Cell culture aging

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Summary

Cellular research in aging has been stimulated by the observation that human diploid cells have a limited number of cell divisions in culture. This loss of cellular proliferation (*in vitro* senescence) has been extensively studied by biochemical, clonal, and genetic analysis. Studies of human skin fibroblast cultures have revealed that *in vitro* senescense is related to *in vivo* human cellular aging. Recently differentiated cells have been proposed for aging studies. These cells may provide additional information on aging since alterations of *in vitro* cellular functions may be related to the *in vivo* behavior of specific differented cell types.

1. Introduction

The conquest of many diseases, the reduction in birth rates and the control of infant mortality have resulted in an increasing percent of our population being comprised of individuals over the age of 65. It is, therefore, not surprising that the field of aging research has displayed tremendous growth in the last few years. Aging research has accelerated in many areas including those concerned with the clinical, biological, social and psychological aspects of aging.

Research on aging has occurred at several biological levels ranging from the whole organism to individual molecules. One area that has attracted considerable interest is cellular research. This was stimulated by the observations of Swim and Parker (1), which were greatly expanded and elaborated by Hayflick (2, 3), that human diploid cells when placed into culture had a limited number of cell divisions. With only a few exceptions these initial observations have been confirmed in numerous laboratories in a variety of human diploid cells obtained from different tissues. The exceptions involve cells such as peripheral lymphocytes which contain a transforming virus (EBV) (4).

2. Studies of human fetal lung fibroblasts at early and late passage

Human fetal lung fibroblasts undergoing cellular senescence (as defined by a loss of proliferative abilities) have been studied intensively in order to understand the mechanisms underlying this phenomenon. Numerous biochemical parameters have been measured during *in vitro* serial passage of these cells. Genetic analyses of cellular senescence have been performed in individual cells by clonal studies as well as somatic cell hybridization.

a. Metabolic time versus calendar time

The number of population doublings that a mass cell culture completes determines its *in vitro* replicative life span rather than the time in tissue culture. When cells are frozen and then thawed, the total number of doublings that the cells undergo before senescence remains the same, regardless of the population doubling level reached prior to freezing (2, 3). Cells that are maintained in tissue culture in a nondividing state (with low serum) have an *in vitro* replicative lifespan that is dependent on the number of doublings, rather than the time in

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tissue culture (5). These observations parallel the results of mammary gland transplant experiments performed *in vivo*. The ability of the transplant to grow *in vivo* is dependent on the cumulative number of cell doublings of the glandular cells, not the calendar age of the transplant (6).

b. Cellular changes with passage

With increasing population doublings, there is an accumulation of non-dividing or slowly dividing cells (7, 8). Many of the cellular alterations observed in mass cultures of serially passaged fibroblasts may be attributable to the presence of these cells. One consistent observation is an increase in cell volumes (9, 10, 11, 12, 13). Separation of cells on the basis of volume reveals that cell fractions with the largest cell volumes contain the highest percentage of slow or nonreplicating cells (13). Similarly, the increased nuclear size of late passage cells correlates with this accumulation of slow or non-dividing cells (14). Attempts have been made to alter the replication potential of mass cell cultures by fractionation on the basis of cell volumes. This technique produces populations enriched for either slowly or non-dividing cells (large) or rapidly replicating cells (small) (15). However, these enriched cell populations rapidly lose their fractionated properties in tissue culture, and soon resemble unfractionated cell cultures at similar levels of *in vitro* passage (15). Many of these slow or non-dividing cells are probably arrested or stopped in the G1 phase of the cell cycle. This has been demonstrated with both flow microfluorometric (16) and microdensitometric (17, 18) studies. The increases in total RNA (10, 19) and protein (19, 20, 21) in fibroblasts at late passage may reflect the increases in cell volumes and cell cycle times that occur with *in vitro* passage.

Numerous other cellular parameters (enzyme activities, cAMP levels, chromosomal proteins, lysosomes, etc.) have been found to change as a function of *in vitro* passage. These have been summarized and reported in table form by Hayflick (22, 23). Subsequent reports include an increased sensitivity to hyperbaric oxygen (24), decreased synthesis of glycosaminoglycans (25), and decreases in high affinity glucocorticoid binding sites (26) in late passage cells. Since most of these biochemical changes are probably secondary to the event(s) which create and increase in slow and non-replicating cells, they are primarily of interest in delineating changes of cellular function with *in vitro* passage. The decrease with passage of glucocorticoid receptors may be of interest since cortisone or hydrocortisone will extend the *in vitro* life span of human fibroblasts (27, 28). However, these hormones inhibit the growth of human skin fibroblasts, indicating their effect is tissue specific, and hence probably not directly related to the general phenomenon of *in vitro* senescence (29).

