Failure of Hydrocortisone or Growth Factors to Influence the Senescence of Fibroblasts in a New Culture System for Assessing Replicative Lifespan

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ABSTRACT It has been reported that the replicative lifespan of human fibroblasts can be substantially extended by supplementing the growth medium with hydrocortisone or increased levels of serum proteins. These observations have been made only on cell populations transferred many times at high cell density, and cumulative population doublings have been recorded, rather than a more direct measure of cell division potential. We have measured the replicative potential of human fibroblasts cultured so as to avoid conditions of high cell density, medium depletion, and departure from exponential growth. Two fetal lung and two newborn foreskin fibroblast strains were serially passaged in the presence or absence of hydrocortisone (HC), epidermal growth factor (EGF), and fibroblast growth factor (FGF) until they senesced. At each passage cells were plated at densities sufficiently low that colony-forming efficiency could be calculated. We determined cumulative population doublings and also estimated the number of cell generations attained under each condition. FGF caused small but possibly significant changes, while HC and EGF failed to substantially alter replicative lifespan. The reported effect of HC on the doubling potential of fetal lung fibroblasts is therefore not an inevitable action of this hormone on the senescence mechanism, but may instead depend for its apparent activity on the passage regimen used. The fibroblast's insensitivity to EGF as a modulator of replicative potential, as compared with the keratinocyte, whose lifespan can be tripled by EGF, implies that the mechanisms limiting the replicative potential of these two cell types are not identical.

Human connective tissue fibroblasts display a limited, though very considerable, capacity to multiply in culture (Hayflick and Moorhead, '61). All cells in any normal serially cultured population eventually lose the ability to undergo further cell division, at which time the population is said to be "senescent." The mechanism by which replicative senescence occurs in cells is unknown, and currently seems as obscure as the systems that control deterministic events in embryogenesis. It has been speculated that senescence is the result of a random accumulation of mutations in information transfer systems, culminating in an "error catastrophe" (Orgel, '73) or, alternatively, that it is a programmed change in gene expression related to an event of terminal differentiation (Martin et al., '74; Bell et al., '78). Neither hypothesis has yet received adequate experimental support.

A useful step toward understanding this remarkable change in cell function would be to determine the degree to which replicative lifespan and, by inference, the senescence mechanism itself are subject to experimental perturbation. The inclusion of higher levels of serum protein (Todaro and Green, '64; Ryan et al., '75) in the medium or the addition of hydrocortisone (Macieira-Coelho, '66; Cristofalo, '70) have been reported to significantly extend the culture lifespan of fibroblasts. Relating these results to a possible change in the timing of senescence is difficult, however, because the number of replications undergone by the cells

Received October 32, 1980; accepted February 26, 1981.

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before they senesced was not directly measured in these studies; cumulative population doublings were recorded, instead.

The division capacity of a cell population is not directly estimated from the number of cumulative population doublings because the latter value is not absolute, and varies greatly with the way the cells are passaged (Todaro et al., '65; Hayflick, '65). It has been theorized (Good, '72), but not generally appreciated, that the actual division capacity of human fibroblasts is likely to be substantially higher than the oft-quoted number of 40 to 60 (for fetal lung cells) which is actually the cumulative doublings in mass undergone by a population serially passaged at a 1:2 split ratio. This is because plating efficiency and colony-forming efficiency at transfer are less than 100%, and some cells are continually leaving the dividing "stem" fraction of the population, even at early stages of a strain's lifespan (Merz and Ross, '69; Smith and Hayflick, '74; Martin et al., '74). A change, caused by experimental alteration of culture conditions, in only the variable of colony-forming efficiency at subculture could result in a substantial difference in cumulative population doublings, and give an incorrect impression that the timetable for senescence had been experimentally altered.

