Relationship Between In Vivo Age and In Vitro Aging: Assessment of 669 Cell Cultures Derived From Members of The Baltimore Longitudinal Study of Aging

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We examined the in vitro proliferative potential of 669 cell cultures established from skin biopsies of members of the Baltimore Longitudinal Study of Aging. The colony size distribution was used to estimate the proliferative life span of the cultures. A significant decline in proliferative potential with donor age was observed for female but not male donors. For both male and female donors, the proliferative potential was significantly greater for donors under the age of 30 years compared with all donors over the age of 30 years. In an attempt to reduce genetic heterogeneity, we examined the proliferative potential of cultures derived at different ages from the same donor. These studies revealed a trend (approaching statistical significance) toward low proliferative potential as donors aged. Interestingly, samples obtained from donors who had a history of skin cancer at the time of biopsy had a significantly lower doubling potential than those from donors who did not. The implications of these results for the use of cells derived from donors of different ages for aging research are discussed.

HUMAN diploid fibroblasts have been extensively employed as a model cell culture system for examining cellular aging (1). Support for the use of these cells in aging studies has come from studies that indicate an inverse relationship between the total number of cell population doublings (“in vitro life span”) and the age of the cell culture donor (2–4). This relationship has led to the use of cells from young and old human volunteers in numerous cellular aging studies (4). In the process of establishing a national resource of skin fibroblast cell cultures from 552 volunteer members of the Baltimore Longitudinal Study of Aging (BLSA) at the Coriell Institute for Medical Research (IMR), we had the opportunity to examine these cultures for their in vitro life-span potential. We have previously demonstrated that the in vitro life-span potential or number of cell population doublings (CPDs) can be estimated with accuracy by measuring the colony size distribution (CSD) of these cultures (5–8). Therefore, we examined both CPD and CSD in a number of these cell cultures, and then, after confirmation of the excellent relationship of these two parameters, we measured CSD on the cultures donated to the IMR repository.

In this article, we report the results of experiments designed to ask the following questions. First, is the relationship between donor age and estimated in vitro life span linear across the age span or is it specific to an age group? Second, is the estimated in vitro life span related to the subsequent longevity of the biopsy donor? Third, could we detect a decline in the estimate of in vitro life span in fibroblasts derived by repeated skin biopsies of the same individuals over time? Fourth, does glucose intolerance, diabetes mellitus, coronary heart disease, or cancer incidence of the subjects affect the estimated in vitro life span of their cultured cells?

METHODS

Human Skin Fibroblast Cultures

Skin biopsies were taken from BLSA volunteers, who come to the Gerontology Research Center of the National Institute on Aging approximately every 2 years for a comprehensive series of physiologic and psychological tests. Six hundred and sixty-nine fibroblast cultures were established from 552 volunteers as previously described (6). Skin punch biopsies were obtained, with a 3-mm punch, from the inner aspect of the upper arm (the portion not exposed to the sun). In all cases, we tried to obtain full thickness biopsies from both male and female donors. The biopsy material was immediately placed in ice-cold cell culture medium and cell cultures were initiated 24 to 48 hours postbiopsy. Of the 552 volunteers, 117 subjects had repeated biopsies at different
ages; 104 subjects had two biopsies, 12 had three, and 1 had four biopsies. Of the 552 volunteers, 392 were men and 160 were women.

BLSA subjects were classified as diabetic if they met one or both of the following criteria: (a) they had a fasting plasma glucose level greater than 126 mg/dl glucose on at least two tests or (b) they were currently in a diabetes therapy program. Glucose intolerance was defined by an oral glucose tolerance test with a peak plasma glucose level less than 200 mg/dl and at 2 hours a level greater than 140 mg/dl or a fasting plasma glucose level in the 110–125 mg/dl range. Coronary heart disease was based on the presence of a history of myocardial disease or abnormalities on an exercise treadmill test consistent with ischemia or q waves on resting electrocardiogram. Cancer diagnosis was based on a review of diagnoses established by health evaluation at each visit to the longitudinal study.

In Vitro Life-Span Measurement

For the total in vitro life span of the cultures to be determined, cultures were serially subcultured until they failed to achieve one population doubling in 4 weeks (4). The number of population doublings attained by a culture was the sum of the number of population doublings attained at each subcultivation.