c. Clonal analysis of in vitro senescence

Clonal studies have demonstrated that individual cells within a mass population of diploid fibroblasts at any passage level have a wide variation in doubling potentials. However, the replication potential of cloned cells does not exceed the doubling potential of the mass culture (30). The distribution of colony sizes has been reported to be an extremely accurate indicator of the passage level of a fibroblast culture (31). Human diploid fibroblasts cloned from any passage level have a biomodal distribution of doubling potentials (32). These experiments indicated that the mass population always contains a subpopulation of cells with a very low doubling potential (six or fewer). Therefore, there may be two kinds of events occurring in cellular senescence: one that results in a gradual decline in proliferation potential, and one that results in an abrupt transition. The frequency of this abrupt transition increases as the proliferative potential of the mass culture decreases (32). Theories of cellular senescence should take these observations into account.

d. Somatic cell genetics

The techniques of somatic cell hybridization have been applied to the analysis of cellular senescence. Three distinct types of cell fusions have been employed: 1) heterokaryons, fused cells in which the nuclei remain separate; 2) synkaryons, cells with fused nuclei; and 3) cells constructed from fused cellular components such as cytoplasts (cells without nuclei) fused with karyoplasts (nuclei with a thin rim of cytoplasm).

Heterokaryon studies have demonstrated that the nucleus of a senescent human diploid cell

(HDC) will inhibit the entry into S of a young HDC, but will not prevent ongoing DNA synthesis (33, 34). Senescent HDC are predominantly arrested in G1 (35).

In heterokaryons between transformed cells such as Hela, SV40 or adenovirus transformed HDC and senescent HDC, the inhibition of DNA synthesis is reversed and both nuclei synthesize DNA (36). Not all transformed cell nuclei will stimulate DNA synthesis in a senescent HDC nuclei. In heterokaryons between human glioblastoma or chemically transformed rabbit kidney cells and senescent HDC, the senescent nucleus inhibits entry of the transformed nucleus into the S phase of the cell cycle (37). A possible explanation for this complex situation is 1) that the senescent HDC produces an inhibitor of DNA synthesis, 2) chemically transformed cells have lost this inhibitor, and 3) virally transformed cells have a viral function that overrides the inhibitor. Interestingly, quiescent early passage HDC cells (quiescence meaning cessation of cellular proliferation due to serum deprivation) exhibit the same pattern of inhibition in heterokaryons as do senescent HDC cells when fused to transformed cell types (38, 39).

For many years individuals have tried but failed to produce a viable synkaryon between a senescent HDC and a early passage HDC (40). In fusions between senescent HDC and Hela cells, the synkaryon appears to be immortal (41). This is in agreement with the heterokaryon studies.

Other attempts to analyze senescence have employed cellular components to create hybrids. Hybrids between whole early and late passage HDC and young and old cytoplasts of HDC indicate that the nucleus controls senescence (42, 43). However, cells made by the insertion of karyoplasts into cytoplasts revealed that the old cytoplasm does have an effect on senescence (44).

e. Relationship between in vitro and in vivo cellular aging

One important question that has been frequently raised concerning the *in vitro* serial passage of human fetal lung fibroblasts is: Does this system accurately reflect human cellular aging *in vivo*? It is clear that lung fibroblasts *in vivo* do not replicate for fifty population doublings. If this would occur, humans would be of incredible mass. Evidence obtained from a variety of replicating cell systems also does not indicate that aging leads to a total loss of replicating ability. Furthermore, serial passage of cells in animals has been accomplished for several cell types and has revealed that cell populations have replicative life spans that exceed manyfold the life span of the animal (45, 46, 47). Therefore, we certainly do not die from a 'Hayflick phenomenon'. However, this does not mean that all types of cellular replication are unchanged with aging or that a decline in the cellular replicative ability of certain cell populations such as immune cells may contribute to *in vivo* senescence.

3. Studies of human skin fibroblasts from young and old donors

We have strongly advocated that studies of human cellular aging conducted in early and late passage human fetal lung fibroblasts be confirmed by parallel studies in early passage young and old donor cell cultures (48). If the changes observed with passage are also observed as a function of the age of the cell culture donor, they are more likely to be related to *in vivo* cellular aging. However, it is further suggested that *in vivo* studies of cell populations be conducted in animals. If the three systems are concordant, it is highly likely that the observed age-related changes will be present in human cells *in vivo*.