In the course of testing the responsiveness of human epidermal keratinocytes in culture to agents suspected to be physiologically important growth regulators, it was discovered that epidermal growth factor (EGF) not only was a potent stimulus of short-term growth, but also could produce a tripling of the replicative lifespan of this cell type (Rheinwald and Green, '77). Because the keratinocytes were plated at low density and grown up into large discrete colonies in each passage, several important parameters of cell multiplication could be quantitated accurately. It was observed that, besides the large increase in colony-forming ability caused by EGF, both cell replicative potential and population doubling capacity were greatly increased. Colony stratification (Rheinwald and Green, '77) and cornified envelope formation (Sun and Green, '76) were both markedly reduced in colonies growing with EGF. Thus it was suggested that the culture lifespan of the keratinocyte is largely determined by the rate at which the cells enter their terminal differentiation program; thereby irreversibly losing their division potential (Rheinwald and Green, '77; Rheinwald, '79).

We sought to determine whether any agents demonstrated to stimulate growth of human fibroblasts in short-term culture might also increase their long-term division potential in a manner similar to the effect of EGF on keratinocytes. We serially cultivated four fibroblast strains at plating densities sufficiently low to permit determination of colony-forming ability at each transfer, and always subcultured the cells while they were growing exponentially so that no fraction of the population would have its growth slowed by density inhibition. We found that, under this regimen, supplementation with EGF, FGF, or hydrocortisone at concentrations optimal for short-term growth stimulation failed to substantially alter the replicative lifespan of the fibroblasts.

**MATERIALS AND METHODS**

**Culture materials, media, and growth factors**

Cells were cultured in 60-mm diameter plastic tissue culture dishes (Lux) with a medium volume of 4.5 to 5 ml, at 37°C in an atmosphere of 92% air/8% CO₂. Medium was F10 (Ham, '63), (Gibco, Cat. No. 430-1200), (to which penicillin and streptomycin (ICN Pharmaceuticals) were added at 0.1 mg/ml) plus 15% fetal calf serum (M.A. Bioproducts). Hydrocortisone (A grade, Calbiochem) was first dissolved in 100% ethanol and then serially diluted in medium plus serum such that the cells were never exposed to more than 0.01% ethanol. Murine EGF and bovine brain FGF were from Collaborative Research, Inc. EGF was added to cultures from a 100 X concentrated solution in water containing 0.1% crystalline bovine serum albumin (Calbiochem). FGF was added to cultures from a 100 X concentrated solution in Earle's salts plus 20% FCS.

**History of cell strains and estimation of cell generations elapsed before lifespan experiments were begun**

Strain A1-F was initiated by M. A. Beckett in this laboratory by plating a single cell suspension of trypsin/collagenase-disaggregated newborn foreskin in F10 + 15% FCS (Rheinwald, '80). Colony-forming efficiencies (cfe) in the primary and secondary cultures were known precisely, so the number of replications the cells had undergone in culture prior to the lifespan experiment could be directly calculated (according to the formula given below) as 21 cell generations.

The other strains had been derived from explant outgrowth cultures and had been serially passaged at high density at least seven times before we received them. From their culture histories we estimated the cumulative cell generations undergone by these strains prior to the
lifespan experiments. We assumed that fibroblasts divide almost once per day in primary explant outgrowth culture and that early in their lifespans they reinitiate growth with an efficiency of about 50% in high density subcultures. The latter assumption is consistent with the data of Todaro et al. (’63) and Hayflick (’65) which show that an increase in the number of times that a population is subcultured during passage to senescence results in a decrease in the apparent replicative potential of approximately one population doubling per additional passage.

Strain 356 was kindly provided by Dr. Lois Jacobs. It was initiated (Jacobs and DeMars, ’78) from an explant outgrowth population of newborn foreskin fibroblasts grown 8 days in primary culture and then serially passaged at approximately 1:3, 1:8, 1:10, 1:6, 1:8, 1:8, and 1:12 dilutions before the lifespan experiment was begun at an estimated 33 cell generations.

