ANALOGY IN GROWTH BETWEEN LATE PASSAGE HUMAN EMBRYONIC AND EARLY PASSAGE HUMAN ADULT FIBROBLASTS

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INTRODUCTION

It was shown by Hayflick and Moorhead (2) that human embryonic lung fibroblasts, after a period of rapid proliferation (phase II) in vitro, enter a period of slow growth (phase III) culminating in death of the population. We have previously described some changes in the division cycle that take place in phase III cultures (4, 5). These changes were manifested by a decreased number of cells entering deoxyribonucleic acid (DNA) synthesis during a 24 hr period, a decreased number of cells dividing between subcultivation and resting stage, and a longer generation time mainly due to a prolonged G_2 period.

Cell lines originating from human adult fibroblasts also go through an initial period of more rapid proliferation followed by a period of slower growth. The cell-doubling potential, however, is decreased in adult human cells compared with cells of embryonic origin (1). We have now found that a line of human adult fibroblasts in phase II shows the same changes in the division cycle as those that take place in late-passage embryonic cells.

MATERIALS AND METHODS

Two fibroblastic lines were used during the first part of their lifetime in vitro when proliferation was more active. This period of active growth lasted 60 subcultures in both lines. In order to make the experiments comparable, they were performed when growth was optimum. This was chosen as a function of the shortest time needed to arrive at resting stage and of the highest saturation density reached without medium replacement after an inoculum of 10^6 cells/60 mm plastic Petri dish. The two lines were the HEB of fetal lung origin (3) and the 2S derived from an adult skin biopsy (6). Since no difference in the growth potential has been found between skin and lung fibroblasts originating from the same type of donor (2), the comparison of adult skin to embryonic lung does not seem unreasonable. The cultures were maintained in Eagle's minimal essential medium (MEM) with penicillin (100 U/ml), Aureomycin or kanamycin (50 μg/ml), and 10% calf serum. Cell counts and determination of cell size were done with an electronic counter as described (7).

Radioautography was done by labeling cultures grown in 60 mm plastic Petri dishes containing cover slips, with 0.01 μc/ml tritium-labeled thymidine (H3-TdR) with a specific activity of 1.9 c/mmole. After the required labeling period, cultures were washed with phosphate-balanced salt solution and fixed for 1 hr with acetic acid:methanol (1:3). The cover slips were then striped with Kodak AR10 film according to a method previously described (5). Each point of the curves corresponds to 100 metaphases counted to determine the percentage of labeled metaphases and 1000 nuclei observed for the determination of the proportion of labeled interphases. These observations were made on duplicate cultures.

RESULTS

Cultures from each line were pooled separately, and several Petri dishes were inoculated with the same number of cells. From the time of subcultivation, duplicate cultures were labeled during 24 hr every consecutive day. Two cultures were continuously exposed to H3-TdR from the time of seeding until no mitotic activity could be observed. Cells in duplicate cultures were counted each day. Labeled cells were processed for radioautographic analysis at the end of the experiment. Fig. 1 shows the cell counts and the percentage of labeled interphases in HEB and 2S cultures in phase II. The growth curves show that embryonic cells have an initial lag of only 1 day and a period of growth lasting 3 days. Resting stage is reached on the 5th day after subcultivation, as can be seen from the curve of the percentage of labeled interphases. Adult phase II cells have an initial lag of 2 days followed by a growth period lasting 6 days and reach resting stage on the 10th day after subcultivation. Furthermore, the growth curve is much steeper in embryonic than in adult cell cultures. Also the latter cultures stop growing at a considerably lower cell density. The curve of the percentage of labeled interphases shows that the maximum number of cells entering DNA synthesis during a 24 hr period was 97% in HEB and 48% in 2S populations. The line indicating percentage of labeled interphases in adult cell cultures fluctuates over a wide range. These fluctuations have only been found previously (5) in phase

