Standardization of Human Diploid Fibroblast Cultivation: Centrifugation Procedure

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The effect of varying centrifugal forces on the growth rate, longevity, and adsorption on glass of human embryonic diploid lung fibroblasts was studied. Cells centrifuged at 120, 500, or 1,500 × g at each passage had similar growth rates but their longevity decreased slightly with increasing force. These forces had no influence on the proportion of cells attaching to the glass. When the material in the first supernatant was recentrifuged at 2,000 × g for 30 min and added to the cells precipitated in the first centrifugation, the longevity of these cells was increased by several cell divisions. Cells which were not centrifuged but added directly from the cell suspension in trypsin to the new culture grew at a slightly slower rate than the centrifuged cells and became senescent at an earlier time. However, the noncentrifuged cells adsorbed to glass better than those centrifuged.

Human embryonic diploid lung fibroblasts (HEDLF) not only represent the possibility of a safer and more standardized host system for viral vaccine production (4) but a sensitive in vitro system for biological testing (J. Litwin and M. Thelestam, Environ. Soc. Newsletter, in press; reference 5) and studying cell aging (3). However, to obtain maximum growth and reproducible results, it is important that the cultivation techniques be standardized. One of the more critical periods in the in vitro life history of cultured cells is the time they are passed from one culture bottle to another. Since the cells are usually centrifuged during this process, the effect of centrifugal force on the growth rate, longevity, and cell attachment was studied.

MATERIALS AND METHODS

Cells. HEDLF cells were grown from human embryonic lung tissue by a procedure similar to Hayflick and Moorhead (2). Only one cell strain was used for the experiments reported here. The growth characteristics of this strain was similar to the WI-38 strain of Hayflick (3) and to an additional ten independent HEDLF strains studied in this laboratory (6).

Medium. Eagle's medium (1) was used in all experiments supplemented with 10% calf serum, 4 mM L-glutamine, 1 mM Na pyruvate, 0.02% Na bicarbonate, 100 μg of streptomycin per ml, and 100 units of penicillin per ml. The complete medium is referred to as the growth medium.

Cultivation and centrifugation. The cells were grown at 35°C in Roux bottles with a growth surface of 264 cm²; 150 ml of growth medium per bottle was used. All cultures were seeded with 10⁶ cells (approximately 4 × 10⁶ cells/cm²). When the cell layer was confluent, the cells were suspended with 50 ml of 0.25% trypsin (1:300, N.B.C., USA) in Eagle's medium without calf serum, pH 7.8, at 4°C (7). The trypsinized cell suspensions were treated by the following procedure.

(i) No centrifugation. The trypsinized cell suspensions were counted and 10⁶ cells were added directly to new culture bottles, without centrifugation. The volume of trypsin added to 150 ml of growth medium varied between 3.5 and 18 ml or a final concentration of 0.006 to 0.03%; trypsin in the medium. In one of these subcultivation series the cells were allowed to attach for about 6 hr, then the medium was changed, and the cells attached to the glass were counted. In the second subcultivation series the culture was allowed to incubate overnight (about 20 hr) before the medium was changed and the cells attached to the glass were counted.

(ii) P cultivation series. The trypsinized cell suspensions were centrifuged at either 120, 500, or 1,500 × g for 20 min at 4°C, resuspended in 10 ml of growth medium, counted, and added to new culture bottles. The cells from a given culture series were always centrifuged at the same force during passage. The cells attached to the glass were counted after overnight incubation.

(iii) S cultivation series. The cell suspensions were centrifuged and counted similarly to the P series. The supernatant was centrifuged at 2,000 × g for 30 min at 4°C. The pellet from this second centrifugation was resuspended in 10 ml of growth medium and the number of cells was counted. Five milliliters from this suspension was added to a culture bottle together with 10⁶ cells from the first, lower speed centrifugation. The procedure was always repeated with each passage. The remaining 5 ml from the higher speed pellet was added to another culture bottle containing only
growth medium. This culture was used to demonstrate the capability of the cells present in the higher speed pellet to grow to a confluent monolayer. The cells attached to the glass were counted after overnight incubation. The cells in suspension were counted with a hemocytometer.

