The Cell Cycle During Growth Inhibition of Human Embryonic Fibroblasts *in Vitro* (37523)

A. MACIEIRA-COELHO AND L. BERUMEN

Institut de Cancérologie et d'Immunogénétique, Hôpital Paul-Brousse, 94800—Villejuif, France

Human embryonic fibroblasts cultivated *in vitro* have been found to accumulate in the G_2 period of the division cycle when the growth curve reaches a plateau (1, 2). Subsequently it has been claimed that stationary cultures of WI-38 cells are arrested in the postmitotic G_1 period (3). This phenomenon was reexamined using different methods which showed that although human fibroblasts stop dividing in G_1 they are delayed in the G_2 period when they approach stationary phase.

Materials and Methods. One human embryonic lung fibroblastic line was used in the present studies between the 5th and 10th passage. The methods used to start and maintain the cultures were described elsewhere (4). Eagle's MEM supplemented with 10% foetal calf serum and 50 μg/ml aureomycin was used. Before the experiments cells were pooled and evenly distributed into new plastic 60 mm Petri dishes. Cell counts were made with an electronic cell counter (5) after suspending the cells in phosphate buffered saline (PBS) free of Ca and Mg supplemented with 0.25% trypsine and 2% ED TA. The technique used for autoradiography has been previously described (1). Samples of 3000, 100, and 1500 cells/slide were used to determine mitotic indices, % labeled mitoses and % labeled interphases respectively. Under the experimental conditions used, cells spending the whole S period in the presence of 0.01 μCi/ml tritium labeled thymidine (H^3-TdR) with a specific activity of 2 Ci/m*M* had 70 grains/cell. Microspectrophotometric analysis was performed as previously described (6).

Results. The amount of cells accumulating in G_2 was measured by three different methods: in the first experiment cell kinetics were analysed on various days after subcultivation, through exponential growth up to resting phase; the second and third experiments followed the events after the cultures were stimulated in resting phase either by medium change or by replating at lower density. In the last experiment microspectrophotometric analysis was performed between subcultivation and resting phase and after stimulating cell division with a medium change.

Analysis of the cell cycle between subcultivation and resting phase. After subcultivation, 0.01 μCi/ml H^3-TdR was added to a group of identical cultures; then duplicate samples were fixed each second hour during a 10 hr period. This procedure was repeated every day until the growth curve reached a plateau. The % labeled mitoses determined during each continuous labeling is illustrated in Fig. 1. If all the cells had spent the same time in the G_2 period the percent labeled metaphases would be represented by a vertical line since 100% of the metaphases would appear simultaneously labeled. However, as cells are heterogeneous the curve will have a slope depending on the difference between the shortest and the longest G_2 period. Cells with the shortest G_2 will appear as the first labeled metaphases while cells with the longest G_2 will be the last to show up as labeled metaphases. When all cells that were in G_2 at the time of labeling divided, the curve of the percent labeled mitoses will reach the 100% level, the average G_2 period being the time between addition of H^3-TdR and the moment when 50% metaphases are labeled. Hence the area confined by the vertical lines

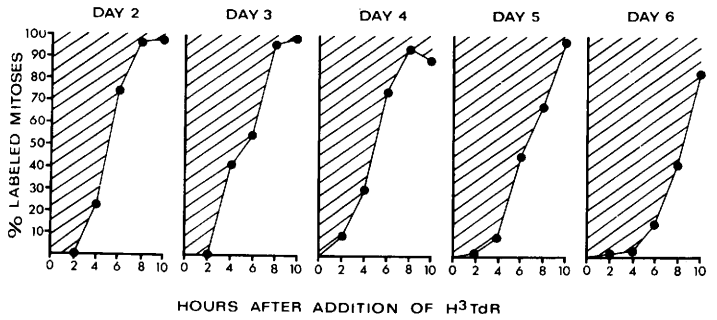

FIG. 1. Percent labeled mitoses during 10-hr periods following the addition of H^3-TdR on different days after subcultivation.

