REVIEW ARTICLE

CELLULAR AGING
CLONAL SENESCENCE
POSTREPLICATIVE CELLS

Cellular Aging—Clonal Senescence

A Review (Part I)

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Some General Considerations of the Pathobiology of Aging

Definition of Senescence

SENESCENCE MAY BE DEFINED as a constellation of deteriorative changes in structure and function of an organism, generally occurring after sexual maturation, which results in a progressive decline in the efficiency of homeostasis and in the success of the reaction to injury. The mechanisms of biologic aging are likely to involve complex sets of interacting phenomena at many levels of organization—populations, organisms, organs, tissues, cells, extracellular connective tissues and fluids, and molecules. In thermodynamic terms, the organism appears to undergo an inexorable increase in entropy. For large populations of mature individuals, the net result is an exponential decrease in the probability of surviving as a function of time; the characteristic age range at which this exponential decline begins and the rates of decline must be subject to genetic as well as environmental controls.

Genetics and Evolution of Longevity

How many informational genetic loci are likely to be involved in the determination of life-span? I have approached this problem by estimating the proportion of known genetic loci which may have relevance to the pathobiology of aging.[1] Two criteria were used in the assessment of relevance: a) loci with allelic or mutant genes which result in abiotrophic (delayed onset) degenerative pathology that overlaps to some extent with one or more aspects of the senescent phenotype as seen in human beings and b) loci thought to be of importance to the pathobiology of aging in terms of pathogenetic mechanisms (for example, loci for DNA repair enzymes). Of the 2336 genetic loci listed in the 1975 edition of McKusick's catalog,[2] 162 (6.9%) were judged to have potential relevance to the pathobiology of aging. Assuming an upper limit for man of 100,000 informational loci,[1,2] this would give an upper limit of 6900 genes of potential relevance to aging. I would guess that no more than about 1% of

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these are likely to be of regular importance in aging as it normally occurs, giving about 70 genes. Although such calculations are highly speculative, it seems clear that the phenotype of aging is under polygenic controls. There is no single gene at which mutation can greatly decrease or increase rates of aging in all of its aspects. There do exist what I have termed *segmental progeroid syndromes*,[1] such as the autosomally recessively inherited Werner syndrome;[3] these result in accelerations of many aspects of aging, but the picture is more that of a caricature than a phenocopy of aging.[1,3]

What *kind* of genes is important to the evolution of varying rates of aging in mammals? As is the case with many morphologic developments, the evidence suggests that the evolution of longevity was comparatively rapid—too rapid to be explained by known rates of amino acid substitutions in proteins.[4-9] Evolutionary biologists have recently highlighted the potentially crucial role of changes in gene regulation presumably brought about, in part, by chromosomal rearrangements.[10] Therefore, it is likely that changes in gene *expression* rather than changes in structural genes have accounted for the evolution of longevity.

How might such alterations in gene regulation be acting? Louis Bolk provided anatomic evidence many years ago that embryologic development in man is comparatively delayed.[11,12] He suggested that such delayed maturation results in enhanced longevity as compared to nonhuman primates. This may well have been the crucial initiating type of event which set the stage for longer life-span. It is probable that subsequent important refinements also took place. For example, more efficient repair of damage to DNA could have evolved, either via greater rates of synthesis of repair enzymes from changes in gene regulation or via mutation at structural gene loci to give catalytically more efficient enzymes, or both.

Cellular Aging—Two Aspects

It is convenient to consider two sorts of questions in dealing with aging at the cellular level. First, one might ask if there are intrinsic limitations in the replicative life-spans of various normal somatic cells, and if so, what relevance such restrictions in cell regeneration might have for the debilities of aging in the organism. If the limited replicative life-span involves individual clones of cells, it could be referred to as clonal senescence, even though, as I shall point out below, the process of clonal senescence might be more accurately pictured as occurring in two stages, with only the second stage representing true senescence. Secondly, one needs to know why and how cells age once they cease replicating. There are two parts to the first question. With respect to the first part, this review will make it

clear that there is a large amount of evidence showing that large populations of normal somatic cells and individual clones of normal somatic cells eventually cease replicating. The answer to the second part of the question is less clear, but arguments will be presented which suggest that for certain aspects of the aging process, the relative rates of clonal attenuation of different cell types may be of considerable significance.

We probably know even less about the answers to the second question (senescence of postreplicative cells), even though critically important cell populations are involved, such as central nervous system neurons and myocardial cells. For this reason and also because of the author's special research interests in an analysis of the replicative life-spans of cultured cells, the bulk of the review will be concerned with Question 1 (clonal senescence).

In Vitro Studies of the Replicative Life-Spans of Somatic Cells

Historical Aspects

In 1912, Carrel published a paper, "On the permanent life of tissues outside of the organism," [13] in which he reported results with chick cells. The text of this paper was much more modest than the title. He stated that "the maximum age that tissues living *in vitro* can reach is still undetermined. Many cultures have died after less than two months, but a few were very active at the beginning of the third month of their life outside the organism." In 1913, Carrel's associate, Albert H. Ebeling, published a paper on "The permanent life of connective tissue outside of the organism," [14] describing a strain of chick embryo heart fibroblast-like cells initiated by Carrel. Again, the language of the text does not match the optimism of the title: "The experiments show that connective tissue can be kept in a condition of active growth outside of the organism for more than eleven months." There followed a paper by Carrel [15] on the "Present condition of a strain of connective tissue twenty-eight months old," in which the same chick embryo heart strain was shown to have undergone 358 passages without attenuation of its growth. Finally, in 1935, Carrel published a philosophic book [16] in which he stated (p. 173) that "colonies obtained from a heart fragment removed in January, 1912, from a chick embryo, are growing as actively today as twenty-three years ago. In fact, they are immortal." The last communication which I can discover on this famous series of experiments appeared in the 1961 edition of Parker's text on tissue culture,[17] in which Eberling is quoted as having maintained a strain of chick connective tissue in a state of active multiplication for 34 years. I take some time to outline this history in detail, as those conclusions had an enormous impact on gerontologic research for

two generations, having been widely interpreted [18] as definitive evidence that the fundamental mechanism of aging cannot exist at the levels of somatic cells. In the intervening years, careful investigations have shown that chick fibroblast-like cells have strictly limited life-spans *in vitro*.[19,20] Two explanations come to mind to explain the Carrel-Eberling results: a) Their famous cell line could have resulted from viral transformation *in vitro* by endogenous or exogenous agents. This seems unlikely, since such spontaneous transformation is not well documented with chick cells. Although Rous-induced tumors were being studied in their laboratory, such transformation seems unlikely in view of the morphologic evidence for apparently normal fibroblast-like cells and colonies; this evidence includes a 1938 photograph provided by Eberling and published in Parker's book.[17] b) More likely is Hayflick's explanation [21] that, during all those decades, their "immortal" cell line was maintained via fresh infusions of viable cells which contaminated the chick embryo extract used for the media.