Selection of an appropriate population of donors for the establishment of skin fibroblast cultures is difficult. The ideal population would be the same individuals biopsied at increasing chronologic ages. Unfortunately, this type of longitudinal approach would require several generations of researchers. While we are conducting a longitudinal approach on a limited scale, most of our studies are cross-sectional in nature, utilizing donors of different ages examined at a single point in time. We have employed a population that has been extensively studied, the members of the Baltimore Longitudinal Study of Aging. These individuals come to the Gerontology Research Center of the National Institute on Aging every eighteen months for a comprehensive series of physiologic and psychologic tests. Included in this battery is a skin biopsy.

a. Total cellular proliferative capacities

Several laboratories have examined the *in vitro* replicative life spans of skin fibroblast cultures as a function of the age of the culture donor. The procedures have been similar, with continuous subcultivation of the culture until the rate of replication declines dramatically in senescence. The major difference between the studies is the population examined. One laboratory has examined normal volunteers, hospitalized patients and post-mortem material (49). Another laboratory examined diabetics, their relatives and control individuals with normal glucose tolerance (50). We have utilized the members of the Baltimore Longitudinal Study (51). While two of the labs have found a significant decline in replicative potential as a function of the age of the cell culture donor (49, 51), the third has found this relationship to hold only for diabetics and pre-diabetics (50). In this study, a 'supernormal' group of individuals with normal glucose tolerance did not display an age-related decline in total proliferative abilities (50). To further investigate this important observation we have established cell cultures from over three hundred volunteer members of the Baltimore Longitudinal Study and will examine these for total replicative abilities. Since extensive glucose tolerance studies have been conducted on this population, we can address the question of whether this relationship holds only for individuals with altered glucose tolerance.

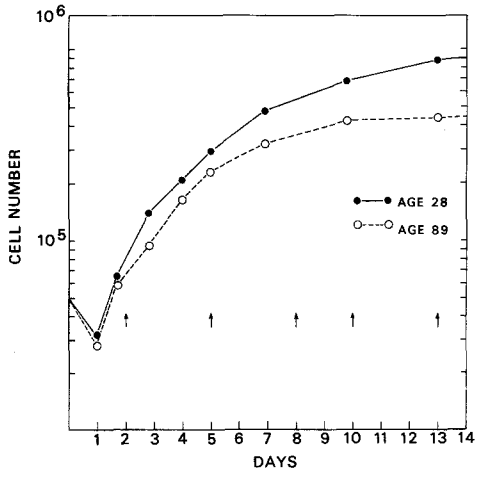

Fig. 1. Cell population growth curves of skin fibroblast cultures derived from a young (●---●) and an old (O---O) donor. Arrows indicate change of medium. From reference 51.

b. Acute cell replication

In addition to measuring the cumulative replication capacity, several other studies were conducted to assess the acute replicative abilities of cell cultures derived from young and old donors (51). Cell cultures were examined at equal levels of early *in vitro* passage. Growth curves were employed to measure the cell replication rate as well as the cell number at confluency. Typical growth curves of a young and an old donor cell culture are seen in Fig. 1. Results of several measurements are summarized in Table 1 and reveal that cell cultures derived from older donors had significantly slower

Table 1. Summary of several studies on skin fibroblast cultures derived from young and old human beings.[a,b]

Donor group	20–35 years (young)	65 + years (old)
Cell population Replication rate (h)	20.8 ± 0.8 (18)	24.3 ± 0.9 (18)
% Replicating cells[c]	87.7 ± 1.6 (07)	79.6 ± 2.5 (07)
Cell No. at confluency ($\times 10^4$ cells per cm^2)	7.31 ± 0.42 (18)	5.06 ± 0.52 (18)
% of cells able to form colony > 16 cells[d]	69.0 ± 3.3 (09)	48.0 ± 4.4 (08)
SCE per cell[e]	67.9 ± 1.6 (07)	56.1 ± 1.4 (06)
Viral plaque forming units per cell[f]	83.7 ± 37.6 (09)	223.2 ± 61.0 (09)

[a] The results of these studies were originally published in references 31, 51, 53, 57.

[b] Numbers within parentheses indicated the numbers of cell cultures examined; values are means ± standard error of the mean.

[c] Determined by incubation of cells for 24 h with tritiated thymidine and subsequent measurement of the frequency of labeled nuclei by autoradiography.

[d] Two weeks after plating at low cell densities.

[e] Cell cultures were incubated for 48 h with 7.5 mg of mitomycin C per millitier of medium.

[f] Cell cultures were infected with vesicular stomatitis virus at low multiplicity of infection for 24 h and then the medium was assayed for viral plaque-forming units as described in reference 53.