RESULTS

Growth results. The subcultivation series which were not centrifuged after trypsinization are shown as part of Fig. 1. It made little apparent difference in the growth rate whether the cells were allowed to attach for 6 hr or overnight before the medium was changed; however, there was a slight benefit in longevity in the overnight subcultivation series. Comparison of these curves with the growth results of cells centrifuged at 120 × g (Fig. 1) shows that the former technique resulted in poorer growth and a shorter longevity.

The two curves showing the growth results of cells centrifuged at 120 × g (Fig. 1) with each passage represent the two different conditions defined as the (P) and (S) subcultivation series. The (S) series was studied to see if the cells or other particulate material remaining in the first supernatant had some influence on cell growth or longevity when added to the culture. It can be seen that the growth rates of the two subcultivation series were similar for the first 60 to 70 days of culture and then the (S) culture showed markedly better growth than the (P) culture and an increased longevity of about eight cell divisions.

Figures 2 and 3 show the growth curves of the subcultivation series centrifuged at 500 × g and 1,500 × g, respectively, under the two conditions given above. In both figures the (S) cultures showed a longevity of about four cell divisions greater than the (P) series although the growth is not apparent.
rates were the same. It can be seen from Fig. 1–3 that the centrifugal forces over the range tested had no influence on the growth rate, but the longevity decreased about three cell divisions with each increment of increase.

Effect on cell attachment. In all of the series studied the best cell adsorption was obtained with the cells which were not centrifuged and the medium changed after overnight incubation. The subcultivation series in which the medium was changed after 6 hr of incubation was much poorer than the former perhaps because not all the cells had attached by this time.

The cell adsorption of the (P) and (S) cultivation series centrifuged at different forces showed very similar results. There appeared to be little difference between the two series and no influence of increasing centrifugal forces within the range studied. The adsorption of cells present in the initial supernatant, which were then centrifuged at 2,000 × g for 30 min were generally lower than the (P) and (S) cultures.

The cells found in the 120 × g supernatant always grew at the normal rate even though they were then subjected to a force of 2,000 × g. However, the cells recovered in the 500 and 1,500 × g supernatant, especially cells from cultures older than the eighth passage, showed progressively poorer growth. The poor growth at higher cell ages may have resulted from the very low cell concentration present although very young cells showed significant growth even at inocula as low as 200 cells per cm² (J. Litwin, XIIth Eur. Symp. Poliomyelitis Allied Dis., in press). Alternatively, as the initial centrifugal force was increased, it is possible that mostly defective, damaged, or slower growing cells were left in the supernatant.

Measurement of cells attached to glass. The number of cells attached to the glass surface was determined by a technique described earlier (J. Litwin, XIIth Eur. Symp. Poliomyelitis Allied Dis., in press; reference 5). Fifteen fields-of-view were selected randomly at corresponding places on each bottle and the number of cells per field-of-view was determined with a Leitz inverted microscope. The total number of cells on the surface was calculated by multiplying the average number of cells per field-of-view by the ratio of the area of the growth surface to the area of the field-of-view.

The estimate of the number of cells attached to the glass was taken into account when calculating the number of cell divisions per passage.

The proportion of cells left in the supernatant at each centrifugal force is given in Table 1.

When the cells were sedimented at a force of 120 × g for 20 min, usually less than 10% of the cells were left in the supernatant; however, there were several passages (9, 10, 12, 13, and 16) in
which more than 10% and as high as 58% of the cells remained in the supernatant.

At a force of 500 × g a much lower proportion of cells was left in the supernatant and at 1,500 × g less than 1% was most frequently found.

**DISCUSSION**

The exposure of cells to centrifugal forces is one of the many manipulations which may introduce cell damage and artifacts into long term studies of cell growth and aging. The ideal situation would appear to be the passage of cells without centrifugation. However, the procedure which we employed in these experiments gave poor growth results. These results may have been due to the residual trypsin to which the cells were exposed during the first day of the culture.