The Experiments of Swim and Parker and of Hayflick and Moorhead

In 1957, Swim and Parker [22] seriously questioned the then prevalent view that cessation of growth of normal human cell cultures was attributable to artifacts of culture. They presented semiquantitative evidence for variations in longevities of cultures of fibroblast-like cells as a function of the tissue of origin. Hayflick and Moorhead [24] and Hayflick [25] subsequently published now classic papers showing that the life-spans of human diploid embryonic lung fibroblast-like cells were 50 ± 10 mass population doublings. They also established that there was a significantly greater life-span of embryonic cells as compared to adult cells. These studies have been extensively reviewed elsewhere.[26-29] Hayflick and Moorhead [24] first proposed that, in contrast to transformed cells, such as the HeLa line, diploid somatic cells have intrinsic limitations of their growth potentials and that the reproducible patterns of "senescence" of such cultures might serve as *in vitro* models for the study of cellular aging.

Numerous laboratories, including our own,[30] have since confirmed the invariability of *in vitro* senescence of normal diploid somatic cells in a great variety of media and culture conditions. Although the most detailed work has been done with human cells, the general rule that seems to be emerging is that all mammalian somatic cells have limited replicative life-spans *in vitro*.

Apparent Exceptions

Are there species whose normal somatic cells can be cultivated indefinitely? This is an important question, since a bonafide exception,

especially in a mammal, would lend strong support to the view, still held by many scientists, that the limited replicative life-span of normal somatic cells is an artifact related to inadequacies of culture media and to cumulative accidents. (One embarrassing argument which critics have developed is that all of the well-documented reports of clonal senescence have utilized serial enzymatic dissociations of cells, especially trypsin.)

There are several reports of predominantly diploid cultures of rat somatic cells of apparently unlimited growth potential,[31-34] but there are no publications establishing euploidy with the newer chromosomal banding methods.[35] In the case of certain Chinese hamster cell lines thought to be diploid or near diploid, for example, banding revealed extensive chromosomal rearrangements.[36] Even with careful use of conventional karyology, what first seemed to be chromosomally normal, apparently immortal, cell lines have turned out to have subtle aberrations, for example, in the case of rabbit fibroblast lines from Puck's lab.[37] We have found subtle chromosomal aberrations via banding procedures in long-term lines from rabbit aorta associated with changes in growth behavior; the latter could be readily missed unless quantitative monitoring is performed.[38] Even in the case of euploidy, a somatic cell line of apparently unlimited growth potential cannot be assumed to represent normal diploid cells. For example, at least a proportion of human lymphoid cell lines are euploid by banding methods,[39] yet there is evidence that they behave as neoplasms when injected into suitable hosts.[41] These various observations, therefore, do not detract from the general conclusion that, at least in the case of mammalian cells, escape from *in vitro* senescence (limited replicative life-span) can be taken as good evidence that one is no longer dealing with normal somatic cells.

There is one apparent exception from nonmammalian tissues which is worth discussing. According to Simpson and Cox,[41] promuscle cells (precursors of skeletal muscle, possibly derived from a stem-cell population of "satellite" cells) from the early tail regenerate of the lizard (*Anolis carolinensis*) do not have a limited life-span *in vitro*. A line established from sexually mature animals was cultured for 350 generations without obvious changes in growth rate; for the first 250 generations, the line retained its ability, when transferred to special medium, to express the differentiated muscle phenotype (multinucleated myotubes). Another line continues to express the muscle phenotype (by periodic testing in the fusion-permissive medium) after more than 300 cell generations in continuous culture; the diploid stem line was thought to have a normal karyotype, although such analysis is exceedingly difficult in that species. In an effort to test the hypothesis that such cell lines had in fact undergone malignant

transformation *in vitro*, cells were injected into the peritoneal cavity of irradiated lizard recipients, but no tumors were observed after 4 months. Incredibly large inocula were reported (2.5×10^{12} to 1.2×10^{14} cells), and there were no control injections of known neoplastic cells. However, skin grafts were indicative of adequate immunosuppression in the host lizards.

Cell Cultures From Donors of Different Ages

One would like to know whether or not cultures established from a given individual to some extent reflect his or her biologic age. With respect to the replicative life-span of human skin fibroblast cultures, we have reported a regression of −0.20 cell doublings per year from ages 0 to 90.[30] However, this study has been criticized in failing to show significant regression in the critical age range from 20 to 100.[42] More recent studies [43] with a larger number of individuals in this age range (N = 104) gave a similar regression (−0.18 cell doublings per year, standard error = ± 0.05), although with a correlation coefficient of only 0.33. The regression was highly significant ($P < 0.001$) despite the great variance. We believe the large variance is attributable to the following methodologic considerations: a) only small samples of skin are explanted (two 1-cu-mm specimens), so that "hot spots" and "cold spots" of cultivable cells may be expected—that is, the distribution of cells capable of growth in culture is likely not to be random in the specimen of skin, but clustered; b) many different batches of newborn calf serum were employed, undoubtedly varying in their amounts of mitogenic substances; c) samples were taken from patients with a great variety of illnesses and some were antemortem and some postmortem. Mitsui and Schneider [44] have rectified the second and third problems and have provided good evidence of a decreased growth potential (with respect to many parameters) of cells from normal subjects ages 21 to 36 compared to normal subjects ages 63 to 92. Earlier studies by Goldstein *et al.*[45] (skin biopsies) and by LeGuilly *et al.*[46] (liver biopsies) showed a decline in growth potential as a function of age of donor, although Goldstein now believes that a statistically significant regression does not emerge from his data when diabetic and prediabetic subjects are separately considered.[47] (We shall discuss the role of genotype below.)

