UNMASKING LARGE AND PERSISTENT REDUCTIONS IN PROLIFERATION RATE OF AGING CELLS

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SUMMARY

We have reported that nontransformed sublines of NIH 3T3 cells that are incubated under the growth constraint of confluence for 10 d or longer exhibit heritable reductions of growth rate upon serial subculture at low density, which simulate the effects of aging in vivo on cell growth. There is also a marked increase in the likelihood of neoplastic transformation. After switching to a new batch of calf serum (CS), we found the reduced growth rate was no longer produced within the previously established timeframe. However, substitution of fetal bovine serum (FBS) for CS during the period of recovery from confluence or the following tests of growth rate resulted in profound inhibition of growth in cells serially subcultured from confluent cultures. In some cases, fewer than one in a thousand cells from subcultures of confluent cultures formed colonies in FBS although they cloned at relatively high efficiency in CS. The reduced growth in FBS was retained in the postconfluent subcultures after many generations of multiplication at low density in CS. Generally, similar results with individual variations were obtained with three other batches of FBS. The numbers of cells per 3-d colony initiated from subcultures of confluent cultures were lower than those of control cultures that had never been confluent. Supplementation of FBS-containing medium with CS fully restored the growth of the postconfluent subcultures to the rate in CS medium, indicating that there is a deficiency of growth factor(s) in FBS rather than the presence of an inhibitor. The results show that prolonged incubation at confluence induces a populationwide heritable increase in requirement for growth factor(s) in short supply in FBS. Because clonal studies have shown that the reduction in growth rate is irreversible and varies in degree from clone to clone, we propose that it arises from damage to DNA at any of many different genetic loci or from chromosome aberrations. Such genetic damage is also consistent with the increased tendency for neoplastic transformation in subcultures from the long-term confluent cultures.

Key words: senescence; growth factors; transformation; genetic damage.

INTRODUCTION

A reduction in the rate of cellular multiplication with increasing age has been reported for fibroblasts, endothelium, and a wide variety of epithelial cells of the mouse (2,14,28,29,31). There is also a reduction in the rate of epidermal cell renewal with age in humans (10). The same variety of cells that exhibit reduced growth rate with age in vivo continue to do so when cells from young and old mice are grown in vitro (15) as do fibroblasts from young and old humans (22,26). This indicates that the reduction in growth rate with age is an intrinsic property of the cells rather than an ambient response to systemic change in the aging organism. We have found that prolonged incubation of NIH 3T3 mouse cells under the growth constraint of confluent cultures results in a reduction in the growth rate of the cells that persists through many generations when they are serially subcultured at low population densities (3,4,18–20). The extent of the reduction in growth rate of the NIH 3T3 cells was about 15–20%, similar to that found in cultured cells from aging mice and men (15,22) leading us to propose that long-term confluence mimics the growth-impairing effect of aging.

Another consequence of long-term confluence is neoplastic transformation of the cells, with the production of multilayered foci and increased population densities at confluence. Because the incidence of the most common epithelial tumors of man increases exponentially with age (5,7,16,17), and cultures of bladder epithelium from old mice are far more susceptible to chemical carcinogens than bladder epithelium from young mice (27), the confluence-induced transformation was taken as another indicator of cellular aging.

Isolation of single cells from long-term, postconfluent cultures and repeated testing of their clonal descendants revealed that both reduced growth rate and transformation are stable characteristics, persisting over many cell generations of serial subculture at low density (4). This finding suggests a genetic origin of impaired proliferation and neoplastic transformation. Mammalian cells are subject to gene amplification and major chromosome restructuring when they are partially but not totally inhibited (11,13,21). Cells with amplified genes proliferate much more slowly than their normal progenitors (9). Growth inhibition of epithelial cells at confluence, followed by further inhibition in suspension for 12 h, results in widespread breakdown in DNA (8), as does removal of growth factors from hemopoietic cells (30). It is not surprising, therefore, that random damage to DNA
should occur in the NIH 3T3 cells kept under the constraint of confluence for extended periods, and that it should result in an irreversibly depressed rate of proliferation.

All the experiments with NIH 3T3 cells described above were carried out in medium with serum from a single batch. When the supply of that serum was exhausted, a new batch was introduced. Within a month or so of standard passage in the new serum, the cells were growing to a higher saturation density than before and producing some small transformed foci in a standard assay. The heritable reduction in growth rate no longer occurred within the standard 2-wk period of confluence. Therefore, we thawed out a cryopreserved vial of the original cells and compared their response to confluence with that of the "old" cells in the presence of either the new calf serum or a batch of fetal bovine serum (FBS). The newly thawed cells proved to be more susceptible than old ones to the growth-imparing effect of confluence, particularly when tested in the presence of the FBS. The effectiveness of FBS in revealing confluence-induced growth impairment was greater when present during subculture from confluence than during the period of confluence itself. Other batches of FBS were consistently more effective than calf serum (CS) in producing growth impairment. The results favor the view that the aging damage produced in cells is not fully expressed as a reduction in proliferation rate unless that rate is measured in medium with suboptimal growth stimulatory activity. In that sense, the altered cells reflect the standard view of the aging process as the result of deteriorative changes in the organism that lead to its increased vulnerability to stressful situations (6,24).