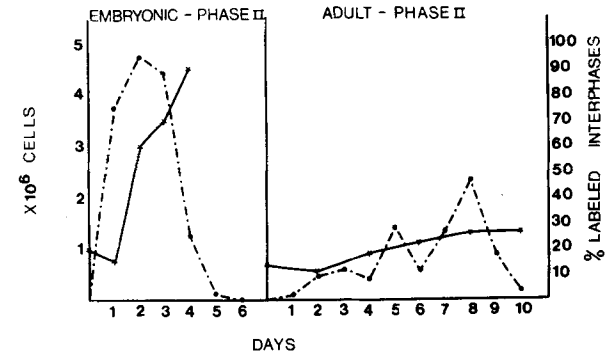

FIGURE 1 Cell counts plotted semilogarithmically (X——X) and percentage of labeled interphases plotted arithmetically (·—·—·—·) during each 24 hr after subcultivation in HEB and 2S cultures.

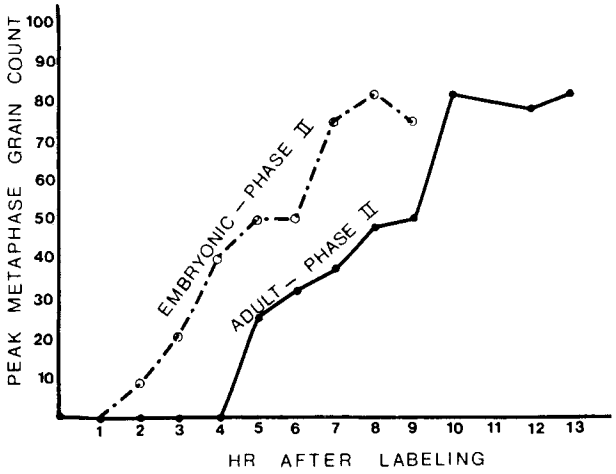

FIGURE 2 Peak metaphase grain counts at each hour after a continuous labeling of logarithmically growing HEB (o—·—·—o) and 2S (·——·) cultures.

III embryonic cells. Adult cells labeled continuously from the time of subcultivation to the time when growth stopped showed that only 66% of the population entered DNA synthesis during one passage.

The time spent in DNA synthesis was measured according to the method of Stanners and Till (9). The interval between the time when the first labeled metaphases appear and the time when the metaphase grain count reaches a plateau on the curve, during a continuous labeling, corresponds to the S period. H3-TdR was added to logarithmically growing cultures, and duplicate samples were removed at 1 hr intervals and fixed. At the end of the experiment the samples were processed for radioautography The grain count distribution obtained from the analysis of 100 labeled metaphases for each hour after addition of H3-TdR was plotted as histograms. The cumulative percentage calculated from the histograms when they were plotted on probability paper gave straight lines. The intersections of these lines with the 50% line on the paper represent the peak values of metaphase grain counts which are shown in Fig. 2. In the embryonic cell population labeled metaphases are first seen from the 2nd hr. The time between the appearance of the first labeled metaphases and the time the peak grain count reaches a plateau is 6 hr (S period). In the cultures of cells of adult origin, labeled metaphases are found only from the 5th hr. The S period, however, lasts the same time as in the embryonic cell cultures. In both populations the peak metaphase grain count reaches the plateau at about the same value (80 grains/metaphase) which is to be expected, since both are human diploid lines and hence have the same amount of DNA per cell. To measure the prolongation of the G_2 period in detail, the percentage of labeled metaphases from the same experiment was plotted against time (in hours). As can be seen in Fig. 3, 100% of the metaphases are labeled from the 5th hr in embryonic cell cultures. On the other hand, in adult cell populations 100% labeled metaphases