Using two independent *in vitro* methods, Martin *et al.*[48] demonstrated a decline, as a function of age, in the replicative potentials of cells of the aorta and adventitial tissues of mice. In one method, autoradiographic analysis of tritiated thymidine labeling patterns in organoid cultures permitted identification of cell types involved in the decline of replicative potential. All such cell types examined (endothelial lining of intima,

medial smooth muscle, adventitial fibroblasts, adventitial adipocytes, endothelium of adventitial blood vessels, adventitial mesothelium) showed a decline in labeling indices. Of interest was the approximately 100-fold range in young mature animals (6-month-old mice) in the labeling indices as a function of cell type (from circa 0.5% for the case of medial smooth muscle to about 50% for the case of adventitial mesothelium). Primary and secondary cloning studies also showed a consistent decline, with age, of clonable cells from the aortic wall.

In preliminary experiments with a most promising clonal cell culture system, Rheinwald and Green [49] reported data suggestive of a decreased replicative potential of epidermal cells from older normal subjects as compared to younger normal subjects.

We can conclude that the replicative potential of cultivated human somatic cells reflects, to some degree, the age of the donor of those cells. One could argue, of course, that in the best studied tissue (dermis), there is only a comparatively small change in growth potential over the life-span and that there is no evidence that such a change is in any way detrimental to the host. A counter-argument could be that any detectable change at all is remarkable in view of the fact that we are constantly selecting for the residual cells most capable of growth. In any case, what we need to do is examine in more detail those cell types and tissues which are likely to have greater relevance for the senescent phenotype, such as cells derived from blood vessels. Especially needed are studies in which *in vitro–in vivo* correlations can be made. This cannot be done in either the case of human skin cultures or fetal lung cultures such as WI-38, since there is no certainty of the cell type of origin and, in any event, it would be quite difficult to isolate many kinds of cells from those particular organs.

Cultures From Donors of Various Genotypes—Interspecific Differences

In view of the power of a comparative biologic approach, I am surprised at how few studies have been carried out in which cell cultures have been studied from species of contrasting life-spans. The rationale is simple: one attempts to establish a correlation between a given parameter in culture and the life-span of the species from which the cells were obtained. One does not require enormous resources of animal colonies. Once explant cultures are established, early passages can be cryobiologically preserved as multiple aliquots. Hopefully, granting agencies will support systematic banking of such materials at such national resources as the American Type Culture Collection and the Institute for Medical Research. The biopsies should be obtained from comparable tissues from comparably aged donors (ideally with samples from both young sexually mature adults and

senescent individuals). There is always the risk that, because of contrasting features of the histology or because of contrasting nutritional needs (including differential effects of various serum mitogens), different cell types may be preferentially selected from different species. This risk should be minimized by restriction of one's studies to a series of mammals. The most convenient source of biopsy is the skin (dermis), from which fibroblast-like cells typically emerge, but the use of better defined cell types, for example, epidermal cells,[49] would be desirable.

Although the parameter of replicative life-span of cultivated somatic cells has had a great deal of attention, there has yet to appear a well-controlled study in which a variety of mammalian species were studied in the same laboratory with comparable media and other techniques. Comparisons with widely different species is unsatisfactory. For example, the comparatively long life-span of the Galapagos turtle [50] is difficult to interpret because this animal is poikilothermic. Cultures from the skin of a kangaroo may yield cell types different from those derived from a rabbit.[51] Numerous laboratories (including my own) can testify that normal diploid mouse fibroblast-like cells have much reduced life-spans in comparison with human fibroblast-like cells, but there is little hard data. The paucity of information is attributable, in part, to the propensity of mouse cells to transform in culture. In our experience, in nonhuman species, spontaneous transformation to cell lines of apparently unlimited growth potential may sometimes occur insidiously, detectable only by quantitative assessments of growth rates in serial passages and by careful chromosomal banding.[38] Thus, it is difficult to interpret studies such as those of Stanley *et al.*,[51] in which the time of onset of transformation was arbitrarily determined by the time of appearance of heteroploidy. In the Stanley study, however, there were some striking discordances between the longevity of a species and the longevity of its cells in culture. These findings deserve to be followed up with experiments in which there are more rigorous controls, especially regarding the exact site of biopsy, developmental stage of the donor, and criteria for spontaneous transformation. Of special interest was their report of a single culture, from the fat-tailed dunnart (an animal with a maximum life-span of only 3 years), which was still diploid and still actively growing after 175 cumulative population doublings.

There have been two other lines of experimentation in which comparisons were made among cultures derived from species of contrasting life-spans. The results are of considerable interest in that both lend support to the intrinsic mutagenesis theory of aging,[52] at least in my broadly interpreted version of that hypothesis.[1] In brief, the idea suggests

that the genetic loci which account for the bulk of the forty- to fiftyfold variance of maximum life-span observable in mammals are those responsible for the quantitative differences in somatic mutation rates and their consequences. In Table 1, I have listed some likely candidates for such loci, notable among which are the DNA polymerases,[53] which might be expected to replicate DNA with varying degrees of fidelity, and the DNA repair enzymes,[54] which might be expected to repair various modalities of DNA damage with differing efficiencies, depending upon the genetically determined amino acid sequences of the given homologous enzymes in the different species.

Given a certain level of environmental premutagens, different animals are liable to metabolize them in different ways. Therefore, mixed-function oxidases (aryl hydrocarbon hydroxylases) and enzymes which inactivate proximal mutagens [55] should be listed.

Finally, it has been shown that abnormal proteins, the products of many varieties of somatic mutations, are preferentially degraded by proteases.[56,57] Therefore, genetic loci coding for such scavenger enzymes are likely to be of significance in modulating the consequences of somatic cell mutation.