IMR-90 was obtained from the Institute for Medical Research, Camden, New Jersey, at nominal population doubling 10. It was initiated (Nichols et al., ’77) from an explant outgrowth culture of fetal lung fibroblasts grown 9 days in primary culture and then subcultured at approximately 1:4 dilution to create the secondary culture population (Institute for Medical Research designation: population doubling level 1). The cells were passaged four more times at approximately 1:6 dilutions, and then at 1:12, 1:20, and 1:20 dilutions before the lifespan experiment was begun at an estimated 41 cell generations.

WI-38 was obtained from the Institute for Medical Research at nominal passage number 19. It was initiated (Hayflick, ’65) from an explant outgrowth population of fetal lung fibroblasts grown for an unknown period of time in primary culture (we guess approximately 9 days), then serially passaged 18 times at 1:2 dilution and twice at approximately 1:4 dilution before the lifespan experiment was begun at an estimated 50 cell generations.

Estimating short-term mitogenic activity of agents

To determine the concentration of agents to be used in the lifespan determinations, the four fibroblast strains were plated at 500 cells per dish in medium containing 1, 10, 100, 10^4, or 10^5 ng/ml hydrocortisone; 3, 10, 20, or 30 ng/ml EGF; and 3, 10, or 30 ng/ml FGF. Cultures were fed 1 week after plating and every 3 or 4 days thereafter until the colonies almost began to merge in the most rapidly growing culture. Then all dishes were fixed, stained, and assessed visually for relative colony size and colony-forming efficiency.

**Determination of colony-forming efficiencies and cell generations during serial culture**

Frozen cell suspensions were thawed, plated at high density, and grown for at least 3 days in F10 + 15% FCS before the low density serial passages were begun. At each passage, cells were disaggregated to single cells with 0.1% trypsin, counted with a hemacytometer, and plated in triplicate at densities of 100, 300, and 10^3 cells per dish. Cultures were fed once weekly and within 2 days of the next subculture. Twelve to fifteen days after plating, before the cells had slowed from exponential growth due to the merging of colonies or to density inhibition in colony centers, one of the cultures originally plated with 10^4 cells was trypsinized, counted, and replaced for the next passage. The remaining dishes were fixed in phosphate-buffered 10% Formalin and stained with 0.2% methylene blue.

Large colonies (defined as those the sum of whose constituents was estimated to account for more than 98% of the cells on the dish) were counted to determine colony-forming efficiencies. In this way, small senescent or slowly growing colonies, whose constituents did not contribute significantly to the size of the cultured population, did not bias the calculation of generations undergone during each passage by the more prolific cells in the population. These large colonies ranged in size from about 1,000 to 5,000 cells during most of the lifespan, sometimes decreasing to about 200 to 1,000 cells in the penultimate passage. In the final passage, maximum colony size was sometimes as small as 16 cells. The total number of colonies (greater than a size of 50 cells) was never more than twice the number of large colonies used for the calculation of cell generations.

The number of cell generations undergone by a strain during a single passage was defined as

\[
\text{log}_2 \left( \frac{\text{# cells in dish at time of subculture}}{\text{# cells plated}} \times \frac{\text{colony-forming efficiency}(\%)}{100} \right)
\]

As a typical result, a culture initiated with 10^3 cells that had a cfe of 12% and contained 2.4 × 10^4 cells at the time of subculture, was calculated to have undergone

\[
\log_2 \left( \frac{(2.4 \times 10^4)}{(10^3)(0.12)} \right) = \log_2(2000) = 11 \text{ cell generations}
\]
The traditionally determined number of population doublings undergone by a strain during a single passage does not take into account colony-forming efficiency and is calculated as

$$\log_2 \left( \frac{\text{number of cells in the dish at time of subculture}}{\text{number of cells plated}} \right).$$

Thus the hypothetical culture described above underwent

$$\log_2 \left( \frac{2.4 \times 10^9}{10^5} \right) = \log_2 (240) \approx 8 \text{ population doublings.}$$

When colony-forming efficiency decreased as senescence was approached, strains were subcultured at $300 \times 10^5$ and $3 \times 10^5$; or at $10^4$, $3 \times 10^4$, and $10^4$ cells per dish. Senescence was defined as the inability of cells plated at $10^4$ per dish to form any detectable colonies during 3 weeks in culture.

