Proliferation Kinetics of Density-inhibited Cultures of Human Cells, 
A Complex in Vitro Cell System

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SUMMARY

Proliferation kinetics and cell cycle parameters have been measured in exponentially growing and density-inhibited (daily medium changes) stable-plateau phase cultures of Chang human liver (LICH) cells. The mean duration of all phases of the cell cycle among the cycling population was approximately doubled in density-inhibited cultures. About 50% of the population was effectively noncycling but still viable. The lower rate of proliferation was balanced by cell loss into the culture medium leading to a constant cell number and a cell turnover rate of 20% per day. Density-inhibited cultures of LICH cells possess a number of the kinetic characteristics of tumors in vivo and may provide a useful in vitro system for a study of the effects of some chemotherapeutic agents, protracted schedules of X-irradiation, and of interaction between the two.

INTRODUCTION

Exponentially growing cultures of established lines of mammalian cells have been widely used to determine the lethal effects of radiation and a wide variety of chemotherapeutic agents on cells. These cultures have the advantages of in vitro systems in that they allow accurate quantification of effects and the determination of precise dose response relationships. On the other hand, both normal tissues and malignant tumors in vivo are complex cell systems which do not fit a simple exponential growth model.

Hahn et al. (11) proposed that plateau or stationary-phase monolayer cultures of mammalian cells might be a more appropriate in vitro cell model for malignant tumors. They found that a small amount of proliferative activity and cell turnover occurred in plateau phase cultures of Chinese hamster cells. We have been studying the kinetics of cell proliferation in various growth phases of an established line of human liver cells (Chang). If the culture medium is renewed daily, these cells will eventually enter a stable plateau phase of growth in which the lowered rate of cell proliferation is quantitatively balanced by cell loss into the culture medium. We use the term "density-inhibition" of growth to describe this phenomenon, with no intention of implying a mechanism for the effect. In contrast to cultures allowed to reach stationary growth by medium exhaustion, proliferative activity and cell turnover rates are relatively high and, with daily medium changes, stable density-inhibited cultures with a constant cell number may be maintained indefinitely.

In this report we present a detailed analysis of the cell cycle and proliferation kinetics of density-inhibited monolayer cultures of Chang liver cells. These cultures represent a complex cell system that possesses several of the characteristics of in vivo systems, in particular a significant population of apparently nonproliferating but viable cells that are capable of unlimited cell division under optimal growth conditions.

MATERIALS AND METHODS

Cells used in these experiments were from a permanent line of human liver cells (Chang or LICH cells) established by Dr. R. S. Chang in 1954 from an explant of normal human liver and maintained in this laboratory since that time (3). These cells have epithelioid rather than fibroblastic morphology and retain certain functional characteristics of hepatic cells (14). They were grown as monolayers at 37°C in an atmosphere of 5% CO₂. The nutrient medium used was Eagle's minimal essential medium with Earle's balanced salt solution supplemented with 8.9% calf serum inactivated at 56°C for 30 min; nonessential amino acids; l-glutamine; penicillin, 50 units/ml; and streptomycin, 25 µg/ml. Stock cultures were maintained with regular medium changes in 75-ml Falcon flasks and were trypsinized and subcultured at low density at 7-day intervals so that the cells remained at all times in exponential growth.

For experiments, cells were grown as monolayers in replicate 25-sq cm plastic Falcon flasks. The flasks were initially seeded with a cell suspension providing approximately 8 × 10⁵ cells/sq cm of surface area. The nutrient medium was changed daily after the 1st day. Experiments utilizing cultures in exponential growth were performed on the 3rd day after seeding, at which time the mean cell concentration was approximately 2 × 10⁶ cells/sq cm of surface area. For density-inhibited cultures, the nutrient medium was renewed daily until the experiments were performed 10 to 14 days after seeding, at which time the cell concentration had reached a stable plateau of approximately 4 × 10⁵ cells/sq cm of surface area.
The proliferation kinetics were determined primarily by autoradiographic techniques. Cultures were pulse labeled for 15 min in medium containing TdR-3H with a specific activity of 2 Ci/mCi at a concentration of 0.5 µCi/ml. The cells were then washed twice with warm fresh medium and subsequently were incubated in medium containing unlabeled thymidine at a concentration of 5 × 10⁻⁴ M. Continuous labeling was carried out with TdR-3H at a concentration of 0.1 µCi/ml.