The first such comparative study along these lines was reported in 1974 by Hart and Setlow.[58] They established early passage cultures from a series of biopsies of superficial limb dermis of a series of mammals which had completed an estimated 1/20th of their characteristic life-spans. The latter ranged from circa 1.5 years (*Sorex cinereus*, the shrew) to 95 years (their estimate for *Homo sapiens*). Unscheduled DNA synthesis—a measure of excision repair—was measured autoradiographically (grain counts of nuclear ^{3}H-thymidine incorporation) at various times after exposure to various intensities of ultraviolet light (primarily 254 nm); normal semiconservative DNA replication was blocked with hydroxyurea. A striking direct correlation was obtained between the extent of excision repair (measured 13 hours after exposure to various ultraviolet light fluences) and the characteristic species life-span. Assuming that the excision repair system is important in the restitution of modalities of injury caused by

Table 1—Examples of Genetic Loci of Relevance to the Intrinsic Mutagenesis Theory of Aging;[52] Listed are Classes of Proteins Specified or Regulated by Such Genes

DNA polymerases
DNA repair enzymes
Activating enzymes for premutagens (e.g., aryl hydrocarbon hydroxylases)
Inactivating enzymes for mutagens
"Scavenger" enzymes which preferentially degrade abnormal proteins (various proteases)

agents other than UV, the results are consistent with the intrinsic mutagenesis hypothesis. One reservation cited by the authors was that individuals with xeroderma pigmentosum, most of whom have defective excision-repair, do not appear to show premature aging in tissues other than in the skin (in which a variety of sunlight-induced neoplasms arise, most of which are of the common age-related types). Recently, however, a correlation has been discovered between the severity of the enzyme defect and the clinical findings of central nervous system impairment;[59] it remains to be seen to what extent these findings and other tissue pathologies are concordant with what is observed in a normal aging population.

In 1975, A. G. Schwartz[60] reported an inverse correlation between species life-span and capacity to activate 7,12-dimethylbenzanthracene to a mutagenic form. Israeli scientists had previously reported[61] that, whereas diploid rodent fibroblasts had such activity, human fibroblasts did not. The methodology was as follows: Early passage normal diploid fibroblasts from a given species were plated on an irradiated feeder layer. (Such feeders are able to metabolize but unable to replicate.) An "indicator" Chinese hamster cell line (V79) was co-cultivated with the feeder layer in the presence of various concentrations of dimethylbenzanthracene (DMBA). Unlike normal hamster cells, the indicator cell line cannot metabolize DMBA and has an indefinite growth potential. The V79 cells were then replated to determine a) clonal viability and b) frequency of clones resistant to 8-azaguanine (a measure of the incidence of forward mutation to deficiency of the enzyme hypoxanthine guanine phosphoribosyl transferase). In interpreting the results of an inverse correlation between species longevity and induced mutation frequency, the author pointed out that varying rates of *inactivation* of the reaction form, as well as varying rates of DMBA *activation* may have been responsible.

Schwartz is currently extending these studies to include a larger series of animals. It would also be of interest to use other markers, such as oubain resistance (an autosomal dominant).[62]

These apparently straightforward studies might be interpreted in a different fashion, and it is for this reason that I discuss them in the context of clonal senescence. Suppose that, in culture, many somatic cell types undergo a differentiation step or differentiation steps analogous to what occurs *in vivo*. This might result in functionally heterogeneous cultures with respect to the parameter being measured (for example, DNA repair or ability to metabolize a premutagen). It is conceivable that, at comparable passage levels, the ratios of "stem cells" to putative differentiated cells might vary as a function of the species of origin in a systematic fashion related to the longevity of these species. *In vitro* differentiations

clearly take place with cultured epidermal cells [63] and myoblasts.[64] We have suggested the possibility that such events may also be taking place with the fibroblast-like cells cultured from dermis. In any case, evidence consistent with extensive epigenetic heterogeneity in such cultures will be discussed later in this review. Hart and Setlow have themselves shown cellular heterogeneity in cultures of human fibroblast-like cells with respect to DNA repair. There was an excellent correlation between the ability of cells to carry out repair synthesis and their ability to carry out semiconservative DNA synthesis.[65] Normal terminally differentiated cells, such as muscle cells have deficient DNA repair.[66] Thus, the results of Hart and Setlow and of Schwartz might be more related to epigenetic than to genetic differences.

Donors of Various Genotypes: Intraspecific Differences

Martin *et al.*[30] found that cultures of skin fibroblast-like cells from 4 patients with Werner syndrome had growth potentials less than two standard deviations below the means of cohorts of age-matched and biopsy-matched controls. Confirmation that skin fibroblast-like cells from that genotype undergo rapid *in vitro* senescence has come from Stecker and Gardner,[67] Nienhaus *et al.*[68] and Holliday *et al.*[69] However, Nienhaus, in repeat biopsies from the skin and breast of a patient previously shown to have skin cultures of very limited life-span,[68] observed life-spans almost as great as those from an age-matched control. In our experience, there may be significant variance from biopsy to biopsy from the same patient.[43] As indicated above, it seems probable that the distribution of cells capable of establishing cultures is nonrandom, so that biopsies from "hot spots" and "cold spots" may occur. Thus, many replicate cultures may be necessary to establish enhanced or diminished growth potential from a given genotype. However, in the case of Werner syndrome, in an extensive series of cultures established from many different skin biopsies taken at autopsy from 1 patient,[70] we have never observed a degree of growth potential even approaching what we observed in numerous controls from a variety of autopsy materials. Therefore, it seems well established that the cells which grow out from the skin of patients with Werner syndrome are unquestionably deficient with respect to growth potential

The rationale for investigating cultures from patients with Werner syndrome was that it was a genetically determined disorder (autosomal recessive) with many features strikingly suggestive of accelerated aging (hence the synonym *adult progeria*).[3] One observes premature graying and loss of hair; cataracts; premature onset and rapid progression of various forms of arteriosclerosis; an increased incidence of neoplasia,

diabetes mellitus, osteoporosis; atrophy of skin and muscle; a striking regional atrophy of subcutaneous areolar connective tissue; ulceration of the extremities; and early death, typically from a myocardial infarction in the middle or late forties. Therefore, the finding of rapid "senescence" of cultured cells from such patients would seem to strengthen Hayflick's hypothesis that aging of cells in culture may serve as a model of *in vivo* aging.[24-28]