at 0 hr and the curves representing the percent labeled metaphases will correspond to the number of cells in G_2 during that period (shaded areas in Fig. 1). The surface of the shaded areas, representing the G_2 compartment for each day after subcultivation, was measured with a planimeter and plotted in Fig. 2, together with the growth curve and the percent labeled interphases found during each 10-hr labeling. Results show that the G_2 compartment is slightly increased during lag phase (1st day after subcultivation) and is smallest when growth is most active (2nd day). Then the amount of cells in G_2 increases progressively until the growth curve reaches a plateau.

Cell kinetics after stimulation of resting phase cultures. 1. Cultures were pooled, subcultivated and counted each day. When a plateau was reached (2 consecutive days with the same cell counts) the medium was changed in half of the cultures and 0.1 μCi/ml H^3-TdR was added to all cultures (0 hr). In each group (with and without medium change), 4 cultures received 0.02 μg/ml colcemide and were fixed 4 hr later; this procedure was repeated every 4 hr during 24 hr. The mitotic indices, % labeled interphases and % non-labeled mitoses were determined for the group kept in resting phase (no medium change) (Fig. 3A) and for the group stimulated with a medium change (Fig. 3B). Results show that only about 5% of the interphases were labeled during the entire experimental period in cultures kept in resting phase, and that nonlabeled mitoses appeared with an irregular pattern during the whole experimental period. In the group stimulated with a medium change the percent labeled interphases started increasing after the 12th hour and the mitotic index after the 20th hour. The percent of nonlabeled mitoses decreased progressively during the 24 hr fol-

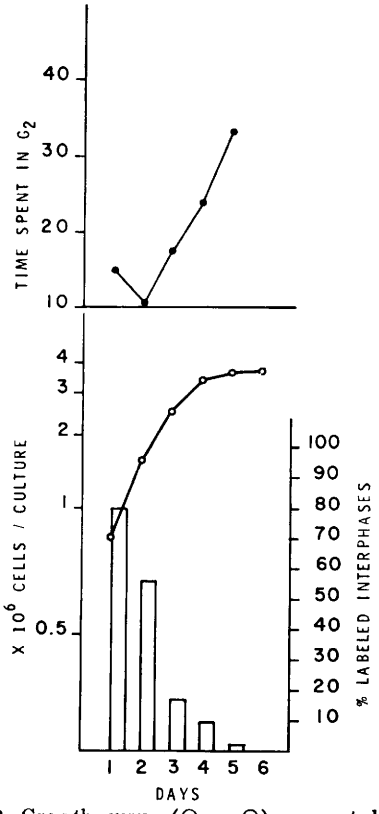

FIG. 2. Growth curve (O——O), percent labeled interphases (bars) and surface (●——●) of the shaded areas illustrated in Fig. 1, in the same experiment.

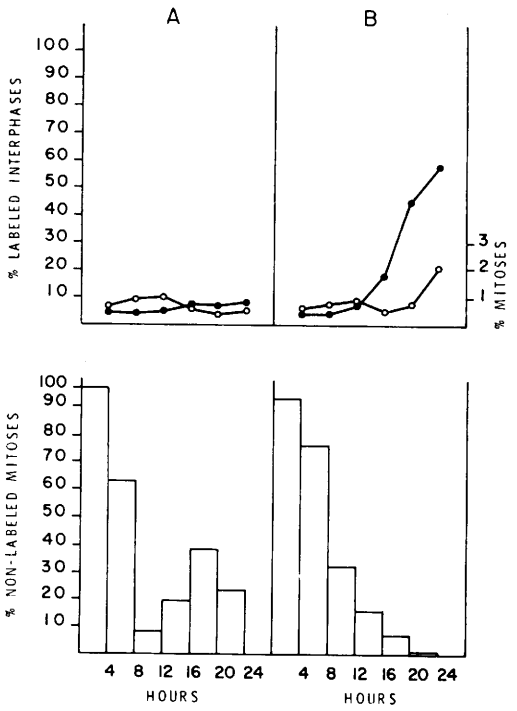

FIG. 3. Percent interphases (●——●), mitotic index (○——○) and percent nonlabeled mitoses (bars) during 4 hour periods following the addition of H^3-TdR to cultures that had reached resting stage (A) and to identical cultures where medium was renewed (B) at the time of H^3-TdR addition (0 hr).