It is estimated that the cells were exposed to between 0.006 and 0.03% trypsin. This concentration was not high enough to suspend all the cells from the glass in buffer and had no influence on cell attachment in complete medium. However, the overnight and 6-hr "no centrifugation" curves (Fig. 1) had the same growth rate in spite of the fact that the former was incubated in the presence of trypsin for a longer time than the latter. If the trypsin was toxic, one would expect worse results with prolonged incubation unless the cells were sensitive to the enzyme for a relatively short time after adsorption on the glass and then unaffected by the enzyme after that. Furthermore, it has been shown that prolonged incubation of suspended diploid cells in 0.25% trypsin at 37 C had little effect on the subsequent growth (6).

In a previous report (5) where the culture was seeded directly from a trypsin suspension and the medium changed after 6 hr, relatively poor growth was obtained. Further, some preliminary experiments had shown that changing the medium within the first 24 hr of these cell cultures resulted in poorer subsequent growth. Thus, it may not have been the presence of trypsin but the early change of medium which caused the reduced growth rate and longevity in these cultures. However, the effect of prolonged exposure to trypsin during the period of cell growth has not been investigated thoroughly.

The cells centrifuged at 120 × g for 20 min had a significantly increased growth rate and longevity (Fig. 1). Adding the cells and material remaining in the low speed supernatant, which was concentrated by centrifugation at 2,000 × g for 30 min, did not affect the growth rate while the cells were young. After 30 cell divisions the growth rate of the culture receiving this concentrated supernatant remained constant, whereas the control culture began to slow down. The cells in the former culture grew eight cell divisions longer than the latter. Although such low forces are frequently recommended when centrifuging mammalian cells, one can expect to lose between 5 and 10% of the cells and occasionally a much higher proportion at this force (Table 1).

Cells subjected to higher centrifugal forces (Fig. 2 and 3) with each passage showed a slight progressive decrease in longevity but no change in growth rate when compared to the 120 × g culture. In each case, however, adding the material from the concentrated supernatant increased the longevity slightly.

The ability of the cells to attach to the growth surface did not appear to be influenced by the centrifugal force applied in this study, indicating no progressive cell damage. However, the best adsorption was obtained from the cells that were not centrifuged and were left to incubate overnight before the medium was changed.

The effect of the concentrated low speed supernatant on the longevity of HEDLF cannot be explained at present, but the following hypothesis is suggested as a basis for further experiments.

The effect probably is not caused by the intact cells left in the supernatant because these cells had been subjected to a force of 2,000 × g for 30 min, and it has been shown that even lower forces can shorten their longevity. It is assumed that the effect may be due to some subcellular particles large enough to be sedimented at this low force which contained enzymes necessary for aging cells. A large amount of extracellular particles and amorphous material can be seen floating in the medium and attached to the glass. This material appears to increase significantly as the cells approach senescence, in agreement with observations made by Hayflick (2, 3) on aging diploid lung fibroblasts. These particles may come either from degenerated cells or fragment off the long processes extending from fibroblast cells. The effect produced by such particles would be expected to be stronger at lower initial centrifugal forces because the particles might not be sedimented at 120 × g and, therefore, most would be left in the supernatant and concentrated by the higher force applied later. When the trypsinized cell suspensions were centrifuged at higher speeds, these particles were sedimented with the cells and diluted out when a small volume of the cell suspension was added to a new bottle.

The cell strain used for this study showed the typical growth characteristics, karyotype, and morphology found in the WI-38 cells (3) and in about ten independent strains of human embryonic lung fibroblasts grown from primary
tissue and studied in this laboratory (6). Therefore, it is assumed that the observations made with this cell strain would apply to others of this type. It is apparent that the centrifugal forces in the range studied had no influence on the growth rate or cell attachment and only a slight influence on the longevity of the cells. Furthermore, it has been shown recently by Wang et al. (9) that centrifugal forces as high as $42,200 \times g$ had little influence on the viability of Burkitt lymphoma cells provided the temperature during centrifugation was kept at 0°C. At 25°C a marked reduction in viability at comparable forces occurred.

In the standardization of diploid cell cultivation techniques for viral vaccine production and other applications, it is important to know within what limits one may handle these cells without introducing too many artifacts. It is clear that older cells are more sensitive to centrifugation than younger ones, but the force can be varied considerably without influencing the growth results. The present results suggest that the lower the centrifugal force the greater is the number of cell divisions diploid cells may undergo before becoming senescent; however, this advantage may be reduced by the occasional high loss of cells remaining in the lower speed supernatants.

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