However, I now have increasing reservations about such a conclusion for the following reasons: a) As pointed out by Epstein *et al.*,[3] Werner syndrome presents more of a *caricature* of aging than a *phenocopy* of aging. If one examines the phenotype critically, one finds many discordances with what is observed in usual senescence. For example, the spectrum of neoplasms is unusual, with a preponderance of non-carcinomatous lesions (sarcomas and meningiomas). Other discrepancies are given by Epstein *et al.*[3] and by Martin,[1] the latter pointing out, among other things, the lack of clinical and neuropathologic evidence for senile dementia. b) The cultured cells from patients with Werner syndrome show a strikingly high frequency of what we have termed *variegated translocation mosaicism*.[70] There is the repeated occurrence of non-constitutional chromosomal rearrangements, with resulting multiple clonal mosaicism of pseudodiploid karyotypes. Although the published evidence dealt with multiple cultures from a single individual, our laboratory has now observed variegated translocation mosaicism in cultures of skin fibroblast-like cells from other patients with Werner syndrome. In contrast, a careful analysis of karyotypes from early, middle, and late passages of control cultures did not reveal such mosaicism.[70] Although the phenomenon is not unique to Werner syndrome (it has been observed in two other instances, including one well-documented series of cultures in a patient who clearly did not have Werner syndrome), it is possible that a characteristic spectrum of chromosomes were involved in cells from patients with Werner syndrome.[70] In any case, the great majority of skin cultures in our laboratory provide stable euploidy throughout their *in vitro* life-spans, with the exception of variable degrees of tetraploidy. This indicates that the life-span of fibroblasts from patients with Werner syndrome may be limited for reasons other than those of normal fibroblasts.

The life-span of fibroblast-like cells from the skin of patients with the Hutchinson-Gilford syndrome (also called *progeria of childhood*) has also been claimed to be limited, but this has not been extensively documented.[71] In our own study, a culture from a 4-year-old girl with progeria ranked 23 of 26 in that particular age-group.[30] Progeria is of special interest because of the spectacular rate of progress of atherosclerosis.[72]

Therefore, it would be of interest to examine the growth potential of arterial endothelium and smooth muscle from such patients. However, as in the case of Werner syndrome, the Hutchinson-Gilford syndrome differs from usual aging in many ways.[1]

Goldstein *et al.*[45] compared the cloning efficiencies of skin fibroblast-like cells derived from normal subjects and from the progeny of conjugal diabetics. They found that the prediabetics had reduced growth potential by that assay. In the study of Martin *et al.*,[30] in which the growth potentials of mass cultures of skin fibroblast-like cells were determined from 100 subjects, 9 patients yielded cultures which fell below the 95% confidence limit of the regression line. Of these, 4 had diabetes mellitus (3 had Werner syndrome, and 1 had juvenile diabetes).

Subsequently, Vracko and Benditt [73] reported decreased growth with cultures from 3 adult patients with insulin-dependent diabetes. More recently, Goldstein [47] claims that a statistically significant negative regression of growth potential dependent on donor age is only evident for the case of diabetic subjects.

These observations have important implications for the pathogenesis of the vascular pathology of diabetics and of the similar pathology found in aged individuals. Some of these implications have been discussed by Vracko and Benditt [73] and Vracko.[74] It is now necessary to extend such studies to diabetics of well-characterized types, since the disorder is very heterogeneous in terms of genetic subtypes.

In the survey I carried out concerning the potential relevance of various genetic syndromes to the pathobiology of aging, the Down syndrome (trisomy 21) ranked at the top.[1] Is there any evidence that somatic cells from such individuals have deficiencies in cell replication *in vitro*? To date, only skin fibroblast-like cells have been examined. Schneider and Epstein [75] found that such cultures from 4 trisomic patients yielded replicative life-spans of 40 ± 1.5 cumulative populations, in comparison to the 51.5 ± 2.0 in age-matched controls. The trisomic fibroblasts appeared to maintain a slower rate of replication throughout the history of the serially passaged cultures.

What Limits the Replication of Life-Spans of Cultivated Cells?

It is convenient to divide the various theories which have been proposed to explain the limited growth potential of normal somatic cells into two broad categories: those which invoke changes in the *structure* of the genetic material and those which invoke changes in the *expression* of the genetic material. The intrinsic mutagenesis theory [52] has already been mentioned as one example of the former class. *A priori*, somatic mutation

would seem to be more likely to be of relevance to the aging of post-replicative cells, since, in proliferating populations, one would expect strong selection against cells which accumulate mutations. Hoehn *et al.*[76] have approached this question by examining the replicative life-span of tetraploid hybrid clones (obtained via Sendai virus–mediated fusion). If dominant mutations were involved, then the availability of four targets per locus would result in more rapid reproductive death of the clone. If random recessive mutations were involved (far more likely, since most point mutations are recessive) then one should expect complementation in tetraploids; such hybrids should therefore have greater life-spans. The results, although preliminary (since the large variance of clonal life-spans requires large sample sizes for analysis), revealed that hybrid tetraploid clones had neither unusually short life-spans nor unusually long life-spans, making somatic mutation an unlikely explanation for the reproductive death of the clones. Littlefield [77] came to a similar conclusion on the basis that he failed to obtain proliferating hybrids between normal fibroblasts and senescent fibroblasts. He correctly pointed out, however, that complementation might not occur if there were very large numbers of somatic mutations.