Abbreviations: PD = population doubling, SCE = sister chromatid exchanges.

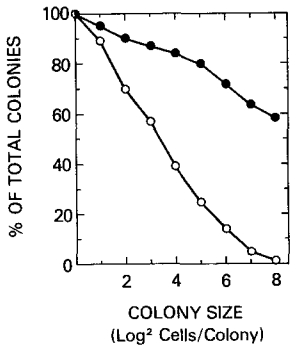

Fig. 2. Percentage of colonies able to attain at least a specified size as compared to colony size. Colony size is expressed as $\log_2$ of the number of cells per colony; Adult human skin cultures from a young (GRC74, age 33 years; (●---●) and an old (GRC20, age 80 years; (O----O) donor were cloned at the 10th population doubling *in vitro*. From reference 31.

replication rates and lower cell yields at confluency. The percentage of rapidly replicating cells in each culture was measured by incubating the cell cultures with tritiated thymidine and counting the number of a radiolabeled cell nuclei by autoradiography (51). Once again, a small but statistically significant lower percentage of rapidly replicating cells was observed in cultures derived from older donors (Table 1).

c. Clonal studies

Another approach to examining acute and chronic replicative abilities of human skin fibroblasts in vitro is to determine the colony size of individual cloned fibroblasts (31). In Fig. 2, typical colony size distributions of fibroblasts derived from a young and an old human donor are presented. Although 60% of the cells from the young donor were capable of forming colonies with 256 or more cells (8 or more doublings), less than 2% of old donor fibroblasts were capable of such division. The results of measurements of colony size distributions for 17 cell cultures are summarized in the Table 1. Once again, a highly significant decrease in replicative capabilities was observed in cultures derived from old volunteers.

d. Macromolecular synthesis

Orgel proposed that with aging there is an accumulation of errors in translation and transcription until an 'error catastrophe' occurs leading to cell death (52). One might therefore predict, on the basis of this hypothesis, that older cells would exhibit less macromolecular synthesis than younger ones. This premise was examined in skin fibroblast cultures utilizing a viral probe to test for impaired macromolecular synthesis, since defective cells were expected to produce diminished yields of infective virus (53). However, infection of skin fibroblast cultures with VSV (vesicular stomatitis virus) resulted in a slight, but not statistically significant ($p < 0.05$), increase in the yield of infective virus from old donor cells (Table 1) (53). These findings indicated that macromolecular synthesis does not decrease significantly as the age of the fibroblast donor increases. Measurement of cellular RNA and protein content also failed to reveal significant quantitative alterations in these macromolecules as a function of donor age (51).

e. Sister chromatid exchanges

The application of the BrdU-differential chromatid staining techniques permits rapid and unequivocal detection of sister chromatid exchanges (SCE) both *in vitro* in cultured cells and *in vivo* in rodent cell populations (54, 55). These cytogenetic events have been demonstrated to be accurate indicators of cellular DNA damage (56). These techniques provided an opportunity to examine the effect of aging on SCE induction in cell populations *in vivo* as well as *in vitro*. In all the systems examined, including human fetal lung fibroblasts at early and late passage, human skin fibroblast cultures from young and old donors, and mouse and rat bone marrow and spleen cell populations from young and old animals, background or spontaneous SCE levels were not significantly different. However, significant decreases in the levels of induced SCE were demonstrated in all cell systems as a function of cellular aging (Table 1) (57, 58, 59). While the exact nature of SCE remains to be elucidated, these studies would suggest that the response of cells to DNA damage is altered with aging.

f. Observed variability in human studies in vitro and in vivo

One consistent observation in studies of human

skin fibroblasts is the considerable variation observed between cultures derived from individuals of the same age. Physiological studies conducted on human subjects have revealed a similar degree of variation (60). Some of this variation may be related to the use of chronological age as the index of *in vivo* age. Research scientists as well as clinicians have long been aware that biological age may not be well represented by chronological age. Another important source of this variability in studies on cultured human cells is the unavoidable genetic heterogeneity of human beings.

Despite the observed variation in skin fibroblast culture studies, statistically significant decreases were observed in the *in vitro* replicative abilities and mutagen-induced SCE levels of skin fibroblast cultures as a function of human aging. The success of these studies may be due to the emphasis placed on standardization of skin biopsy procedures, explantation and subcultivation protocols, utilization of the same medium, and the performance of all determinations on parallel old and young donor cultures. It is important to obtain cells from a nonhospitalized, normal population since disorders such as diabetes can alter the *in vitro* life span as well as other parameters (50).

