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EXISTENCE OF AN UPPER-LIMIT TO ELONGATION OF THE PREREPLICATIVE PERIOD IN CONFLUENT CULTURES OF C3H/10T½ CELLS

David Miska and H. Bruce Bosmann

Department of Pharmacology and Toxicology and University of Rochester Cancer Center, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

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SUMMARY: Cultures of C3H/10T½ CL8 mouse embryo fibroblasts show a high degree of postconfluence inhibition of cell division in the absence of serum depletion. Confluent cultures had their serum concentrations increased from 3 to 10% at 6¼, 6½, 8, 15, 22, and 28 days postplating, stimulating synchronized DNA synthesis as measured by the uptake of [^{3}H]thymidine into acid-insoluble material. The prereplicative period, from serum increase to the beginning, or median, of synchronized thymidine uptake, increased with culture age. This movement into longer prereplicative times occurs in exponentially declining increments, indicating that an upper limit exists.

Much work has been done recently on defining the nature of cellular quiescence in vitro, and the conditions required for movement into, and out of, this state. The most widely used indicator of quiescence is a lengthening of the time it takes for cells to move from mitosis to the beginning of DNA synthesis relative to cycling cells. There is disagreement as to whether this indicates a slowing down of traverse through this phase (G_1), or if the extra time is required to prepare cells that have entered a discrete resting state (G_o) for reentry into the cell cycle (1).

A characteristic feature of normal, untransformed fibroblasts grown in vitro is their ability to show density-dependent regulation of growth (2). A number of explanations have been proposed, such as contact inhibition between cells (3), loss of mechanical tension at confluency (4), and diffusion, or nutrient, limitations (2,5).

Augenlicht and Baserga (6) and Rossini et al. (7) showed that confluent cultures of WI-38 human diploid fibroblasts, kept for increased periods in the quiescent state, acquired longer prereplicative times following a medium change However, quiescence in their system was achieved by allowing the cells to remain in the original medium used for plating, or by replacing it with medium containing a sharply reduced serum concentration. This suggests that nutrient depletion played a major role in the growth arrest seen in these cultures.

Reported here are results from a similar investigation using cultures of C3H/10T½ fibroblasts, a mouse embryo cell line that shows a high degree of

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postconfluence inhibition of cell division (8), even with frequent medium renewals during confluency that contain the same serum concentration used during the growth phase. Growth arrest in these cells is seen as being less dependent upon nutrient restrictions and may indicate a qualitative difference in their resting state from WI-38 cells. However, confluent cultures of C3H/10T½ are shown to express a similar lengthening of the prereplicative phase with increased culture age. There is also an apparent limit to progression into this state.

MATERIALS AND METHODS

C3H/10T½ CL8 mouse embryo fibroblasts were kindly provided by Drs. Charles Heidelberger and Sukdeb Mondal. Stocks were grown and maintained as described previously (9). Frozen cell stocks from passages 6 or 7 were thawed and seeded into 150-cm^2 Costar plastic tissue culture flasks containing Eagle's basal medium with Earle's salts, supplemented with 3% heat-inactivated fetal calf serum (Gibco). When the cultures were judged to be in the late growth-phase, they were rinsed with Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution and trypsinized with a 0.1% trypsin solution (2.5% stock trypsin from Gibco) made from the same salt solution, whose activity was quenched with serum approximately 6 min later. Samples were counted in a Coulter Counter, model ZB, and the cells were suspended in medium with 3% heat-inactivated fetal calf serum at 25,000 cells/4 ml, in a side-port reservoir bottle. The suspension was stirred with an umbrella stirrer (Fisher Scientific Co.) at 1 rpm, and 4-ml aliquots were dispensed into 60 mm dia Costar plastic tissue culture dishes using a Manostat repeatable pipetter. The medium was replaced every 3 days with 5 ml of the same. All cultures were maintained at 37° in a humidified incubator with a 5% CO_2 in air atmosphere. No antibiotics were used. The cells tested negatively for mycoplasmic contamination using Hoechst 33258 stain (American Hoechst Corp., N. Sommerville, N.Y.).

The cultures became confluent 6 days after plating. At 6¼, 6½, 8, 16, 22, and 28 days after plating, groups of 48 dishes were administered 0.39 ml serum, shifting the concentration of serum from 3 to 10%, to stimulate a wave of DNA synthesis. The last medium change for these groups, as well as their unstimulated controls was made 48 h earlier. The time course of DNA synthesis was determined at 3-h intervals after stimulation by measuring [^{3}H]thymidine (2 Ci/mmol, Amersham) incorporation into the acid-insoluble material of three stimulated and three unstimulated dishes, according to the method of Bertram and Heidelberger (8). Representative dishes were counted in order to calculate the cpm/1000 cells in the labeled dishes.