At the appropriate times after pulse labeling or continuous exposure to TdR-3H, the cultures were trypsinized and the cells were washed twice with balanced salt solution and placed in a hypotonic solution (0.95% sodium citrate) for 15 min. They were fixed in a solution of 3 parts methanol to 1 part glacial acetic acid, dropped onto clean wet slides, and allowed to air dry. For autoradiographs, the slides were dipped into Ilford G-5 liquid emulsion diluted 1 to 2 with distilled water. The emulsion was exposed for 5 days at 4°C, and the autoradiographs were developed and stained through the emulsion with hematoxylin and eosin. A minimum of 1000 cells from 20 to 30 random high-power fields were scored to determine the percentage of labeled cells. Cells in visible mitosis in these fields were counted for a minimum of 1000 cells from 20 to 30 random high-power fields. For the estimation of percentage labeled mitoses, at least 50 random mitotic figures were scored and labeled fractions were noted.

The biochemical separation and counting of radioactivity in DNA, RNA, and protein fractions was carried out by a modified Schmidt, Thannhauser, Schneider technique, as previously described (17).

RESULTS

Chart 1 shows the changes in cell density with time that occurred in LICH cell cultures maintained under differing nutrient conditions. After seeding at low concentration, the cells in both cases multiplied exponentially following a short lag period. When the nutrient medium was not renewed (lower curve), the cultures entered a plateau phase of growth at a density of 6 to 10 × 10⁴ cells/sq cm of surface area. This cell density was maintained for 3 to 5 days before cellular death occurred. When the nutrient medium was renewed daily; however, the cell density further increased until a new plateau was reached at approximately 4 × 10⁵ cells/sq cm of surface area. If daily medium changes were continued, these cultures could be maintained indefinitely with an approximately constant cell number. This report concerns this latter type of plateau phase (density-inhibited) culture.

Neither the rate of cell multiplication during exponential growth nor the final cell density at the plateau was significantly increased by renewing the nutrient medium at 12- instead of 24-hr intervals, by adding 10 instead of 5 ml of medium at each change, or by increasing the concentration of serum in the medium up to 20%.

The changes in the rates of DNA, RNA, and protein synthesis per cell during growth and entry into density inhibition are shown in Chart 2. Although all synthetic processes declined to some extent, the most marked decrease was in the rate of DNA synthesis, which consistently fell to 20% or less of the normal rate of cells in full exponential growth.

The duration of the various phases of the cell cycle was derived from PLM curves, such as those shown for exponentially growing and density-inhibited cultures in Chart 3 (22, 23). The mean duration of the S phase is estimated from the width at the midpoints of the ascending and descending slopes of the 1st wave of labeled mitoses, the G₂ phase (+1/2M) from 0 time to the midpoint of the 1st rise in labeled mitoses, and the total generation time from the midpoint of the 1st rise to that of the 2nd rise in labeled mitoses. The duration of G₁ is derived from the differences between the generation time and the other phases of the cycle. In this study, these parameters were actually estimated by a computer fit of the PLM data by Dr. A. J. Vallerone of the Statistical Unit, Institute Gustave Roussy, Villejuif, France. The values obtained, along with the other kinetic data, are given in Table 1. As can be seen, the plating efficiency of cells in stationary phase cultures declined by less than 10% compared with that of exponentially growing cells, indicating that most of the cells remained viable in terms of their potential ability for unlimited proliferation (colony-forming ability).

The cell turnover rate in density-inhibited cultures (Table 1) was estimated by hemocytometer count of the number of cells sloughed into the culture medium (floating cells) during 12- and 24-hr intervals. These estimates would be
inaccurate if significant lysis of sloughed cells occurred during these intervals. As a test of this possibility, all cell multiplication was arrested by irradiation of cultures with 10,000 rads, and the number of floating and attached cells were counted 24 hr later. All of the original population could be accounted for at this time, indicating that lysis of sloughed cells did not occur within 24 hr.