lowing the medium change; this percent is identical in both groups during the first 4 hr; however, during the two subsequent 4-hr periods, the percent of nonlabeled mitoses is higher in the stimulated cultures.

2. Combined cultures were subcultivated and left until a plateau was reached (the same cell count on two consecutive days). After the cells were again combined and subcultivated, 0.01 μCi/ml H^3-TdR was added to all cultures. Starting at the time of subcultivation and each fourth hour thereafter, colcemide was added to 4 cultures which were fixed 4 hr later; this was done up to the 12th hour after subcultivation (Fig. 4). Mitotic indices were below 1% and most of the mitoses were nonlabeled up to 12 hr after subcultivation. Nonlabeled mitoses decreased progressively during the experimental period. As expected, these results are identical to those obtained in the stimulated group of the preceding experiment (Fig. 3B), since in both cases the cultures were in resting phase at the beginning of the experiment; in one they were stimulated by addition of fresh medium and in the other by subcultivation.

Microspectrophotometric measurements. Figure 5 illustrates the growth curve of human fibroblasts each day after subcultivation. On the 4th day when the cultures were in resting phase the medium was renewed. A net increase in the cell number was observed 48 hr after changing the medium which is to be expected since the peak mitotic index is seen between 24 and 30 hr after stimulation (7). Under these conditions cultures again enter resting phase 48 hr after the medium renewal (7). Microspectrophotometric measurements of the DNA content in Feulgen stained cells were done on the 1st, 3rd, 5th, and 8th day (Fig. 6) which corresponds respectively to dividing cultures, resting phase cultures, and cells that had been stimulated 24 hr and 48 hr previously. Proliferating cultures, 24 hr after subcultivation (1st day) have 43% of the cells with a G_2 DNA content. Proliferating cultures 24 hr after being stimulated in resting phase (5th day), have 21% of the cells with a G_2 DNA content.

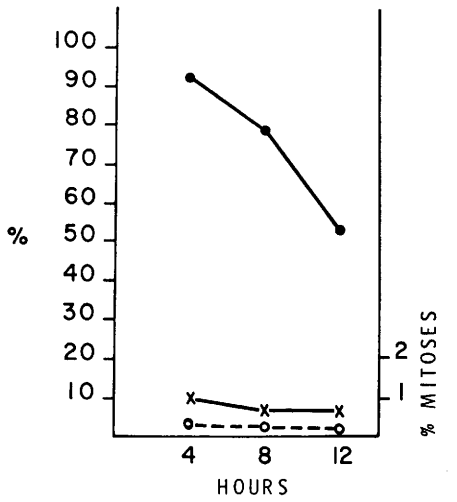

FIG. 4. Percent nonlabeled mitoses (●——●), mitotic index (x——x) and percent labeled interphases (○- - -○) during 4-hr periods following the subcultivation of resting stage cultures. H^3-TdR was added at the time of subcultivation (0 hr).

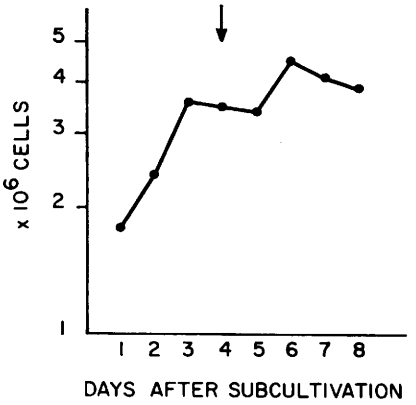

FIG. 5. Growth curve of human embryonic fibroblasts. Microspectrophotometry was performed on the 1st, 3rd, 5th and 8th days (Fig. 6). The arrow indicates the time when medium was changed.