RESULTS

Postconfluent cultures of C3H/10T½ cells are highly growth-inhibited and show a synchronized wave of DNA synthesis upon being harvested and replated (8). In the experiment presented here, confluent cultures of C3H/10T½ cells were shifted from 3 to 10% serum at different times after attaining confluency. This resulted in synchronized waves of DNA synthesis with lengthened prereplicative periods, as measured from the time of serum step-up to the beginning or median time of incorporation (Fig. 1). Unstimulated controls had incorporation levels (not shown) throughout the 48 h that were similar in magnitude

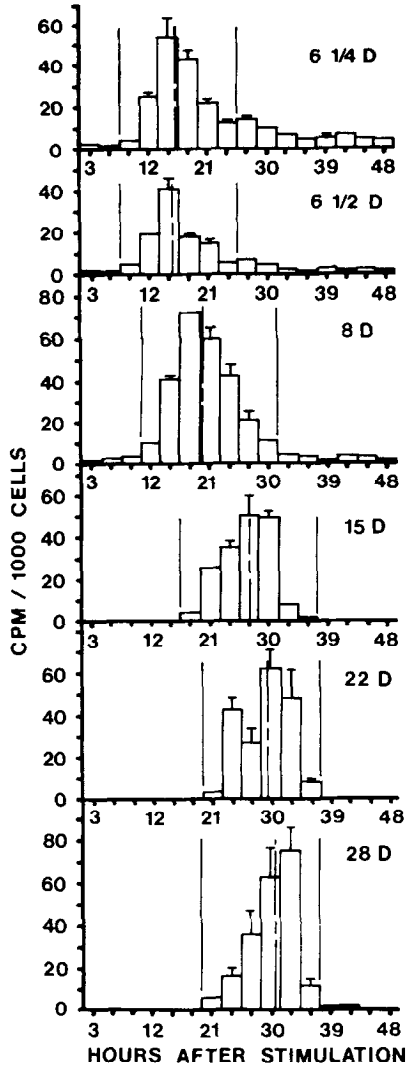

Figure 1. Incorporation of [^{3}H]thymidine into acid-insoluble material at 3-h intervals (30-min pulse) after the addition of 0.39 ml serum (shift from 3 to 10%) to confluent cultures. The culture age (days) upon stimulation is given beside each histogram. Each bar represents the mean cpm/10^3 cells from three dishes; standard errors greater than 1.0 are shown.

The times between the solid vertical lines on each histogram are periods for which the incorporation values represent a synchronized wave of stimulation relative to untreated controls (not shown). The median time for incorporation between these limits is also shown.

to those values for the incorporation periods in stimulated cultures preceding and following the synchronized wave of incorporation (delineated in Fig. 1 by solid vertical lines). Incorporation levels in the controls, and the pre- and postwave periods in stimulated cultures, decreased with culture age, an indication of higher levels of growth inhibition being attained.

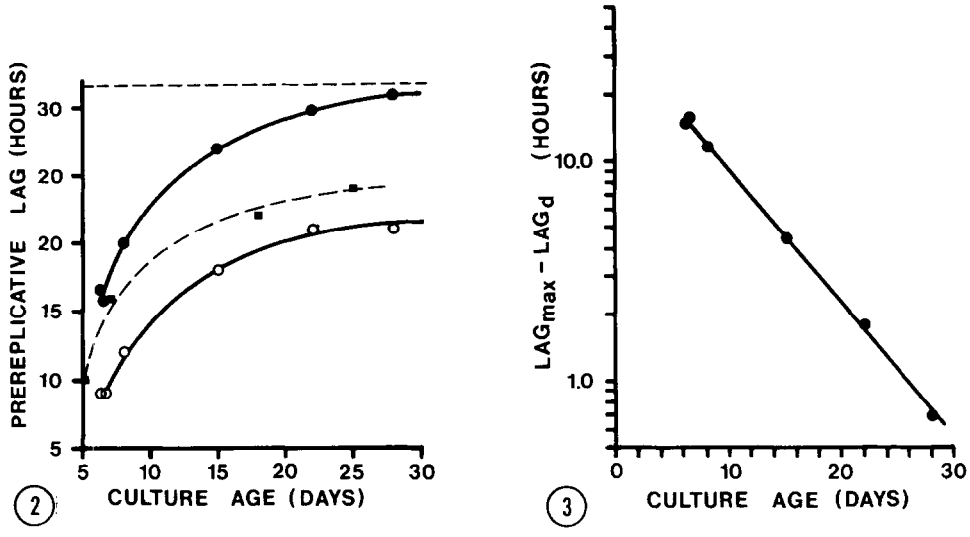

Figure 2. The median (●) and beginning (○) of synchronized [^{3}H]thymidine incorporation from each histogram in figure 1 relative to culture age at the time of serum addition. The dashed horizontal line is the asymptote to the median curve (LAG_{max} = 31.6 h), determined as described in figure 3.