It is reasonable to assume, since they had survived to the age of 65, that our old population was comprised of relatively vigorous individuals. Therefore, we may have underestimated the extent of the *in vitro* alterations that occur as a function of *in vivo* aging.

It would be of interest to determine if the *in vitro* data obtained from human volunteers in the Baltimore Longitudinal Study will have *in vivo* predictive value. Will the old donor whose cells replicate well in tissue culture have relatively good immune function and wound healing? Will the young donor whose cells proliferate poorly have impaired immune function and delayed wound healing? These are some of the questions that can be addressed with integrated *in vitro* and *in vivo* studies.

4. Other cell culture systems

Fibroblasts are clearly not ideal for aging studies. They are undifferentiated, have few specific functions or products and their *in vivo* origin is difficult to ascertain. Therefore, many laboratories have searched for differentiated cells that have specific functins where comparisons between *in vivo* and *in vitro* observations can be facilitated.

2. Lens epithelial cell

The lens epithelium is an interesting system for aging studies since it is comprised of cells with different *in vivo* life spans and different division potentials. Like the cultured human fibroblast, the cultured lens epithelial cell has a limited growth potential (61). One interesting observation in these cultures is that lens epithelial cells derived from adults appear to dedifferentiate in early passage culture to resemble cells derived from human embryonic lens. However, after additional culture, these cells developed alterations that resembled adult rather than embryonic cells in situ (62).

b. Epidermal keratinocyte

Epidermal keratinocytes obtained from skin biopsy samples can be cultured successfully *in vitro* for extended periods (63). Furthermore, they retain some of their differentiated cell properties, such as the formation of a cornified envelope and the development of curved ridges resembling dermatoglyphs (64, 65). They are particularly amenable to *in vivo* and *in vitro* comparisons since they can be easily identified *in vivo* and retain many of their functions *in vitro*.

c. Neurons and glial cells

Although there are early reports that human glial cells have a finite *in vitro* replicative life span (66), more recent advances in tissue culture suggest that these relatively undifferentiated cells were fibroblasts rather than glial cells. Primary cultures of neurons, obtained from embryonic or neonatal brain, are differentiated cells which maintain many of the characteristics of neurons *in vivo*. These cells divide only a limited number of times or not at all *in vitro* (67). Primary cultures of pure glial cells (atrocytes and oligodendroglia) have recently been isolated from neonatel rat brain and characterized (68). Cell culture aging has not yet been studied in these differentiated cell populations.

d. Vascular endothelial cells

Several laboratories have been able to culture endothelial cells. These cells, at least those obtained from fetal bovine origin, have a limited *in vitro* replicative life span and patterns of senescence which resemble those seen in human fibroblasts, i.e., decreased replication rate and increased cell volumes with passage (69). However, unlike fibroblasts they do retain differentiated functions such as expressing factor VIII antigen throughout their *in vitro* life span. This cell should provide particular usefulness since it plays a vital role in the common age-related disorder, atherogenesis.

e. Arterial smooth muscle cells

Another important cell type which may play a prominent role in atherogenesis is the smooth muscle cell (70). These cells have a limited proliferative capacity in cell culture which is inversely related to the age of the cell culture donor (71). Studies *in vitro* have demonstrated a decreased degradation of low density lipoproteins as a function of the age of the cell culture donor (72). These proteins play a crucial role in the transport of cholesterol, and may therefore provide a clue to the increased incidence of atherogenesis with aging.

5. Future directions

Most studies of *in vitro* cellular aging have involved human fibroblasts. This was due to their ease of culture and their availability. The development of new tissue culture media and improved culture techniques has led to a proliferation of new cell types which can be cultured *in vitro*. Therefore, future research in aging may be able to examine those cell types which play important roles *in vivo*.

Previous emphasis in cell culture aging studies has been placed on cellular division. This was one of the easiest parameters to measure in a cell type with few differentiated functions. While cell division may play an important role in the immune system, it is unlikely to be a ratelimited phenomenon in other tissues. Therefore an attempt should be made to focus on differientated functions in cell types which deteriorate with age.

Cells derived from the brain are obvious choices for study since a number of age-related alterations occur in this organ. The cell of choice is clearly the neuron. Reports indicate that this cell is amenable to tissue culture and retains mrophological and biochemical functions. However, primary cultures can currently be established only from embryonic or neonatal nervous tissue. It would be of great interest to examine this cell type and determine if the same changes that occur with aging *in vivo* occur in this cell type *in vitro*. If there is a correspondence between *in vivo* and *in vitro* findings, tissue culture of isolated neurons might provide insight into the mechanisms of human cellular aging and perhaps provide the clue to the etiology of one of leading causes of disability in the elderly, senile dementia.

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