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Influence of Cell Density on Growth Inhibition of Human Fibroblasts *in vitro*.* (32142)

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The division cycle of phase II human fibroblasts during logarithmic and postlogarithmic growth *in vitro* was studied previously(1,2). It was shown that the percentage of cells synthesizing deoxyribonucleic acid (DNA) between subcultivation and confluency was maximal during the second day after subcultivation. This fraction declined later, eventually reaching zero when the population became crowded(3,2). An increasing proportion of cells was delayed in the G2 period of the cell cycle(2) as the cultures became confluent. The previous work was done always with the same initial inoculum under conditions where high mitotic indices were obtained together with a relatively long logarithmic growth.

The present work extends the previous findings by studying the influence of cell density on the division cycle and analysing in more detail the periods of the cell cycle affected by cell growth inhibition.

Materials and methods. Cell culture. A cell strain (HEB) derived from human embryonic fibroblasts(2) was used, maintained in Eagle's minimal essential medium(4) supplemented with 10% calf serum, streptomycin (50 μg/ml), penicillin (100 U/ml) and aureomycin (50 μg/ml). Methods used for subcultivation are the same as described by Hayflick and Moorhead(5). Cell counts were done electronically in a Celloscope (AB Lars Ljungberg & Co., Sweden) as described by Santen(6). The terminology used to designate how the cells were subcultivated is the following: a 2:1 split means that cells contained in 2 dishes were plated into one new dish, a 1:1 split means that the cells from 1 dish were plated into 1 new dish, a 1:2 split means that cells from 1 dish were plated into 2 new dishes, a 1:4 split means that cells from 1 dish were plated into 4 new dishes.

Autoradiography. Tritium-labeled thymidine (H3-TdR) with a specific activity of 1.9 C/mM was used at a concentration of 0.01 μC/ml, previously shown to be nontoxic(2). For the experiments cells were pooled and subcultivated into new 60 mm plastic Petri dishes containing coverslips. The techniques used in the autoradiographic procedure have been described(1). To determine the amount of labeled interphases, 1000 cells were analysed. Mitotic indices and the percentage of labeled mitosis were obtained from the analysis of 3000 cells. Mitotic indices are expressed as percentage mitoses of the total number of cells.

Results. Influence of inoculum on amount

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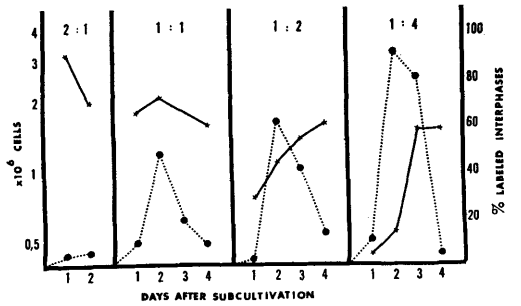

FIG. 1. Cell counts (×——×) plotted semilogarithmically and percentage labeled interphases plotted arithmetically (● . . . . . ●) at different days after subcultivation with 2:1, 1:1, 1:2 and 1:4 splits.

of cells synthesizing DNA. Cultures in the 17th passage were subcultivated so that one group resulted from a 2:1 split, another group from a 1:1 split, a third from a 1:2 split and a fourth from a 1:4 split. H3-TdR was added to 4 cultures in each group at the time of subcultivation and to 2 cultures from each group on successive days thereafter. At each day after subcultivation the duplicate cultures labeled for 24 hours at this time were fixed. Duplicate cultures labeled at subcultivation were also fixed at the end of the experiment in each group. Cells were counted each day after subcultivation up to the time when the cultures were in stationary phase. Fig. 1 shows the cell counts and the percentage labeled interphases in each group. The cell counts obtained in the first day after subcultivation correspond well to what would be expected from the different split ratios. The counts obtained at the time of confluency seem to show that in spite of the different inocula the cultures had the same density at the time when DNA synthesis approached zero. It can also be seen that the maximum number of cells synthesizing DNA during a 24-hour period, increases as the inoculum decreases. Analysis of cultures labeled continuously from the time of subcultivation to the time of confluency (Table I) reveals that the total proportion of cells synthesizing DNA during that period also increases as the inoculum decreases. The comparison between the data presented in Table I and Fig. 1 shows that in the 1:1 split 21% of the cells synthesized DNA during the time between subcultivation and confluency, while during the first day after subcultivation 10% of the cells synthesized DNA, during the second day 48%, during the third day 20% and 10% during the fourth day. The same comparison made in the 1:2 split shows that a total of 75% of cells synthesized DNA between subcultivation and confluency, while 62% synthesized DNA during the second day, 42% during the third day and 14% during the fourth day. In the 1:4 split 97% of the cells synthesized DNA between subcultivation and confluency, 11% during the first day, 91% during the second day and 80% during the third day. The sum of the proportion of cells synthesizing DNA during the different 24-hour periods after the 1:1, 1:2 and 1:4 split ratios was 88, 118 and 182% respectively. Thus these sums are larger than the total proportion of cells entering DNA synthesis during the time between subcultivation and stationary stage in the respective experiments (Table I). In the 1:2 and 1:4 split experiments the sums are also higher than the total population. This must mean that in each of the 3 cases some cells either entered the S period at least twice of detached after going through the S period. It is evident from the cell counts after the 1:1 split that cell detachment took place in this group.