MATERIALS AND METHODS

The 173~ subline of NIH 3T3 cells was used in this study (19). It had been cryopreserved in liquid nitrogen after 250 three times weekly passages at low population density in a medium consisting of 90% molecular, cellular and developmental biology medium (MCDB 402) (23) and 10% (vol/vol) CS (HyClone Laboratories, Logan, UT). After thawing, the low density seeding used as control cultures were 2 × 10^4 cells per 100-mm dish (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ). Every 2 to 3 d after detachment by a 5-min incubation with 0.01% trypsin and 0.5 mM ethylene diamine tetraacetate (EDTA) in Tris-saline buffer, the cells were seeded at 10^5 per 60-mm dish for 3 d to be trypsinized and reseeded at 10^5 per 100-mm dish in MCDB 402 with 10% CS for 2 or more days to allow recovery from the direct inhibitory effects of confluence. After the recovery period, the cells were again trypsinized and reseeded at 10^5 per 100-mm dish in MCDB 402 with 10% CS, and stained with 4% Giemsa at pH 7.0. They were also trypsinized, counted electronically, and reseeded at 2 to 4 × 10^6 cells in 100-mm dishes in MCDB 402 with 10% CS for 2 or more days to allow recovery from the direct inhibitory effects of confluence. After the recovery period, the cells were again trypsinized and reseeded at 10^5 per 100-mm dish in MCDB 402 with 10% CS, and counted every day for 4 d to establish growth curves, although the results are in several instances tabulated as the ratio of counts at Day 4 to Day 1. The cells were also seeded at 50 cells per 60-mm dish for 3 d to be fixed and stained for counting the number of cells per colony microscopically, or incubated 6 d and stained to determine colony-forming efficiency and morphology. In some experiments, cells from the 1° assay were reseeded for secondary (2°) and tertiary (3°) assays under the same conditions as the 1° assay, but were always terminated at 2 wk.

OT or "old thaw" refers to cells that were thawed after cryopreservation in liquid nitrogen, and kept in serial three times weekly subculture at low density for 3 mo, before starting the present experiments. NT or "new thaw" refers to cells that were thawed and subcultured once for 2 d before the present experiments began. A fresh thaw of cells was later initiated as described in the "Results" section. Population doublings per day (PD/D) were calculated from the ratio of the count on a given day divided by the count on a previous day or on the number originally seeded.

FBS is substituted for CS as the primary variable of the culture medium in the experiments described here. The most dramatic difference in concentration of major proteins in the two serum types is gamma globulin, which is, on average, about 10 mg/ml in CS, and 15- to 50-fold lower in FBS (data analysis from HyClone Laboratories). The first batch of FBS was from Sigma Chemical Co., St. Louis, MO. The next three batches were from HyClone and designated a, b, and c, whereas the Sigma batch was designated 2.

RESULTS

Growth rates of cells in standard low density passages. Beginning in the third passage after thawing, the growth rate of the NT cells in 10% FBS (NT-FBS) fluctuated between 1.3 and 1.5 PD/D while the same cells in 10% CS (NT-CS) started at 1.6 PD/D and gradually increased to 1.9 PD/D (Fig. 1). The OT cells in CS (OT-CS) varied in growth rate between 1.9 and 2.1 PD/D. Despite the passage-to-passage fluctuations, there was no simultaneous overlap of growth rates among the three categories until the NT-CS cells caught up to the OT-CS cells at about 56 d. A more detailed picture of growth rates can be seen in the growth curves of Fig. 2, started on Day 19 after thawing the NT cells. The doubling times were approximately 14, 11, and 10 h, respectively, for the NT-FBS, NT-CS, and OT-CS cultures.

Population densities of cells in long-term cultures. High population density at confluence is an index of neoplastic transformation in a culture. All three groups of cells when seeded in 2% CS for a 1° assay (10^5 cells per 21-cm^2 dish) reached confluence within a week and increased more slowly afterwards (Fig. 3). The number of NT-CS cells per dish slowly increased from 5.5 × 10^6 at 10 d to 9.54 × 10^6 at 6 wk. The NT-FBS cells increased from 4.25 to 4.89 × 10^6 between 14 and 28 d while the OT-CS went from 9.51 to 16.6 × 10^6 in the same period. There was a significant increase in the 14-d density of cells from the 1° to the 2° assay in all cases except the 2° assay of the NT-FBS cells derived from a 2-wk 1° assay (Fig. 4). The increases in cell yield between 1° and 2° assays were particularly prominent in the OT-CS population, especially in the 2° assay of cells that had been held for 4 wk in 1° assay. By this criterion there was increasing transformation in the order NT-FBS, NT-CS,