The data derived from PLM curve (Table 1) indicated that the S phase was considerably prolonged in stationary phase cultures. For confirmation of this finding, exponentially growing and density-inhibited cultures were pulse labeled with TdR-3H under identical conditions, and the grain-count distributions overlying S-phase cells were scored on autoradiographs. The mean ± S.E. grain counts were 35 ± 2.1 and 14 ± 1.2, respectively. These results indicate that the mean rate of DNA synthesis in S-phase cells of density-inhibited cultures, as measured by grain counts, was approximately 50% of that in exponential cultures, which is consistent with the PLM data.

As can be seen in Chart 3, the percentage labeled mitotic cells during the 1st wave of labeled mitosis in stationary cultures did not reach 100%. This was a consistent finding; the PLM never exceeded 80% in several experiments. This finding suggested that some cells were held in G2 for relatively long periods compared with the mean G2 time. For an estimate of the duration of G2 in this population, PLM experiments were performed in which the cultures were continuously incubated with TdR-3H. The mean results from 3 such experiments are shown in Chart 4. As all cells that pass from S through G2 into mitosis during the course of an experiment would be labeled, unlabeled mitosis would represent only those cells in G2 at the beginning of the experiment. As shown in Chart 4, 100% labeling had not occurred by 30 hr, indicating that some of the cells remained in G2 for 30 hr or longer before dividing. This finding is reflected in the G2 time estimate in Table 1.

Additional experiments were performed to estimate the size of the population of cells held for prolonged periods in G2 which were capable of proliferation. The protocol was similar to that in the above-described experiments except that, after 6 hr, the cultures were trypsinized and cells were seeded at low concentrations in fresh medium. Under these conditions, exponential cell multiplication begins after a 6- to 8-hr lag period. If a large number of the cells held in G2

![Chart 2. Change in DNA, RNA, and protein synthetic rates in cells during various growth phases. Nutrient medium was changed daily throughout the course of the experiment. The cultures were pulse labeled for 15 min with the appropriate labeled precursor: Δ, TdR-3H; O, uridine-3H; ©, leucine-3H; x, cell count.](chart2.png)

![Chart 3. PLM curves for exponentially growing (O) and density-inhibited (●) cultures. Cultures received a 15-min pulse of TdR-3H at Time 0.](chart3.png)

<table>
<thead>
<tr>
<th>Cell culture</th>
<th>Plating efficiency (%)</th>
<th>Pulse-labeling index (%)</th>
<th>Mitotic index (%)</th>
<th>$T_c$ (hr)</th>
<th>$T_{ci}$ (hr)</th>
<th>$T_s$ (hr)</th>
<th>$T_{si}$ (hr)</th>
<th>Growth fraction* (%)</th>
<th>Cell turnover (loss) per day$^*$ (%)</th>
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<tr>
<td>Exponential</td>
<td>60-80</td>
<td>35-40</td>
<td>2.5</td>
<td>28</td>
<td>10.5 ± 5.1$^*$</td>
<td>13.0 ± 4.5</td>
<td>4.0 ± 0.7</td>
<td>≈100</td>
<td>≈0</td>
</tr>
<tr>
<td>Density-inhibited</td>
<td>55-75</td>
<td>15-20</td>
<td>1.5</td>
<td>55</td>
<td>27 ± 14</td>
<td>19 ± 9</td>
<td>8 ± 10</td>
<td>≈50</td>
<td>≈20</td>
</tr>
</tbody>
</table>

$^*$ Cycling cells.
$^*$ Calculated by the method of Mendelsohn (20); see "Discussion."
$^*$ Estimated from measurements of number of cells sloughed into culture medium per day.
$^*$ Mean ± S.E.
were capable of dividing under optimal growth conditions, the PLM should decline after the lag period, as these G2 cells enter mitosis and dilute the labeled population. Some estimate of the size of this population could be made from the magnitude of this decline. In reality, the PLM remained at between 65 and 80% during the 10-hr interval after trypsinization and then rose, reaching 100% 15 to 20 hr later. These results indicate that, although a few G2 cells still moved slowly into mitosis, a large population of G2 cells capable of entering mitosis under optimal growth conditions but delayed or prevented from doing so under the conditions in density-inhibited cultures, did not exist. Most of the non-S-phase cells in these cultures were therefore in G1.

