, 1.2 \( \times 10^4 \) cells/sq cm). These results indicate clearly that growth rate and cell density are not correlated in a simple manner in SV40 3T3 cells grown under the conditions adopted in our experiments. The recorded fluctuations of the rate of cell proliferation as a function of the initial plating conditions and of the time spent by the cells in culture were well reproducible. They offered the opportunity to investigate in some detail the effects of proliferation and density on amino acid transport in these cells and to attempt a discrimination between these 2 regulatory processes.

Amino Acid Transport, Cell Proliferation, and Cell Density. Chart 2 shows data from experiments in which inocula of increasing numbers of SV40 3T3 cells were seeded into partner culture wells and allowed to grow for variable time intervals as follows: 22 hr (Chart 2a); 44 hr (Chart 2b); and 53 hr (Chart 2c). The activity of System ASC, as estimated by L-serine uptake under appropriate conditions of transport assay (see "Materials and Methods"), decreased as a function of increased cell density (following a complex exponential trend) at all the growth intervals selected for transport measurements. This result is to be contrasted by the variable profiles of the growth rate within the corresponding time intervals. In Chart 2, a and b, the growth rate quotient increased markedly as a function of cell density whereas in Chart 2c it decreased.

When all the data of L-serine uptake by System ASC were plotted against cell density, they were best fitted by a single curve (Chart 3a), irrespectively of the parameter (initial cell plating density, period of cell growth) that has been varied to attain the final density of the culture. The curve was described by a double-exponential equation, whose parameters were calculated imposing a 90% confidence limit (8). A similar plot constructed for growth rate quotients against cell density yielded scattered data. Dispersion of the data was higher for sparse than for crowded cell cultures (Chart 3b).

DISCUSSION

Untransformed 3T3 cells growing in culture are subject to density regulation of growth (7, 28, 30). The results recounted

![Cell Density, Growth Rate, and Amino Acid Transport](chart.png)
in this paper show that some form of modulation by density of the rate of proliferation is retained, at least transiently, in SV40-transformed 3T3 cells (Chart 1). This is particularly true for subconfluent cultures, whereas sparse cultures exhibit marked fluctuations in their growth rate quotients as the incubation proceeds in a defined, unchanged medium. The latter behavior suggests that, in sparse SV40 3T3 cells, factors other than density play a role in modulating the proliferation rate of cell population. These factors are likely to include the number of cells initially plated, the time spent by the cells in the same culture conditions (growth period), and perhaps the production of endogenous self-addressed growth factors (29). In the same biological model (SV40 3T3 cells), density-dependent transient deceleration of growth has been revealed by total serum withdrawal (33); moreover, density-dependent growth-inhibiting processes occurred following serum step-down versus serum addition to the culture medium (4). These results and those presented in this paper provide some evidence that the loss of growth control by density in SV40 3T3 cells is likely to be incomplete.

In SV40-transformed 3T3 cells, a reduction of amino acid transport was associated with an increase of cell density (2, 21, 22). As shown in Chart 2, a pronounced decrease in neutral amino acid transport by System ASC was not accompanied by a parallel change in the proliferation rate of cell population, but again it decreased as a complex function of density (Chart 3). Depending on the initial cell-plating density, amino acid transport and growth rate quotient of SV40 3T3 cells followed comparable trends only in cells incubated for relatively long periods (53 hr in our conditions), whereas the 2 phenomena changed in opposite directions (i.e., growth rate quotient increased and amino acid transport decreased) in cells incubated for shorter periods of time (44 hr or less). These results clearly indicate that changes in amino acid transport are not strictly

Chart 2. Proliferation rate and amino acid transport in SV40-3T3 cells versus cell density. The cells were initially plated over a 20-fold range of increasing densities and each set of subcultures was allowed to grow for 22 (a), 44 (b), and 53 (c) hr. Growth conditions were as described in Chart 1. For each growth time interval, the growth rate quotient \( R \) has been calculated by the equation given in the text, and L-serine uptake (A, initial rate) was measured as follows. After a 30-min depletion, the cells were washed and incubated for 1 min at 37° in EBSSG containing the labeled amino acid (final concentration, 0.1 mm) and excess 2-methylaminoisobutyric acid (see text). Computer-drawn curves for L-serine uptake versus cell density were obtained by a double-exponential equation of the type \( y = Ae^{-at} + Be^{-bt} \) when a 90% confidence limit for the final estimates of parameters was imposed. Computer-drawn curves for growth rate quotient versus cell density were the best fitting to the experimental points according to a logarithmic equation of the type \( y = A + B \ln x \).

Chart 3. Density-dependent changes of amino acid transport and cell growth rate. In a, all the data of L-serine uptake of Chart 2 are plotted versus cell density. A double-exponential equation of the type presented in the legend to Chart 2 best fitted these data as indicated by the value of its residual variance (the lowest among those found for other nonlinear regressions tested). In b, all the values of growth rate quotient of Chart 2 yield scattered data when plotted against cell density.
linked with the rate of cell growth in this biological model and suggest that an increase in transport activity is not a prerequisite for optimal cell proliferation. They also imply that cell growth does not regulate amino acid transport by System ASC in SV40-transformed 3T3 cells, as it does in normal cells in culture [e.g., lectin-activated lymphocytes (33)]. This Na⁺-dependent agency whose activity is markedly dependent on cell density (2) suffers no interferences by such widely distributed regulatory processes as adaptive and hormonal controls (12). The transport of neutral amino acids by System A, as assessed by L-proline and glycine uptake, was also found dependent on cell density in SV40 3T3 cells (2, 22). Its activity appeared not to be correlated with the growth rate of cell population, but the relevant results (not shown) are somewhat handicapped by the strong interference of adaptive regulation which, under the experimental conditions adopted, tend to mask the transport response under study. A lack of correlation between changes in transport of Site A-reactive amino acids and rate of cell proliferation has been reported by Robinson and Smith (24) in density-inhibited Shionogi 115 cells stimulated to grow by testosterone addition and by Bush and Shodell (44) in SV40 3T3 cells depressed in their growth rate by serum withdrawal. These results are related to other observations indicating that the transport of such nutrients as hexose and phosphate is uncoupled from the rate of cell growth (11, 32).

It has been suggested that a density-dependent modulation of nutrient transport in normal cells in culture is possibly related to cell-cell contacts (18). That this is unlikely for SV40-transformed 3T3 cells is shown in Chart 3, where the steeper part of the curve relating amino acid transport to cell density (maximal change in transport for minimal change in density) corresponds to very low cell densities and its steepness attenuates before microscopic evidence for visible contacts among the cells is established. It has been also proposed that an alteration in membrane transport of critical nutrients may result in differential growth rates of malignant cells (13). Again, our results seem to indicate that fluctuations of the growth rate of SV40 3T3 cells that occur in response to different cell-culturing conditions are not associated with corresponding changes in the rates of uptake of neutral amino acids by the Na⁺-dependent System ASC.

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