There has been great interest in the "error catastrophe" theory of Leslie Orgel [78,79] as an explanation of *in vitro* cell aging; in its modified form,[80,81] somatic mutations also are considered to occur and to play an important role in the ultimate reproductive death of cells. Orgel called attention to the potential for positive feedback if a cell made a mistake (either during transcription or translation) in the synthesis of protein which itself was used in the synthesis or in the posttranslational modification of other proteins. For example, an aberrant RNA polymerase or tRNA methylating enzyme might readily result in an amplification in the proportion of abnormal proteins within a cell, whereas this would not be expected in the case of an aberrant collagen molecule. In his original publication, Orgel [78] deduced that the error frequency would increase exponentially, leading to an error catastrophe and the death of the cell. In a subsequent correction [79] he concluded that such a catastrophe need not occur. Theoretical aspects have been further discussed in the literature,[82-85] with the weight of the arguments, it seems to me, being against the Orgel hypothesis. At the experimental level there is also conflicting evidence. Holliday and Tarrant [80] have shown that, in MRC-5 cells (derived from a human fetal lung), the proportion of heat-labile glucose-6-phosphate-dehydrogenase (G6PD) rises from 4% to 20% as the cultures undergo senescence. This was interpreted as evidence in favor of Orgel's hypothesis, since it is known that abnormal proteins, including mutant forms of human G6PD,

are typically heat labile. Increasing levels of heat-labile enzyme have also been reported by Goldstein.[86-88] Of interest also are reports of high baseline levels of heat-labile enzyme in cultures from patients with progeria and Werner syndrome.[69,86-88] However, Pendergrass *et al.*[89] failed to uncover any heat-labile G6PD in their aging cultures of a human skin fibroblast cell line, even though such could be demonstrated with model systems. Moreover, they could find no evidence of increasing titers of abnormal enzymes using an immunologic probe. Since Orgel's hypothesis predicts an involvement of *all* proteins by the time cultures are fully senescent, they concluded that an error catastrophe had not occurred in their cultures. Evidence against the error theory as an explanation for the limited replicative life-span of cultures has also been presented by Ryan *et al.*,[90] by Tomkins *et al.*,[91] and by Holland *et al.*[92] The latter experiments were especially compelling pieces of negative evidence, in that a sensitive viral probe was employed. Since polio viruses utilize host synthetic machinery, increasing yields of mutant particles might be expected in aged cultures if their protein synthetic apparatus showed decreasing fidelity. Holland *et al.*[92] found no change in the yield of guanidine-resistant mutants in aged WI-38 cells.

As mentioned above, errors in protein synthesis could result in somatic mutations; this might be the case if abnormal DNA polymerases were involved. Fulder and Holliday[93] concluded that such was the case in aging cultures of MRC-5 and that, moreover, the kinetics of accumulation of mutant cells was exponential, arguing in favor of a protein error catastrophe theory as the underlying mechanism producing the mutations. However, I do not believe the evidence for mutagenesis was compelling. The phenotype, cytochemically detectable "high activity" variants for G6PD, could have resulted from a variety of mechanisms other than mutation—for example, the rates of synthesis, activation, or turnover of G6PD in postreplicative cells (largely arrested in G_1 of the cell cycle) might result in higher specific activities. With increasing passages of cultures, there is an increased heterogeneity of cell size, with an increasing proportion of G_1 (or G_0) cells, many of which have an abundance of cytoplasm and, therefore, an abundance of G6PD; such cells are more likely to score cytochemically as high activity cells. In any case, the authors themselves stated that there appeared to be a "continuous spectrum of phenotypes." Finally, it is quite possible that the apparently clonal foci of the high enzyme variants could simply be explained by focal piling up of cells, a common feature of fibroblast cultures; this is suggested by Figure 1c from their paper.

More important evidence for somatic mutation in old cultures is given

by Linn and Holliday,[94] who found that comparatively crude extracts of DNA polymerase from late passage senescent MRC-5 cells exhibited greater infidelity when replicating synthetic templates than did DNA polymerase from early, vigorously proliferating passages. On the other hand, in preliminary experiments with senescent cultures of chick embryo fibroblasts, Fry and Weisman-Shomer [95] found no evidence of increased infidelity. Although there was physical chemical evidence that the properties of this polymerase from old cultures was distinctive, I believe that the data of Fry and Weisman-Shomer is most consistent with an isoenzyme shift and therefore a change in gene expression rather than a change in gene structure.

Several authors have stressed the occurrence of gross chromosomal aberrations in senescing cultures of human diploid fibroblasts.[96-99] However, none have presented compelling evidence that such changes cause the cultures to cease replicating. As discussed above, we have failed to find a significant frequency of such chromosomal aberrations in fibroblast cultures from normal donors, even during their terminal phases of senescence.[70]

Regarding models based upon changes in gene expression, one possibility is that there is a genetic program which acts as a biologic clock and which counts cell divisions, replication ceasing after 50 ± 10 doublings (the "Hayflick limit"). A molecular mechanism for such counting has even been considered.[100] It is now clear that there cannot be any such simple biologic clock. While it is true that the Hayflick limit applies to many *mass* cultures of human embryo fibroblast-like cells, there is marked variation in the life-spans of individual clones within such cultures.[101-102] Serial subcloning experiments show that variations in growth rates are reproduced among the progeny of clones in a fashion that essentially rules out a simple clock mechanism.[102] Such experiments show bimodal distributions of the growth of subclones, with a gradual attenuation of clonal growth such that ever increasing proportions of cells are of the slower growing class. Similar results have been obtained for serial subclones grown on feeder layers, which insures a more optimal nutrition.[103] Martin *et al.* interpreted their results as consistent with the *in vitro* differentiation of stem cells into terminally differentiated, postreplicative cells.[102,104] On direct microscopic observation, they could sometimes observe asymmetric mitosis, one daughter cell continuing to replicate, the other transforming into a large, postreplicative cell type. On the basis of error theory, one would have to postulate an asymmetric distribution of abnormal proteins to one of two daughter cells—a highly improbable event.

Martin *et al.* speculated that the postreplicative "fibroblast" might be a

type of histiocyte or macrophage.[102] Subsequent work, however, failed to reveal any C3 or IgG receptors on such cells.[104] Thus, there is as yet no evidence to indicate that there are qualitative, or even quantitative, changes in gene expression in the large, postreplicative "senescent cell."