The change in percentage labeled cells in density-inhibited and exponential cultures during continuous incubation with TdR-3H is shown in Chart 5. The increase in labeling index during the 1st 15 to 20 hr in density-inhibited cultures could be interpreted as being linear, as indicated by the broken line. Density-inhibited cultures of LICH cells grew essentially as monolayers, with no evidence of multilayered sheets as occur with some highly tumorogenic lines. Cell loss appeared to occur singly, with no evidence of necrosis or of areas in which the entire sheet sloughed off into the medium. By microscopic inspection, however, density-inhibited cultures appeared to contain 3 cell populations, namely, a firmly attached dense monolayer, a 2nd loosely attached layer of rounded-up cells overlying the 1st layer, and floating cells. Preliminary experiments have been performed to determine the changes in percentage labeled cells in these subpopulations during continuous incubation with TdR-3H. Replicate density-inhibited cultures were incubated with TdR-3H, and the cell populations were separated and processed for autoradiography 2, 12, and 24 hr later. Floating cells were collected from the overlying medium. Serum-free medium was added to the culture, and the culture was shaken vigorously to detach the loosely attached rounded-up population; the firmly attached dense monolayer was collected by trypsinization. The results of 1 experiment are shown in Chart 6. The plating efficiencies of the cells in this experiment were: floating cells, 7%; loosely attached cell, 44%; and dense monolayer, 65%. In 3 such experiments, the initial labeling index was much lower among the floating and loosely attached cells; but a significant number of labeled cells had appeared in the loosely attached population by 12 hr and increased over the next 12 hr at essentially the same rate as did those in the monolayer. Labeled cells were not present in significant numbers in the floating population at 12 hr but were present at 24 hr.

DISCUSSION

The results indicate that density-inhibited cultures of LICH cells are a dynamic, metabolically active cell system. Although proliferative activity and DNA synthesis are considerably reduced, other synthetic rates continue at a relatively high level (Chart 2). In particular, the rate of protein synthesis per cell in the stable, density-inhibited cultures was 50% or more of that in cells during rapid exponential growth. The depression in uridine incorporation may be due primarily to decreased transport into the cell (6). The plating efficiency or the ability of individual cells to proliferate under optimal growth conditions also remained high in density-inhibited cultures (Table 1). The reduced rate of cell proliferation in density-inhibited cultures, leading to a cell turnover rate of approximately 20% per day under conditions of a steady-state balance in cell density, is due to 2 factors: (a) the length of the cell cycle in the cycling population was doubled in density-inhibited cells; and (b) a significant fraction of noncycling cells were present. We calculate from the data in Table 1 that about 50% of the cells in density-inhibited cultures were effectively
noncycling. First, the duration of the S phase was about 19 hr or 35% of that of the mean generation time (55 hr) of cycling cells, as determined from PLM data, whereas only 15% of all the cells in the culture were in the S phase at a given time (pulse-labeling index). Second, the ratio of labeled cells/total cells at equilibrium was 0.15 compared with the ratio of labeled mitoses/total mitoses of 0.28 ± 0.02 to 0.30 (Chart 3). The ratio of these ratios is an accepted method of calculating the growth fraction in a mixed population (20). On the other hand, the results in Chart 5 indicate that all cells in density-inhibited cultures had gone through a part of the S phase at least once within an 86-hr interval (time for 100% labeling plus S-phase time).