**RESULTS**

*Growth stimulatory activity of agents in short-term culture*

Relative growth rates of the four strains cultured with various concentrations of HC, EGF, and FGF were assessed visually in stained cultures grown from single cells into large colonies. The fetal lung strains varied in different experiments, showing either slight or no apparent stimulation of growth with 10 to 100 ng/ml HC. In the presence of HC concentrations $\geq 100$ ng/ml the cells were less elongated and they packed together more closely, even in small colonies. IMR-90 and WI-38 were only slightly inhibited by high HC concentrations of 1 to 10 $\mu$g/ml. The newborn foreskin strains 356 and A1-F varied in different experiments, showing either no effect or a slight inhibition of growth with 100 ng/ml HC. Their morphology was altered by HC in the same way as the fetal lung strains, but to a more severe degree and at lower concentrations.

In other experiments, WI-38 and IMR-90 cultures were refed twice over a 4-day period after they had reached confluence, and the saturation density was then determined. Cultures grown with 100 ng/ml HC consistently had about 50% more cells at saturation than controls, an effect of this hormone that has been reported by others (Castor, '62; Grove et al., '77; Rosner and Cristofalo, '79). HC at 100 ng/ml (0.3 $\mu$M), was near the peak of a fairly broad concentration range of mild stimulation for the fetal lung strains, and close to the 0.14 $\mu$M concentration now routinely used in studies of the effect of hydrocortisone on growth of WI-38 in high density culture (Rosner and Cristofalo, '79). Because HC did not stimulate clonal growth of the newborn foreskin strains at any concentration, its effect on their lifespan was not examined.

EGF, 20 ng/ml, and 10 ng/ml FGF were found to be the concentrations giving maximal growth stimulation in low density cultures of all four strains. EGF was slightly stimulatory to all strains. FGF stimulated the fetal lung strains slightly and the foreskin strains more strongly, the latter appearing (and confirmed by cell counts of some cultures) to have two to three times as many cells per colony as the controls after 12 days. Significant mitogenic effects of EGF and FGF on human fibroblasts, even in the presence of a high serum concentration, have been described by others (Carpenter and Cohen, '76; Gospodarowicz and Moran, '75).

*Replicative lifespans in the absence of added mitogenic agents*

Strains were serially passaged, as described under Materials and Methods, five to seven times for 62 to 76 additional cell generations until they failed to grow when subcultured (Fig. 1). Based on our estimates of the number of cell generations that had already elapsed for each strain before the beginning of the experiment, this represented the final 61% of the lifespan for IMR-90, 57% for WI-38, 67% for strain 356, and 79% for A1-F. In terms of population doublings, the strains underwent an additional 45 to 52 doublings before they senesced. All strains grew with a nearly constant doubling time of 26 to 29 hr throughout their respective lifespans, until the penultimate or final passage. As shown in Figure 2, the colony-forming efficiencies of each strain varied greatly during the course of the lifespan experiments. However, they were generally 10 to 30% during most of the lifespan and rarely dropped below 5% until the cells were close to senescence, when even the colony-forming cells were no longer dividing at their maximal rate.

The fetal lung strains IMR-90 and WI-38 had total lifespans of 113 and 112, and the newborn foreskin strains 356 and A1-F had total lifespans of 96 and 97 cell generations, respectively, in the absence of added growth factors. The absolute numbers for lifespan might not be expected to be precisely reproducible for each strain because the estimates of the efficiency of large colony formation at each passage are somewhat subjective. However, this number is easily determined within a factor of two at each
passage, so the uncertainty is less than 1 cell generation out of the 11 to 13 that elapse per passage. Because any error is unlikely to occur systematically in one direction, it should cancel out over the course of several passages. A different person in the laboratory carried A1-F to senescence under the same regimen six months before this set of experiments was conducted and also determined its lifespan to be 95 cell generations (M.A. Beckett, unpublished observation). In any event, for this study of growth factor effects the precise number applied to the lifespan is less important than the differences observed under the various conditions of growth.