CSD Measurements and Conversions to Estimated In Vitro Life Span

CSDs were determined as previously described (5–8) on early passage (usually before population doubling 10). In brief, subconfluent cultures were removed with trypsin and resuspended in the appropriate medium. Thirty 60-mm-tissue culture dishes containing 5 ml of medium were seeded with 15–20 cells each. The dishes were incubated undisturbed for 14 days at 37°C and 98% humidity in an atmosphere with a ratio of 5% CO2 to 95% air. The dishes were fixed with 1% glutaraldehyde and stained with 0.5% crystal violet. The distribution of colony sizes was determined by direct microscopic count with a dissecting microscope.

CSDs were determined in minimum essential medium with Earle’s salts, supplemented with nonessential amino acids and 10% fetal bovine serum. To establish the relationship between CSD and in vitro proliferative potential, we performed a CSD analysis, approximately every 10 population doublings, on 83 cultures that were serially subcultivated to the end of their in vitro life span. We compared the percentage of clones with 16 or more cells with the number of population doublings remaining (PDR) at the time of cloning by linear regression analysis to derive a relationship: PDR = a(X) + b. Here X is the percentage of clones with 16 or more cells and a and b are constants derived from a least-squares curve fit of the data from the 83 cultures. We used this relationship to estimate the total in vitro life span of the remaining cultures studied by adding the estimated PDR to the population doubling level at the time of cloning.

Statistical Analysis

Statistical analyses were performed with computer program SPSS 9.0 (SPSS; Chicago, IL). Subject comparisons were done by using a one-way analysis of variance (ANOVA) for age and estimated cell life span (Table 1); sex comparisons for disease frequency used a chi-square test.

Linear regression was performed to determine the relationship between estimated life span and actual life span and to investigate the effects of donor age on the estimated life-span measurement by using the nonlinear program in SPSS. A piecewise regression analysis of the form \( y = a + b_1 \times \text{age} + b_2 \times (\text{age} - k) \times (\text{age} > k) \) was used to determine if and at what age \( k \) a change in slope occurred in the

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Table 1. Characteristics of Subjects at the Time of Initial Biopsy

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<tbody>
<tr>
<td>Subjects</td>
<td>392</td>
<td>160</td>
<td></td>
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<tr>
<td>Age</td>
<td>59.1 (16.7)</td>
<td>54.2 (16.6)</td>
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<td></td>
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<tr>
<td>Coronary heart disease (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>66.3</td>
<td>84.4</td>
<td></td>
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<tr>
<td>Possible</td>
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<td>11.3</td>
<td>.000</td>
<td>33.9 (10.3)</td>
<td>.26</td>
</tr>
<tr>
<td>Definite</td>
<td>15.3</td>
<td>4.4</td>
<td></td>
<td>34.3 (11.5)</td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Normal glucose</td>
<td>38.0</td>
<td>66.9</td>
<td></td>
<td>35.1 (11.1)</td>
<td></td>
</tr>
<tr>
<td>Glucose intolerance</td>
<td>50.3</td>
<td>31.9</td>
<td>.000</td>
<td>34.6 (9.7)</td>
<td>.16</td>
</tr>
<tr>
<td>Diabetes</td>
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<td>1.3</td>
<td></td>
<td>37.3 (13.2)</td>
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<td>Nonskin cancer (%)</td>
<td></td>
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<tr>
<td>By time of biopsy</td>
<td>1.0</td>
<td>1.9</td>
<td>.33</td>
<td>N 35.1 (10.7)</td>
<td>.38</td>
</tr>
<tr>
<td>Ever</td>
<td>14.3</td>
<td>8.8</td>
<td>.26</td>
<td>Y 32.1 (13.0)</td>
<td></td>
</tr>
<tr>
<td>Skin cancer (%)</td>
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<td></td>
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<tr>
<td>By time of biopsy</td>
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<td>1.9</td>
<td>.94</td>
<td>N 35.2 (10.6)</td>
<td>.02</td>
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<tr>
<td>Ever</td>
<td>22.4</td>
<td>18.1</td>
<td>.08</td>
<td>Y 28.5 (12.3)</td>
<td></td>
</tr>
</tbody>
</table>

Notes: The male–female comparison for age was by a one-way analysis of variance; all other sex comparisons were by chi-square test. Life span by disease comparisons were by one-way analysis of variance for each condition. N = did not have skin cancer; Y = had skin cancer.
relationship between in vitro life span and age. Using a maximum likelihood analysis, the program estimates the age at which the slope of the line changes. An ANOVA with Scheffe’s multiple comparison procedure was used to make comparisons among the age decile groups. In addition, an ANOVA using a contrast statement tested the hypothesis that the third decade was the same as all of the other age deciles.