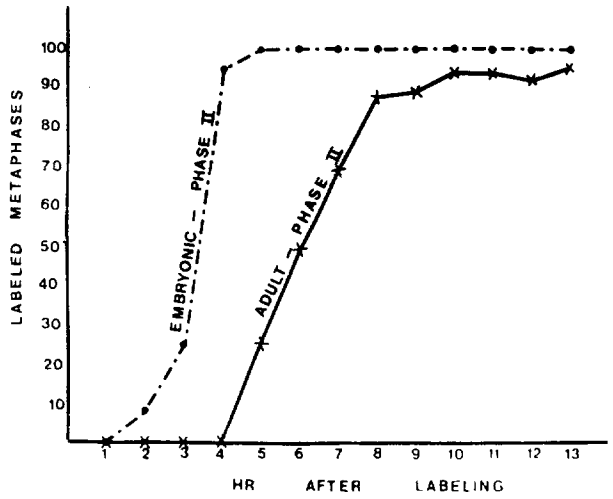

FIGURE 3 Percentage of labeled metaphases in the same experiment as the one shown in Fig. 2. HEB (·—·—·—·) and 2S (×——×) cultures.

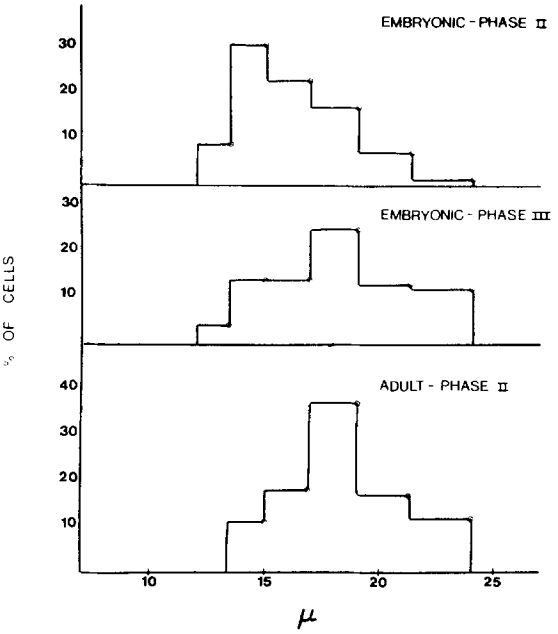

FIGURE 4 Distribution of cell diameters (expressed in microns) found in phase II and III HEB and phase II 2S populations.

were never seen within the experimental time. This shows that in adult cell cultures in phase II there are cells with a G_2 period of at least 13 hr. The fraction of cells delayed in the G_2 period is perhaps insufficient to explain the difference in doubling times observed in the growth curves shown in Fig. 1. The difference in the doubling times could also be due to a prolongation of the G_1 period and is certainly influenced by the decrease of the dividing population in the adult cell cultures.

It was shown by Simons (8) that human fibroblasts in phase III are larger than cells in phase II. For this reason we compared the size distribution of phase II and III embryonic cells with that of phase II adult cells. As is shown in the histogram in Fig. 4, the distributions found in phase II adult cells and in phase III embryonic cells are skewed to the right. Furthermore, the modes coincide in the two latter cultures and are higher than the mode found in phase II embryonic cells.

The adult cell line also eventually entered a

period that preceded cell death, where the rate of growth and the percentage of labeled interphases per 24 hr declined.

DISCUSSION

The problem of knowing why human embryonic lung fibroblasts die invariably in vitro after a constant number of generations, whereas cultures from other tissues and species are able to grow indefinitely, is obviously an important one (2). Hayflick and Moorhead postulated that the phenomenon is due to aging in vitro (2). This idea was further supported by the fact that cultures originating from adult human donors have a decreased cell-doubling potential (1). The present work also suggests that adult cells are already "aged" as compared to embryonic cells. Adult cultures also go through phases II and III. The data obtained indicate, however, that phase II adult cell cultures present some growth patterns that were found only in phase III embryonic populations (4, 5, 8). These patterns show a wide fluctuation and a decreased number of cells synthesizing DNA during a 24 hr period, a decreased number of cells entering the division cycle between subcultivation and resting stage, a lower saturation density, a longer G_2 period, a longer population-doubling time, and also an increase in cell size. It is interesting to note that these changes are independent of the time spent in DNA synthesis. The time of mitosis was also shown to be unchanged in embryonic phase III cells (5). Thus it seems that the mechanisms involved in the slow-down of cell division associated with aging in vitro may be located in the preparation for DNA synthesis and mitosis rather than in these processes themselves.

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