Fig. 1. Replicative lifespans of human fibroblasts in the presence or absence of hydrocortisone, EGF, or FGF. 10^4 cells of each strain were plated per 60-mm dish, grown for 12-15 days to form large, discrete colonies, and subcultured, before the population slowed from exponential growth again, at 10^4 cells/dish. Cultures were refed 1 week after plating and again within 2 days of subculture. Strains were passaged until they no longer formed colonies. Colony-forming efficiencies were determined by counting colonies in duplicate stained cultures, and this number was used to calculate the cell generations undergone in each passage as described under Materials and Methods. The number of cell generations in the final passage (---) was estimated by counting the average number of cells in stained dishes in any small terminal colonies that had formed. The uncertainty in this estimate is indicated by the error bars around the final points. (A) IMR-90, (B) WI-38, (C) 356, (D) A1-F. (•) no additions, (○) +100 ng/ml HC, (▲) +20 ng/ml EGF, (△) 10 ng/ml FGF.

Fig. 2. Colony-forming efficiencies of human fibroblasts during their lifespans when cultured in the presence or absence of hydrocortisone, EGF, or FGF. See legend of Figure 1.
TABLE 1. Replicative lifespan and population expansion capacity of fibroblast strains grown in the presence or absence of growth promoting agents

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. cell gens. at start of experiment</th>
<th>Agent present</th>
<th>Remaining lifespan until senescence</th>
<th>Cell gens.</th>
<th>% of control</th>
<th>Population doublings</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMR-90</td>
<td>41</td>
<td>None</td>
<td></td>
<td>72</td>
<td>(100)</td>
<td>52</td>
<td>(100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HC</td>
<td></td>
<td>78</td>
<td>108</td>
<td>53</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EGF</td>
<td></td>
<td>78</td>
<td>108</td>
<td>50</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FGF</td>
<td></td>
<td>62</td>
<td>88</td>
<td>40</td>
<td>77</td>
</tr>
<tr>
<td>WI-38</td>
<td>50</td>
<td>None</td>
<td></td>
<td>62</td>
<td>(100)</td>
<td>48</td>
<td>(100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HC</td>
<td></td>
<td>61</td>
<td>98</td>
<td>48</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EGF</td>
<td></td>
<td>47</td>
<td>(100)</td>
<td>28</td>
<td>(100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FGF</td>
<td></td>
<td>43</td>
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<td>22</td>
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<td></td>
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<td>(100)</td>
<td>46</td>
<td>(100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HC</td>
<td></td>
<td>68</td>
<td>109</td>
<td>48</td>
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</tr>
<tr>
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<td></td>
<td>EGF</td>
<td></td>
<td>63</td>
<td>(100)</td>
<td>52</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FGF</td>
<td></td>
<td>72</td>
<td>114</td>
<td>52</td>
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</tr>
<tr>
<td>A1-F</td>
<td>21</td>
<td>None</td>
<td></td>
<td>76</td>
<td>(100)</td>
<td>48</td>
<td>(100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EGF</td>
<td></td>
<td>74</td>
<td>97</td>
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</tr>
<tr>
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<td></td>
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<td></td>
<td>83</td>
<td>109</td>
<td>54</td>
<td>113</td>
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</tbody>
</table>

*WI-38 population obtained from a different source, as described in text.

The effect of added mitogens on replicative lifespan

Each strain was carried under control and experimental conditions at the same time and subcultured on the same day (as long as it was practical to do so) in order to minimize effects of unknown variables. The results of these experiments are given in Figures 1 and 2, and summarized in Table 1. The replicative lifespan of WI-38 was essentially unchanged and that of IMR-90 increased by only 6 cell generations (8% of the portion of the lifespan traversed during the experiment) by hydrocortisone. In terms of population doublings, the lifespans of WI-38 and IMR-90 in the presence or absence of HC were virtually identical. In a different experiment, the effect of HC on lifespan was investigated using a WI-38 population from the culture collection of Dr. Howard Green, which had originally been obtained directly from Dr. Leonard Hayflick. An estimate could not be made of the number of cell generations that had elapsed in this population at the time our experiment was begun because sufficiently detailed records of passage were not available. This WI-38 population grew for four additional passages with colony-forming efficiencies of 4 to 7% until the onset of senescence 47 cell generations later in the absence, and 43 cell generations in the presence, of HC.