The preliminary data on the 3 subpopulations in density-inhibited cultures (Chart 6) offer further insight into the kinetics of this system and the nature of the noncycling population. Most proliferative activity, as measured by TdR-3H incorporation during continuous exposure, occurred in the basal, firmly attached population. The progressive appearance of labeled cells, first in the loosely attached and later in the floating populations, suggests that, following division, cells move up into the more superficial loosely attached population where they are held in G1 until they eventually slough off into the culture medium. This superficial layer may therefore represent the effectively noncycling population which derives from the proliferating basal layer and from which cell loss occurs. This would be consistent with the findings of Castor (2) that cell division in a multilayered population occurred primarily in the basal layer.

In density-inhibited cultures of LICH cells, the mean duration of all phases of the life cycle in the cycling population was approximately doubled, compared with those of exponentially growing cells. A similar increase in the mean duration of S and G2 was observed in density-inhibited cultures of Chinese hamster cells (11) and human hematopoietic cells grown in suspension in vitro (29). There was no evidence from the PLM curves of interruptions or discontinuities in the S phase such as had been described in certain cell systems (12). Most of the viable non-S-phase population in density-inhibited LICH cell cultures was in G1, which appears to be the case for many stationary populations in vitro and in vivo (9, 11, 21, 26, 29). Some cells capable of mitosis were, however, held up in G2 for very long periods of time (Chart 4), and under conditions of poor nutrition (medium exhaustion) the population of cells arrested in G2 may become more important (11, 18, 24, 30).

Variations in the duration of G1 similar to those we observed have been reported in a transplantable hepatoma grown in vivo (25).

These density-inhibited cultures should not be confused with the transient “stationary phase” which occurs with established cell lines in which the monolayer is allowed to reach a growth plateau or “saturation density” by medium exhaustion (1, 24, 31) (Chart 1). In such cultures the nutrient medium is never renewed after initial seeding of the cells, and the brief stationary phase before cell death is characterized by a much lower cell density and almost complete absence of proliferative activity (4, 11, 18, 27). Cells particularly sensitive to the contact inhibition of cell division, such as established mouse 3T3 cell lines, also appear on the basis of limited data to have very low rates of cell proliferation and turnover at stationary density (19), and the inhibited cells all appear to be arrested in G1 (21).

The inhibitory phenomenon in 3T3 cell cultures, however, differs significantly from that we observed with LICH cells. The proliferative activity during growth of individual colonies of 3T3 cells (as measured by TdR-3H uptake) occurs around the edges of the colony (8), while we have found that with LICH cells it is much more evenly distributed throughout the colony. The stationary density for LICH cells with regular medium changes was about 10 times higher than that for 3T3 cells (28) but was considerably lower than that for many permanent cell lines, as well as most transformed cells (19). The plateau phase in LICH cell cultures also differs from that usually associated with transformed or highly tumorigenic heteroploid cells in which high cell densities and multiple layering of cells occurs (7).

Do the kinetics we have observed in density-inhibited LICH cell cultures resemble those found in tumor systems in vivo? Like these cultures, the growth of most tumors appears to be associated with an accumulation of cells in a G1-like phase of the life cycle. With solid tumors, however, the decline in growth rate with increasing size appears to be due primarily to a decline in the growth fraction and increased cell loss rather than to a measurable change in the life-cycle parameters of proliferating cells (9, 13, 16). On the other hand, there is a strong correlation between the changes in life-cycle parameters associated with growth into density inhibition in LICH cell cultures and those found with the growth of ascites and leukemic cells in vivo (5, 10, 15, 26). In these tumors, the decline in growth rate is due primarily to a lengthening of all phases of the cell cycle, including S, rather than a decrease in the growth fraction. The PLM curves for large ascites tumors, compared with those for small, exponentially growing tumors, show changes similar to those found with LICH cells in vitro (5, 26).

Obviously, density-inhibited monolayer cultures are an imperfect model for human cancer. They do, however, offer
George F. Zinninger and John B. Little

all of the advantages of an \textit{in vitro} system, including the ease of handling, high plating efficiency, reproducibility, and ability to obtain quantitative data, while offering some of the kinetic characteristics of complex \textit{in vivo} systems. They would appear to be a useful model for a study of the effects of some chemotherapeutic agents, particularly S-phase-active agents of protracted schedules of X-irradiation, and of the interactions between the two.

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