It has been pointed out that the serial subcloning studies referred to above (clonal attenuation) formally demonstrated that the process of *in vitro* aging was stochastic.[104] This does not at all argue in favor of error theories and against the terminal differentiation theory, since the process of differentiation is itself presumably subject to probability laws.[105]

Martin *et al.*[104] suggested that the limited replicative life-span of cultivated somatic cells (clonal senescence) might best be regarded as a two-stage process. In Stage I, there is a process of clonal attenuation in which there is a gradual slowing of growth associated with a stepwise *in vitro* differentiation leading to a postreplicative, terminally differentiated cell. (It is possible that, *in vitro*, one is dealing with a distorted caricature of normal differentiation as it would occur *in vivo* for the given cell type.) In Stage II, true senescence would occur in postreplicative cells, at rates depending upon cell type and culture conditions. This senescence might best be defined as a deleterious change in the structure and function of cells with which there are associated abnormal molecules, most of which presumably result from posttranslational modifications. Such a model is simplified in that various types of cell–cell interactions which modulate these processes undoubtedly occur.

In order to test the terminal differentiation hypothesis as an explanation for why cells stop cycling *in vitro*, Norwood *et al.*[106,107] performed heterokaryon experiments modeled after those of Harris and co-workers.[108] It will be recalled that these workers fused bonafide terminally differentiated postreplicative cells (such as nucleated hen erythrocytes and rabbit peritoneal macrophages) with cells capable of undergoing DNA synthesis and found that, in all cases, DNA synthesis was reinitiated in the nucleus of the terminally differentiated cells.[108] When Norwood *et al.*[106] made such heterokaryons between human skin fibroblasts and either isologous or homologous early passage actively replicating human skin fibroblasts, they found the opposite result: not only did the young cells fail to reinitiate DNA synthesis in the old cell, but the DNA synthesis of the young cell was itself suppressed. When the experiments were repeated,[107] however, with replicating cells of a type comparable to those used by Harris *et al.*[108]—namely those from transformed, heteroploid or "neoplastoid" cell lines[107]—the results observed by Harris were obtained, at least for the first 24-hour pulse period after fusion: DNA synthesis in the senescent nuclei was reinitiated by SV40 fibroblast and by HeLa cells

(although in the latter, suppression of DNA synthesis in both nuclei was observed in subsequent labeling periods). The two types of experiments suggested to us some type of regulatory defect in the senescent cell and not an irreversible change in gene structure. More recent experiments of Norwood *et al.*,[109] however, raise the possibility that HeLa cells and SV40-transformed fibroblasts may differ from normal young actively replicating fibroblasts in having more efficient repair mechanisms (scavenger proteases and DNA repair enzymes). In brief summary, young fibroblasts were treated with either mitomycin C, a DNA cross-linking agent, or with a combination of amino acid analogs, the resulting population having very low replicative activity, comparable to what is observed in senescent cultures. In both instances, the results of heterokaryon experiments with young diploid fibroblasts or with HeLa cells were strikingly similar to what was observed in such crosses with senescent cells: The DNA synthesis of normal cells was extinguished, while the DNA synthesis of the drug-treated cells was reinitiated by HeLa cells. Norwood and colleagues noted that the drug-treated cells differed from the senescent cells with respect to other properties and therefore could not be regarded as phenocopies of cells which have become spontaneously "senescent" as a result of serial *in vitro* culture.

One can conclude this section by stating that we still do not know what limits the replication of cultivated normal somatic cells.

In Vivo Studies of the Replicative Life-Spans of Somatic Cells

Segments of mouse skin have been successfully serially transplanted to immunologically compatible hosts in attempts to discover if there is an intrinsic limitation in the ability of the constituent cells to undergo regenerative hyperplasia and to determine if there are differences in the life-spans of skin obtained from old versus young donors.[110,111] A serious problem in those experiments was that for most cell types which proliferate within the heterogeneous tissues it was not possible to differentiate an origin from host versus an origin from donor; this might be especially crucial in the case of vascular tissue. Nevertheless, evidence was presented by Krohn that there appears to be an eventual limit to the life-spans of such grafts, although in such cases the time of survival was clearly greater than that of the typical host life-span. Moreover, in the case of the strain A mouse (but not of the longer-lived CBA strain), skin from young donors lived substantially longer than skin from old donors.

A more satisfactory *in vivo* system for the evaluation of the proliferative life-spans of serially transplanted tissues was developed by Hoshino and Gardner[112] and Daniel.[113,114] The methodology, originally developed by

DeOme,[115] involved the surgical transfer of mouse mammary gland tissue into an inguinal fat pad from which resident mammary tissue had been previously removed. In the first reported experiments, Hoshino and Gardner [112] concluded that transferred mammary parenchyma could likely survive indefinitely (they made seven passages to a total of almost 4 years). Daniel [113,114] challenged this conclusion on the grounds that not enough data were presented and that the transplantation intervals were excessively long. Daniel quantitated the growth rates of transplants and serial passages; in all cases, the growth rate declined with time, the oldest transplant line being lost after seven generations in slightly more than 2 years. With two lines of experiments, it was shown that the life-span is related more to the numbers of cell divisions than to chronologic time, paralleling the case for *in vitro* clonal senescence [116,117] (although some contrary evidence for the case of cell culture had been published [118,119]).

Young *et al.*[120] failed to demonstrate any difference betwen the life-spans of serially passaged mammary glands from old mice (26-month-old BALB C) versus young mice (BALB C). However, these authors pointed out that they were assaying for ductal elongation, a process which ceases after normal development, with expansion of the gland to fill the entire fat pad. Since the duct system is mitotically quiescent in virgins, one might not expect significant differences in growth potential between young versus old donors.

In a series of excellent experiments by both Harrison [121,122] and Micklem and Ogden,[123] using serially transplanted hematopoietic cells, rather similar conclusions were reached, even though, in that system, there is abundant post-developmental cellular proliferation. Although lines of serially transferred hematopoietic cells eventually ceased replicating, there was no evidence that the life-spans of such cells from old donors was less than those from young donors. It seems to me that the hematopoietic system is unlikely to be rate limiting in the aging process, since natural selection, in the face of such challenges as hemolytic parasitism and repeated trauma and hemorrhage, must have resulted in an enormous reserve capacity of stem cells; any differences between old and young would therefore be very difficult to demonstrate. Micklem and Ogden[123] themselves suggest that the failure to discover any differences may be due to a pool of very primitive stem cells which divide very seldom and thus are not subject to aging changes.