EGF had no effect on the replicative lifespan of A1-F, and increased the lifespans of IMR-90 and strain 356 by only five cell generations (8%). In terms of population doublings, EGF had almost no effect on IMR-90 and A1-F, and caused only a 9% increase for strain 356.

The largest effects were produced by FGF, which increased the replicative lifespan of strain 356 by 9 cell generations (14%) and of A1-F by seven cell generations (9%). However, FGF produced a 10 cell generation (14%) decrease in the lifespan of IMR-90. The number of population doublings achieved by each strain was altered to similar extents.

No consistent correlation was detected between changes in colony-forming efficiency produced by an experimental factor and changes in replicative lifespan (Fig. 2). HC caused a generally higher colony-forming efficiency in WI-38 and in the later passages of IMR-90. IMR-90 and A1-F grown in the presence of FGF displayed consistently lower colony-forming efficiencies.

DISCUSSION

Determination of cell replicative potential vs population doubling capacity

Both the quantitation of replicative lifespan and the understanding of the senescence mechanism in diploid human cells are complicated by the fact that all cells in a cultured population are not an equal number of generations from replicative senescence (Merz and Ross, '69; Smith and Hayflick, '74; Martin et al., '74). Some daughter cells are always produced, even by very early passage populations with a long remaining lifespan, that either abruptly stop dividing or else cycle slowly through several divisions before becoming senescent. As was demonstrated by Hayflick ('66), the lifespan of a population is determined by the replicative potential of its longest-lived constituents. Our passage regimen favored low density colony-forming ability and rapid growth, but it did not appear to select against these longest-lived
cells. Smith and Braunschweiger ('79) showed that IMR-90 and WI-38 cells serially cultivated at plating densities even lower than our system generally achieved the same number of population doublings as did high density cultures. Our control IMR-90 and WI-38 populations underwent 52 and 45 population doublings, respectively, during the course of our experiment. Using the traditional convention of discounting division that occurs in the primary cultures, and assigning one population doubling for every 1:2 split thereafter, the IMR-90 cells had undergone about 24 and the WI-38 cells about 23 population doublings before our experiment. Thus total lifespan, in terms of population doublings, was 76 for IMR-90 and 68 for WI-38 — values equal to or greater than those obtained by Smith and Braunschweiger ('79), Cristofalo ('70), and Nichols et al. ('77) for these strains.

Our subcultivation protocol offers some advantages as an alternative to the traditional regimen (i.e., serial subcultivation at 1:2 or 1:10 dilutions with growth back to saturation density each time). By plating cells at low density in a good clonal growth medium and subculturing before the colonies become densely packed and slow their growth, senescent and slower-growing end-stage cells do not accumulate, and the population remains enriched in presenescent cells with the greatest degree of "stem" character. Because the colony-forming efficiency of the cells is determined at each passage, the actual division potential of a cell population can be estimated more precisely. Variations in colony-forming efficiency during the lifespan of a strain, or as the result of the presence of an experimental factor, can be taken into consideration in the calculation of cell generations. Also, because each passage generally consists of growth of several hundred cells to about $5 \times 10^5$, more than ten generations elapse per passage. The entire lifespan of even a long-lived fetal lung or newborn foreskin strain (A1-F, for example) can be traversed and quantitated in about ten passages over a period of five months, and the cells are subjected to much less manipulation, exposure to trypsin, and fluctuations of pH and temperature.