The longitudinal repeated biopsy analysis was completed by using a mixed-effects model and MIXREG (9). The basic form of the model was estimated as in vitro life span, \( i = (\beta_0 + r_0) + (\beta_1 + r_1) \times \text{time} + \beta_2 \times \text{age first biopsy} + e_i \), where “time” is the time from the first biopsy and “age first biopsy” is the age when the first biopsy was taken. Here, \( \beta_0 \) and \( \beta_1 \) are standard regression coefficients for the fixed group effects. The random effects are represented by \( r_0 \) and \( r_1 \), which are the deviations from the betas for each individual. The mixed-effects model estimates the betas, \( r_0 \) and \( r_1 \). The \( r_1 \) were found not to be significant, so the fixed part of the model adequately explained the data.

RESULTS

Sample Characteristics

Subject characterization is presented in Table 1. The men were older than the women, and they had more coronary heart disease, diabetes, and glucose intolerance. Men and women showed the same frequency of skin and nonskin cancers at the time of their first biopsy and subsequently thereafter.

Relationship Between CSD and In Vitro Life Span

In order to use CSD in place of in vitro life span, 83 skin fibroblast cultures from BLSA volunteers ranging in age from 19 to 78 years were measured by both methods. A highly significant correlation coefficient of 0.89 (\( p < 0.0001 \)) confirmed the excellent correlation between estimated life span determined by CSD and in vitro life span. (Figure 1).

Relationship of Donor Age and Estimated In Vitro Life Span

A significant age effect was observed from a cross-sectional analysis of the first CSD measurements from 552 subjects. However, the relationship was weak, accounting

![Figure 1. Relationship between estimates of in vitro life span and actual in vitro life span. Each data point represents one actual life-span measurement and the corresponding estimated life span, using the colony size distribution assay. PDL = population doubling level.](https://academic.oup.com/biomedgerontology/article-abstract/57/6/B239/564318)

![Figure 2. Estimated in vitro life span as a function of donor age for A, female and B, male volunteers. PDL = population doubling level.](https://academic.oup.com/biomedgerontology/article-abstract/57/6/B239/564318)
for less than 1% of the variance ($p = .03$ and $r^2 = .009$). No difference was found in estimated cell life span for sex (men 35.9 and women 35.3; $p = .75$), coronary heart disease diagnosis, diabetes mellitus, or nonskin cancers. A significantly lower estimated cell life span was noted for those subjects who had skin cancers prior to the time of their biopsy but not when subjects were included who subsequently developed skin cancers (Table 1). Although no sex difference was observed, there was a trend for an interaction between age and sex ($p = .08$). On the basis of this trend, men and women were examined separately.

The results of estimated in vitro life span on 552 cell cultures derived from individual BLSA female and male volunteers are presented in Figures 2A and 2B, respectively. A significant decline in the projected in vitro life span is observed in cultures derived from female BLSA volunteers as a function of their chronological age; $p = .02$, $r^2 = .04$ (Figure 2A). However, no significant relationship was observed in cell cultures derived from male volunteers; $p = .35$ (Figure 2B). When cell cultures derived from repeated biopsies on the same individual as well as cultures derived from individuals in their 90s are included in the analyses, the significant ($p = .01$) negative linear regression remains for cell cultures derived from women but is not present for cell cultures derived from men.

Two approaches were used to examine whether young subjects behaved differently than older subjects. The results listed in Table 2 indicate that the greatest differences in the estimated in vitro life span of cell cultures exist between those cultures derived from individuals in their 20s and those from donors in the later decades of life. A one-way ANOVA revealed that there was a significant ($p < .01$) decrease in estimated in vitro life span between cultures from subjects in their third decade (average age 26.3 years) and those cultures established from older donors. With the use of a piecewise regression, a break in the regression line was found at 31.1 years of age, with a 95% confidence interval based on a bootstrap of 27.7–34.5 with $r^2 = .15$ when women and men were considered together (Figure 3). For subjects older than 31.1 years of age, there was no change in in vitro life span with increasing age.

**No Age Effect in the Establishment of Cell Cultures**

One possible explanation for the observed differences in the estimated in vitro life span by CSD between cell cultures derived from donors aged 20–29 years and those derived from other age groups could be the differential success in establishing these cultures. However, an examination of the success of explanation versus age of the donor revealed no significant age trend in those skin biopsies that produced no cell growth (Figure 4).