It remains possible, however, that in certain special genotypes, the pool of hematopoietic stem cells and the kinetics of their proliferation might be such that differences could be demonstrable between old and young. Harrison's data in fact suggest that this might be the case for strain DBA/2J mice.[122]

In a most elegant series of experiments, Williamson and Askonas [124] have monitored the life-span of a clone of antibody secreting cells upon serial passage to irradiated syngeneic mice. Selection and propagation of a single clone of antibody-forming cells was achieved by transfer of limited numbers of spleen cells and identification of characteristic secreted antibody (to dinitrophenyl bovine γ-globulin) via isoelectric focusing on polyacrylamide gels, the specific antibody molecules being visualized by ^{131}I hapten binding and autoradiography. There was a relatively constant and high production of antibody throughout the first four transplant generations, followed by an exponential declines during transplant generations 5, 6, and 7. The authors suggested that the proliferative potential of memory cells was responsible for a characteristic life-span, clonal senescence ultimately ensuing as a result of terminal differentiation to plasma cells. They calculated that, beginning with an original precursor stem cell, perhaps 90 cell doublings occurred before senescence, which they pointed out was equivalent to the replicative life-span of human diploid fibroblast cultures, if one made certain assumptions suggested by Martin *et al.*[30] Since there is no reason to expect different stem cell lines to have comparable replicative life-spans (especially from different species), such apparently concordant numerology cannot be taken as an argument that the *in vivo* senescence observed by Williamson and Askonas and the *in vitro* senescence observed by Hayflick [24,25] and Martin *et al.*[30] are comparable processes. However, there are similarities in the kinetics of the process, and the theory which Williamson and Askonas invoke to explain the *in vivo* clonal senescence (terminal differentiation) is exactly that developed independently by Martin and his colleagues [102,104] to explain *in vitro* clonal senescence. (Terminal differentiation was also suggested as a mechanism of limiting *in vitro* life-span by Cristofalo.[29])

Asynchronous Clonal Senescence and the Pathogenesis of Age-Related Disorders

In this section, I make some largely speculative suggestions as to how the phenomenon of clonal senescence might contribute to the pathogenesis of diseases which dramatically increase in incidence and severity in association with aging.

Although virtually all of my arguments so far have gone toward convincing the reader that the proliferative potential of various cell types declines as a function of age, I am more impressed with the fact that the senescent phenotype is characterized by a *proliferation* of a variety of cell types. Some of these are listed in Table 2.

It would be of great interest to investigate other anatomic and functional aberrations of the aged from the point of view of cell *proliferation* rather than cell degeneration, which has been the traditional point of

Table 2—Examples of Cell Proliferations Which Accompany Senescence in Human Beings

Cell type which proliferates	Associated age-related disorder
Arterial myointimal cells	Atherosclerosis
Cartilage cells	Osteoarthrosis (osteoarthritis)
Fibromuscular stromal cells and glandular epithelium	Benign prostatic hypertrophy
Epidermal melanocytes	Senile lentigo ("liver spots")
Epidermal basal cells	Seborrheic keratosis (verruca senilis)
Sebaceous glandular epithelium	Senile sebaceous hyperplasia
Adipocytes	Regional obesity
Fibroblasts	Interstitial fibrosis of parenchymal organs (e.g., thyroid)

departure. For example, is it possible that the age-associated increasing serum levels of amyloid [125] and the presumptively associated amyloid deposits in senescent individuals could be associated with proliferations of reticuloendothelial cells? Could certain of the immunologic dysfunctions of the aged be related to selective proliferations of subsets of suppressor cells or to proliferative imbalances of various cell types involved in the coordination of their immune response? [126] Most importantly, are the remarkable age-related increases in the incidences of various carcinomas related to multifocal selective hyperplasias? Aging might well be the most powerful and universal promoting agent in the two-step process of carcinogenesis.[127]

How can one explain the apparent paradox of selective cell proliferations (Table 2) in the face of steady, age-related decline in replicative potentials of various cell types? In the case of atherogenesis (which is a strongly age-dependent process), my late colleague Curtis A. Sprague and I have proposed a model based upon the hypothesis that cell proliferation is subject to negative feedback controls.[48,107,128] We had shown that the growth potential of presumptive smooth muscle cells from the abdominal aorta, a region subject to severe atherosclerosis, was reduced in comparison to the growth potential of comparable cells from the thoracic aorta, a region relatively free of atherosclerosis. We imagined that since myointimal cells are closely related to medial smooth muscle cells, they are subject to comparable regulatory controls and that diffusible macromolecules from the quantitatively larger mass of medial smooth muscle would predominate in such control. We further hypothesized that, under equilibrium conditions, loss of differentiated smooth muscle cells was compensated by the release, from feedback inhibition, of regional "stem cells" capable of yielding new differentiated progeny. If there were regions within the vascular tree in which there was a comparatively rapid rate of clonal senescence of medial cells, with depletion of stem cells (as

our experiments suggested), the effete differentiated smooth muscle cells would no longer be replaced at suitable rates, leading to a decline in the local concentrations of diffusable inhibitory macromolecules. This could then set the stage for multifocal proliferations of myointimal cells. It should be noted parenthetically that such proliferations could, in principal, be monoclonal or oligoclonal [102] and would therefore be consistent with the findings of Benditt and Benditt [129] for the case of small human atheromas.

The fundamental concept in the above discussion is that paradoxical proliferations might occur in situations in which there is *asynchronous* clonal attenuation and clonal senescence occurring among related families of differentiated cell types. I propose that such asynchronous clonal senescence is a common consequence of aging in complex, multicellular organisms.

It is now essential to rigorously pursue the purification and characterization of various cell type–specific mitotic cell cycle inhibitors, or chalones.[130-133] One of the earliest clues that negative feedback might exist for the maintenance of proliferative homeostasis derived from experiments with aortic smooth muscle-intimal explant cultures.[134] Therefore, it is remarkable that almost 40 years elapsed before even preliminary attempts were made at isolating a crude putative aortic smooth muscle chalone.[135] In the interim, there have been major advances in protein chemistry and in our ability to culture and identify specific cell types and tissues for the bioassay of such materials.[38]

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