Data from table 1 by Rossini et al. (7) are also plotted (■): quiescent WI-38 fibroblasts were stimulated to divide with fresh medium containing 10% serum at different times after plating. In this case, the ordinate represents time necessary to label half of the cells that are ultimately stimulated (autoradiography).

Figure 3. The median incorporation times from the histograms in figure 1 (LAG_d) were each subtracted from the limit to elongation of the prereplicative period (LAG_{max} = 31.6 h) that gave the best linear fit when these differences were plotted semilogarithmically. The half-time approach to this limit under the given culture conditions is 5.0 days.

The relationship of culture age to prereplicative lag time is presented in Figure 2. The curves for the median and beginning times of incorporation follow a similar curvature.

Figure 3 shows that the median incorporation times exponentially approach a maximum (LAG_{max} = 31.6 h) with a half-time of 5.0 days. The length of G_1 during the growth phase is the shortest interval between M and S that would be expected--5.4 h for Swiss 3T3 cells grown in 10% serum (10), but with a similar population doubling time as for C3H/10T½ cells grown in 3% serum. Extrapolating this line to times before confluency (about 6 days) would be inappropriate because it would indicate a virtual elimination of the G_1 phase.

DISCUSSION

These data indicate that confluent cultures of C3H/10T½ cells can acquire lengthened prereplicative times, following a mitotic stimulus, when kept for increased periods in the confluent state. A similar phenomenon has already

been described using cultures of WI-38 cells kept quiescent by nutrient deprivation (6,7), and a temperature-sensitive cell cycle mutant of Chinese-hamster ovary cells (11). Increased lag times before mitosis have been observed following partial hepatectomy (12), and isoproterenol stimulation of salivary glands (13) in old versus young rats. This has been suggested to reflect the duration that the cells have spent in a nonproliferative state before the mitotic stimulus (14). Increased prereplicative lag times may therefore be a general occurrence in cultures that become quiescent by different means, and may be representative of cellular behavior in vivo.

The culture medium in our experiments was changed regularly, maintaining the same serum concentration used during the growth phase. Medium taken from confluent cultures allowed cell growth in freshly seeded cultures (data not shown), arguing that these cells are either capable of acquiring longer lag times during confluence in the absence of a nutrient deficiency, or that the requirement for nutrient factors is different in quiescent cultures.

These results further show that there is a maximum attainable prereplicative lag that is approached in exponentially declining increments with increased culture age. A limit to the "deepening" of the quiescent state in WI-38 cultures is also evident in Figure 2, where data from Table 1 by Rossini et al. (7) are plotted.

This steady-state level of preparedness for cell division may indicate that a basal cell size, protein mass, level of a specific protein, or some other cellular activity is necessary to maintain a viable state. Cell size has a role in the emergence of serum-stimulated 3T3 cells from the quiescent state, with, in general, larger cells expressing shorter lag times before entry into the S-phase (15). A concentration-dependent elongation of the lag phase by cycloheximide has been shown in quiescent cultures of 3T3 cells when administered with, or shortly after, serum stimulation, and was correlated with a decrease in protein synthesis (16,17). Cellular protein has been shown to decrease in human fibroblasts as the quiescent period is extended (6,18). The mean half-life of protein in such cultures is approximately 20 h (19). It has also been suggested by a number of authors that a critical amount of a short half-life protein (approximately 2 h) is required for cellular entry into the S-phase (16,17,20).

The movement of quiescent cultures of C3H/10T½ cells into longer prereplicative times approaches the maximum with a half-time of 5 days under the given culture conditions, considerably longer than the 2- or 20-h half-lives mentioned. However, protein levels are a function of synthesis as well as of degradation. If a threshold requirement for protein exists before the cells

can enter the S-phase, the temporal approach to this level has been shown to be a function of both processes (17).

An important question that remains unanswered is whether the percentage of cells in this system that respond to stimulation decreases with lengthened periods of confluency, as in the human cell cultures (6,7). An age-related decrease in the proliferative fraction is seen in regenerating livers (12) and isoproterenol-stimulated salivary glands of rats (13). However, no loss of growth potential was seen with Chinese hamster ovary cells kept at their non-permissive temperature (11). Further experiments will be necessary to categorize the proliferative response of C3H/10T½ cells.

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