**No Seasonal and Yearly Variations on Measurements of the Effect of Age in In Vitro CPD Levels**

Because these cell cultures were established over a 10-year period, a number of parameters were measured to ensure that we were not introducing a systematic bias. No sig-

![Figure 3. Relationship between estimated in vitro life span and age. A piecewise linear regression was fitted to the data with the following model: estimated life span = $a + b1 \times$ age + $b2 \times$ (age - $k$) \times (age > $k$), where $k$ is an age where there is a change in the slope of the line; $k$ was found to be 31.1 years, and the slope above that age did not differ from zero.](image1)

![Figure 4. Percentage of total biopsies explanted that failed to grow as a function of decile of age.](image2)
significant differences were found in CSD and estimated in vitro life spans as a function of the year of explanation and season of explanation (Figure 5).

**Comparisons Between Cell Cultures Obtained From the Same Individual on Repeated Biopsies**

Examinations of multiple cell cultures derived from the same individuals (Figure 6) revealed a slight decline in projected in vitro life spans as a function of the time between biopsies, which approached significance ($p = 0.02$). The decline, $-0.67$ population doubling per year, suggests a very substantial decline over an individual's life span. However, because the mean time between biopsies was exactly 5 years, perhaps a longer interbiopsy time might detect less decline in the projected in vitro life span in cell cultures consistent with the cross-sectional observation. Of interest, the greatest decline in proliferative abilities between cell cultures derived from repeated biopsies occurred in those from donors who were 20 to 30 years of age (Figure 6B).

**Does Cell Life Span Predict Life Expectancy or Skin Cancer?**

The large variability in cell proliferative potential across the life span raises the question of whether cell life span would predict life expectancy, and from Table 1 whether there is an association between cell life span and the development of skin cancer. Cell life spans were divided into quartiles with breaks at 27.0, 35.5, and 43.0 population doublings. A Kaplan–Meier analysis found no difference in life expectancy (log rank, $p = .72$; Figure 7A). Likewise, no difference was found between cell life span and the development of skin cancer (log rank, $p = .45$; Figure 7B).

**DISCUSSION**

Previous studies on the relationship between donor age and proliferative potential of the donor's skin fibroblasts have given mixed results. In general, studies that compared the proliferation potential of cells from young donors and older donors have shown a significant decline in proliferative potential in cultures derived from older donors (reviewed in 1). However, another recent study involving cell lines from the members of the BLSA found no significant decline in in vitro life span with donor age (10). Several studies based on relatively small sample sizes and employing cells other than human fibroblasts have shown a strong steady decline throughout the ages studied (11–15). In this regard, it appears that there is a more severe decline in proliferation potential of certain cell types such as vascular endothelial cells (11,12), and vascular smooth muscle cells.
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(14), for example, than of fibroblast like cells of the skin. This may reflect the lower inherent proliferation potential of these cells compared with skin fibroblasts or alternatively a higher rate of cell turnover in vivo for certain types of cells.

The current study extends, on a greater scale, the previous observations that cell cultures derived from young adults aged 20 to 29 years have significantly longer in vitro life spans than those derived from older adults (aged 65 years or older) (2–4). However, we did not find a significant trend after age 29 in men, and only a modest (but significant) decrease in the in vitro life span of cultures derived from aging women. In a study of in vitro life spans of cultures derived from donors spanning the human life span by Martin and colleagues (3), the largest differences in in vitro life span were between those cultures derived from fetal material and the youngest age groups and those derived from individuals over the age of 29 years. Our previous report, which showed a relationship between in vitro life span and donor age involved only the two ends of the age spectrum—a young group, ages 21 to 35 years, and an older group, ages 65 years and older (4).

The proliferative potential of cell cultures established from female donors showed a significant decline as a function of donor age. It is unknown why the fibroblasts derived from female donors showed an age-related decline in proliferation whereas those derived from male donors did not. However, it should be noted that the proliferative potential of cells derived from female donors in their third decade of life was substantially higher than that derived from male donors. Furthermore, cells derived from women in their ninth decade of life had a lower proliferation potential than those derived from men. This finding suggests that the initial doubling potential of cells from women is higher than that for men but declines more rapidly with age. One possible reason for this more rapid decline is that women tend to have more sun exposure on their upper arms than men (16).