Initiation of the division cycle after subcultivation. Confluent 10th passage cultures were split 1:4 and H3-TdR was added at the time of subcultivation. Duplicate cultures were fixed at different intervals thereafter starting one hour after subcultivation. Cultures were analysed for the percentage labeled interphases, percentage non-labeled mitoses and the mitotic indices (Table II). It can be seen that mitoses are present from the first hour on, after subcultivation. The latter are all unlabeled up to the 6th hour

TABLE I. Percentage Labeled Interphases Found After a Continuous Labeling from Subcultivation to Vesting Stage. Same experiment as in Fig. 1.

Split ratio	%
2:1	5
1:1	21
1:2	75
1:4	97

TABLE II. Time of Appearance of Labeled and Unlabeled Cells at Different Times After Subcultivation, in Presence of H3-TdR. Results are based on analysis of 6,000 cells for each hourly sample.

Hours	% labeled interphases	% non-labeled mitoses	Mitotic index
1	2	100	.7
2	5	100	.4
3	5	100	.8
4	5	100	1.1
5	7	100	.7
6	7	100	.9
7	7	95	1.4
8	7	92	1.2
9	6	73	1.5
10	6	50	1.2
11	6	47	1.6
12	7	50	1.3
13	9	16	.5
14	8	23	.6
15	11	21	.6
23	38	0	.6
24	39	0	.6
25	45	0	1.0

after subcultivation, and continue to register until the 15th hour after subcultivation, the 50% point being reached at the 10th hour after subculture. The percentage labeled interphases is very low until the 15th hour after labeling when it starts to increase.

The delay in the G2 period during cell crowding. Todaro, Lazar and Green(7) have found that a mitotic wave could be obtained in stationary cultures, with medium change. We used this observation to find out if the mitotic wave obtained among confluent cells also in our system is made up of cells stimulated from the G2 period or from the G1 period(2). Fresh medium was added to confluent cultures in the 9th passage and at the same time H3-TdR was also added (0 hour). Duplicate cultures were fixed at different intervals thereafter and the percentage of labeled and non-labeled mitoses, mitotic indices and percentage labeled interphases were determined (Fig. 2). The initial mitotic indices and percentage labeled interphases show that the cultures were in resting stage at the time of medium change. The mitotic indices start to increase 18 hours after addition of fresh medium reaching a peak 30 hours after medium replacement, then declining. Unlabeled mitoses were first registered 24 hours after feeding the cultures and the peak observed in the mitotic index curve coincides with the peak of unlabeled mitoses. Since the cultures were continuously in the presence of H3-TdR the unlabeled dividing cells reached mitosis without going through S and hence were cells that initiated their cycle from the G2 period. The percentage of labeled interphases started to rise during the first 6 hours after addition of new medium and reached a peak coinciding with the mitotic index peak.

Discussion. It was previously shown that there is a progressive decline of DNA synthesis in human fibroblastic cultures approach-

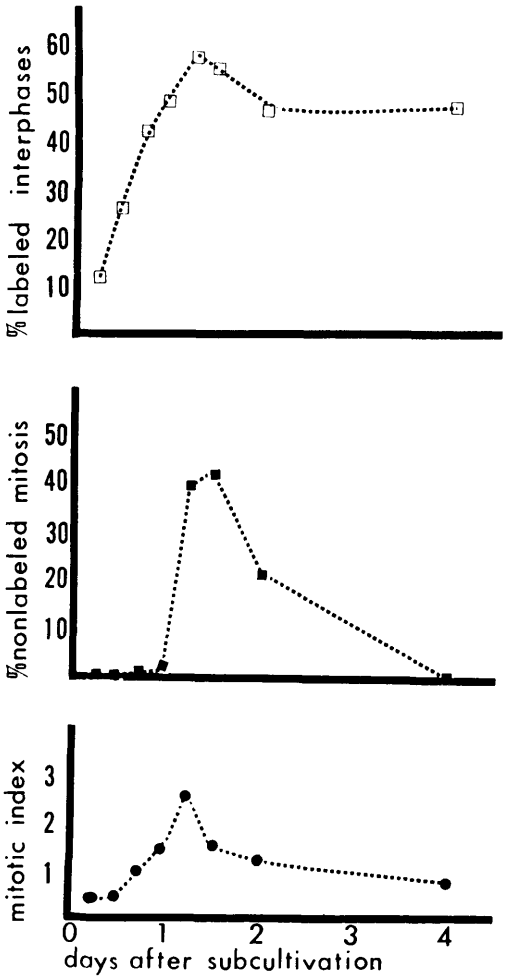

FIG. 2. Mitotic index, % non-labeled mitoses and % labeled interphases at different times after medium change in stationary cultures.

ing confluency(3,2). This is an expression of the inhibition of the cell division cycle in crowded cultures (cell cycle inhibition). The present paper supplies further evidence for the latter phenomenon and shows that the density at which cells are plated influences the capacity of the population to enter the division cycle (Fig. 1 and Table I). When one compares the total proportion of cells that synthesized DNA during the time between subcultivation and confluency (Table I) with the proportion of cells that synthesized DNA during the different 24-hour periods after the 1:1, 1:2 and 1:4 splits (Fig. 1), one must conclude that in each case there were cells which either entered the division cycle at least twice, or detached from the substrate after going through the S period. Also in each case there is a fraction of cells that does not divide, varying in size with different inocula. Although the present data do not include a determination of the extent of cell detachment after the 1:2 and 1:4 splits, previous cell counts made from the supernatants of routinely maintained cultures (1:2 splits) have shown that cell detachment is minimal under similar conditions.