Cultures in resting phase, (3rd and 8th day) have 5 and 8% of the cells in G_2.

Discussion. A block in the G_2 period of the division cycle was first shown in mouse epidermis (8). G_2 delayed cells can also be found *in vitro* and we have previously described that human fibroblasts approaching resting phase accumulate between the end of DNA synthesis and the initiation of mitosis. The results reported above indicate that in fact human fibroblasts have an increased G_2 period when the growth curve reaches a plateau and that cultures stimulated in resting phase have an important fraction of cells that had already synthesized DNA when the stimulus was applied. The results obtained with spectrophotometric measurements reveal that in resting phase only 5–8% of the cells are in G_2 which appears to contradict the results obtained with autoradiography. Microspectrophotometry, however, just reveals the amount of cells during a particular instant, and in this respect it can be compared with a pulse label; it can only indicate the real number of cells delayed in G_2 if there is a complete block. The situation is similar to a motion picture. If the reel speed has been reduced it is immediately evident that normal motion has been slowed. Alternatively, if one simply looks at individual frames intermittently by stopping the projector, then there is no feeling for the reduced reel speed. An exact picture can only be obtained by the combination of the two methods: autoradiography and microspectrophotometry. Thus it seems that the accumulation in G_2 during cell crowding is due to a slowdown in the flow of cells rather than a complete block and that human fibroblasts become arrested in the G_1 period.

The fact that fresh medium, which can partially reverse cell cycle inhibition was able to accelerate the cells delayed in G_2 suggests that both phenomena (inhibition of division during cell crowding and G_2 delay) may be related. An acceleration of G_2-delayed cells can also be induced by viral infection of cell cycle inhibited hamster fibroblasts (9).

"Young" human fibroblasts show a delay in G_2 only when the growth curve reaches a plateau, "aging" human fibroblasts, however, have cells delayed in G_2 even before the cell population approaches confluency (1, 2, 10). Another feature of aging is a decrease in the cell density at which proliferation stops (1, 2, 4) and we have suggested that aging *in*

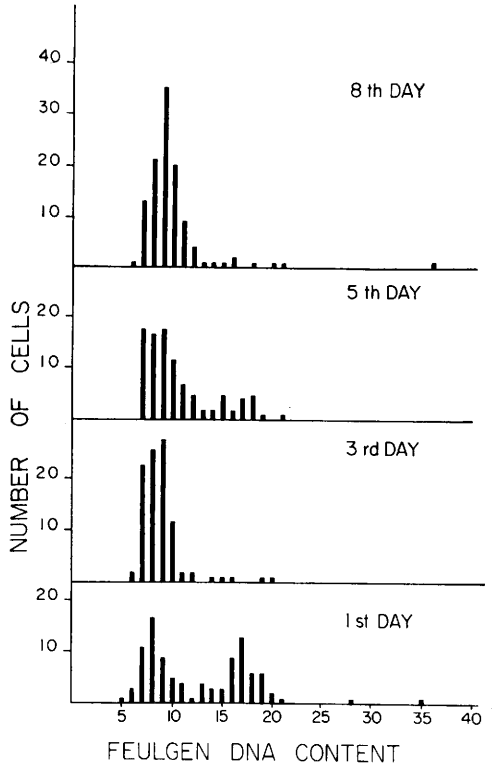

FIG. 6. Distribution of cells with different DNA contents, measured by microspectrophotometry, at different days after subcultivation.

vitro might be associated with an increased sensitivity to inhibition of division (1, 11). If this is the case, increased accumulation of G_2 cells in aging may be a reflection of an increased sensitivity of old cells to cell crowding.

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