We observed lifespans of about 112 cell generations for two fetal lung fibroblast strains and about 96 cell generations for two newborn foreskin fibroblast strains. We were able to measure with our system the entire lifespan of one strain (A1-F), starting with the colony-forming cells in the primary culture initiated from a disaggregated skin cell suspension. The accuracy of the numbers we arrived at for the other three strains depends upon the accuracy of our estimate of cell generations undergone by the strains in primary explant culture and in numerous high density serial passages before we could begin counting replications more precisely. They are likely to be within ten generations of the numbers we would have obtained by carrying them according to our system beginning with the primary culture. These values of about 100 are still slight underestimates of the number of cell divisions that fibroblasts from fetal and newborn tissues can undergo in culture because the calculation does not take into consideration the frequency with which nondividing cells arise in the large colonies. The four strains grew with population doubling times of 26 to 29 hr during most of their lifespans. Intermitotic times for WI-38 and IMR-90 growing under similar favorable conditions have been calculated to be 18 to 20 hr (reviewed by Grove and Cristofalo, '77). Therefore, not all divisions give rise to two cells with further division potential. A factor of 1.1 to 1.4 could probably be applied to our numbers to correct for this. Our experimentally determined values for lifespan are not far from that theorized by Good ('72) to correct traditionally determined lifespans for some of these factors. Because it more closely approximates the true division potential of the longest-lived cells in a population, replicative lifespan determined by our system is likely to be particularly useful for comparing cells from donors of different ages or genotypes.

The effects of hydrocortisone and growth factors on fibroblast lifespan

It seemed probable to us a priori that the passage regimen chosen for these experiments would be much less likely than the traditional one to show apparent differences in lifespan between strains or experimental conditions that result from different colony-forming efficiencies or behavior in high density culture. Of course, such differences would not necessarily be related to the onset or progression of the senescence mechanism. In order to maximize the probability that we would be able to observe the effect of an agent that could directly perturb the senescence mechanism in cells otherwise growing in near-optimal conditions, we used concentrations of growth factors that produced a stimulatory response in short-term culture. This was based on the assumption that the optimal hypothetical concentration for extending lifespan would be close to the optimal mitogenic concentration. There is no theoreti-
The culture lifespans of fibroblasts and keratinocytes are subject to regulation by different factors

We first became interested in carrying out this analysis on human fibroblasts to determine to what extent the extraordinary effect of EGF on the replicative potential of human keratinocytes was specific for that cell type. In a passage regimen identical to the one used in this study but with a different culture medium, newborn foreskin epidermal keratinocytes can undergo more than 150 cell generations in the presence of EGF, but less than 50 without it (Rheinwald and Green, '77). The present study shows that newborn foreskin dermal fibroblasts have twice the lifespan of newborn foreskin epidermal keratinocytes in the absence of EGF, and that EGF has no effect on the replicative potential of fibroblasts. This result supports the interpretation made earlier (Rheinwald and Green, '77; Rheinwald, '79) that EGF exerts a specific effect on the keratinocyte, inhibiting the mechanism that triggers cells of this type to permanently depart from the dividing state and enter their terminal differentiation program. This mechanism may work in conjunction with the senescence mechanism in the keratinocyte, or it may be entirely independent. In the latter situation, the onset and timing of replicative senescence would be masked when EGF is not present because cells stop dividing prematurely for the purpose of differentiation. The data presented here provide no support for the hypothesis that a similar terminal differentiation program is responsible for loss of replicative capacity in the fibroblast, although a program may exist which is not subject to regulation by any of the agents tested in our study.

ACKNOWLEDGMENTS

We are grateful to Dr. Lois Jacobs for helpful discussions and for informing us of her unpublished observations. We thank Mr. Michael Beckett for excellent technical assistance and Ms. Lynne Dillon for preparing the manuscript. This study was presented at the 1980 Tissue Culture Association meeting, and has appeared in abstract form (Didinsky and Rheinwald, 1980). The research reported here was submitted as a senior honors thesis by J.B.D. to Harvard University. This project was supported by grant R01-AG-02048 from the National Institute on Aging.
LITERATURE CITED