It therefore seems likely that several S periods per cell rather than excessive detachment were responsible for the discrepancy between the percentage of labeled cells in the 24-hour samples and the samples obtained after continuous exposure to H3-TdR.

Regardless of the different inocula, a ceiling existed beyond which the cells did not go on in the division cycle. The same finding was described with growth curves for L cells(8). The same ceiling was reached in the present work between 1.5-2 $\times$ 10^6 cells/50 mm Petri dish. When the culture vessels were seeded with a higher cell inocula (2:1 split) the cell number decreased to the level of the ceiling evidently because a proportion of the originally attached cells detached. The highest density reachable by the same cell strain can vary with the culture conditions. We could obtain higher densities with HEB cultures carried without interruption since it was started *in vitro*(2) than in cultures obtained later from frozen cells. Since the only other parameter that varied in these cases was the lot of calf serum used, the latter can also be implicated in the observed difference.

It should be pointed out that our experiments were done under conditions where cell cycle inhibition takes place. The dynamics of cell growth under abolition of cell cycle inhibition by a perfusion system as described by Kruse and Miedema(9) remains to be studied.

The present work shows that after subcultivation cells initiate their division cycle from the G1 and G2 periods (Table II). The latter group was to be expected from the previous findings that some cells are delayed in the G2 period when the population approaches confluency. The mitoses appearing during the first hour after subcultivation could be from cells that were already in mitosis prior to the subculture as it was previously found that mitosis lasts about 1 hour in HEB cultures(1). But all mitotic cells appearing without label later than the first hour are probably from cells that were in the G2 period at the time of subcultivation. At the 10th hour 50% of the mitoses were labeled. This corresponds very well with the average S + G2 periods (6 + 4 hours) obtained previously in HEB cultures (1). These findings imply that the lag phase seen constantly during the first 24 hours after subcultivation in the growth curves obtained with this type of cells cannot be due to a complete standstill in the cell cycle of the entire population(1,2) since some cells resume the division cycle as soon as they have attached to the substrate.

The data plotted in Fig. 2 supply further evidence on the delay of cells in G2 period during cell cycle inhibition. The shape of the mitotic wave obtained after medium change is identical to the one described by Todaro, Lazar and Green(7). It is interesting that in the 3T3 line used by these authors all cells initiated their cycle after subcultivation or medium change from the G1 period(10,7). The curve representing non-labeled mitoses (Fig. 2) shows that the mitotic wave obtained with the addition of fresh medium to confluent HEB cultures is

made up of cells arrested in the G1 as well as the G2 period. It is seen that as long as 2 days after labeling there are still unlabeled mitoses. Since H3-TdR was present during this entire period, these cells must have been retained for at least 2 days in the G2 period. The fraction of cells delayed in the G1 and G2 periods does not seem to be constant, because in experiments parallel to the one represented in Fig. 2, we obtained a variable amount of cells stimulated from the two periods. It could be that the delay in G1 and G2 during cell cycle inhibition depends on the amount of cells present by chance in one of these periods at the time the critical density is reached, which in turn might be correlated with the size of the original inoculum, the presence of fresh serum in the medium or other unknown factors.

Summary. The influence of cell density on DNA synthesis of human embryonic fibroblasts was studied by measuring the incorporation of tritium-labeled thymidine with autoradiographic techniques. It was shown that as the inoculum decreased an increased proportion of cells entered the S period during the time between subcultivation and confluency. The amount of labeled cells after subcultivation and after induction of division in stationary cultures was measured. Evidence was obtained that cells in crowded cultures are delayed in the G1 and G2 periods.

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Effects of Saline Loading on Distal Renal Tubular Sodium and Water Reabsorption.* (32143)

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There is considerable evidence to indicate that saline loading in the dog results in a depression of proximal tubular reabsorption of sodium and water(1-3). Clearance studies have suggested that there may be decreased distal tubular reabsorption as well(4,5), as demonstrated in rat micropuncture experiments(6,7). However, direct micropuncture observations on distal tubular effects in the dog have not yet been reported.

To elucidate the effects of volume expansion by saline loading in the distal nephron of the dog, stop flow studies performed early and late in the course of a progressive saline diuresis were compared with regard to the sodium/creatinine clearance ratio (U/P Na/U/P creatinine) and U/P creatinine values. The former expression compares the clearance of sodium with the clearance of the glomerular substance creatinine, and represents the fraction of the filtered load of sodium excreted in the urine. The latter expression is an index of tubular water movement without regard to solute movement.

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