When taken altogether, the data showed no significant decline in doubling potential in subjects over the age of 30 years. A plausible explanation for this is that, during development, growth, and maturation, individuals experience a significantly higher cell turnover rate than they do in later life. A similar relative decline is observed in the Syrian hamster fibroblast in vitro life span after early adulthood (17). Physiological studies of aging (18) also indicate that peak performance for many parameters starts declining during the fourth decade of life (30–39 years) and might be a result of an earlier decline in proliferation potential. In fact, certain physiologic functions that feature declines in cellular proliferation function may peak at ages 11 to 19 years and start declining in the 20s (18).

Our observed lack of correlation between in vitro proliferative capacity and longevity is not surprising because life expectancy is clearly defined by a myriad of factors; although proliferative capabilities may play an important role, it is unlikely that they alone would determine mortality.

Our studies of cell cultures derived from individuals with diabetes is at variance with a previous report that demonstrated decreased in vitro life spans in these cultures (19,20). The reason for this discrepancy is not known. However, it is possible that differences in biopsy technique, cell culture conditions, or both may be responsible.

Interestingly, we found that fibroblasts from donors who had had skin cancer before biopsies were taken had a significantly lower doubling potential than those taken from donors who had not been diagnosed with skin cancer. Even though the biopsies were taken from the inner aspect of the upper arm, it is possible that a subset of individuals had such high sun exposure that even this usually protected area suffered actinic damage. This observation is consistent with the findings of Gilchrest (16), who found that sun exposure (significantly correlated with skin cancer) resulted in a decreased in vitro life span. Finally, our comparison of cell cultures from survivors and deceased BLSA members sug-
gests no relationship between life expectancy and proliferation potential.

The lack of a significant trend in in vitro life spans in cultures from adults over the age of 29 years might suggest that these cultures may not be good models for studying aging. However, the enormous variation observed between cultures derived from different human volunteers reflects the difficulty of studying a genetically diverse population of human volunteers.

In an effort to address the genetic heterogeneity issue, we estimated the cell proliferative life span of biopsies taken from the same donor at different ages. The maximum time between biopsies was 9 years. The moderate, but not yet significant, decrease in proliferative capabilities of cell cultures derived from the same individuals from repeated biopsies is intriguing and suggests that increasing the time of this longitudinal study might result in a highly significant correlation. A number of studies comparing fibroblasts from young and old donors and human fetal fibroblasts at early and late passage have shown much more consistent differences in the latter experimental paradigm (1,4). However, the consistent difference observed between cell cultures derived from individuals in their 20s and those in later decades does provide a reasonable in vitro model system for studying cellular aging. There have been numerous reports demonstrating a significant difference between cell cultures derived from these young (aged 20–29 years) and old adults (aged 65+ years). However, investigators should be cautious when comparing all cultures in the intermediate decades.

The techniques used may also influence the results. In one large study that combined samples from living donors with those from cadavers, a significant negative correlation of doubling potential with donor age was seen (3). However, when the data were disaggregated, separating the live donors from the cultures established from cadavers, there was no correlation in the data from the living donors but the correlation remained for the cultures from cadavers. One explanation for this result is that larger biopsies were taken from the cadavers, perhaps decreasing the sample to sample variation (G. Martin, oral communication, July 1999). The studies discussed herein measured or estimated total proliferation potential of the cultures in population doublings. Population doublings are a very crude measure of proliferative ability. For example, a change of 10 population doublings in the proliferation potential of two biopsies might mean that all the cells in one biopsy were capable of exactly 10 fewer doublings than those of the other. At the other extreme, it could be that the proliferation potential of the cells is not changed but that only 1 out of 1000 cells is capable of being stimulated to divide at all. The actual case is undoubtedly somewhere in between the two extremes. However, it is easy to see that a change of only 10 population doublings could have very profound effects on the ability of a tissue to respond to insult.

A more sensitive way to measure changes in proliferative ability is to measure the proportion of cells in a biopsy that can form colonies of a given size when the cells are plated at clonal density directly upon dissociation of the tissue. A loss of clonal growth ability in human bone marrow cells has been demonstrated by using this technique (21,22). In addition, this method has been used to show that dietary restriction decreases the rate of loss of proliferative ability in mouse skin cells and other cell types (23). Other investigators were unable to detect the protective effects of caloric restriction (24) when they used the more crude population doubling method.

Acknowledgment

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