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Evidence for a relationship between longevity of mammalian species and life spans of normal fibroblasts *in vitro* and erythrocytes *in vivo*

(cell ageing/replicative potential/cell cultures/phase III/chromosomes)

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ABSTRACT **The replicative life spans of mammalian fibroblasts *in vitro* were studied in a number of cell cultures representing eight species. Emphasis was placed on determining the population doubling level at which phase III (a period of decrease in the rate of proliferation) and chromosomal alterations occur. All the cell cultures studied went through a growth crisis, a period of apparent growth cessation lasting for at least 2 weeks. In most cultures, the crisis represented the end of their replicative capacities, but in some cultures cell proliferation was resumed after the crisis. A predominantly diploid chromosome constitution (more than 75%) was demonstrated prior to the growth crisis. In cultures in which cell proliferation was resumed after the crisis, a nondiploid constitution prevailed in all cases except the rat (with 90% or more diploid cells all the time). The growth crisis occurred at population doubling levels that were characteristic for the species and was shown to be related to the species' maximal life span by a strict power law, being proportional to the square root of the maximal life span. Based on data in the literature, the same relationship was also valid for the lifespans of circulating mammalian erythrocytes *in vivo*. These results may indicate the prevalence of a common functional basis regulating the life span of fibroblasts and erythrocytes and thus operating in replicative as well as postmitotic cells *in vitro* and *in vivo*.**

A hypothesis has been put forward by Hayflick that there may be a direct relationship between species longevity and the replicative potential of its normal fibroblasts *in vitro* (1). This is based mainly on the recorded data in the literature for the three most studied species in this respect, the two mammals, man and mouse, and the chicken. The primary purpose of this investigation is to verify whether or not this hypothesis holds true when the number of mammalian species studied is expanded. According to Stanley *et al.* (2) it does not, but their results are not well founded.

That normal human fibroblasts in culture have a limited replicative life span is well established (3–6). In the case of cultures derived from human embryos, which are frequently used for studies on *in vitro* life spans, cell proliferation is usually sustained for 40–70 population doublings (PDs) (3–6). Because there is an inverse relationship between the replicative potential of human fibroblast cultures and donor age, this cellular system has been suggested as a possible model for ageing *in vitro* (7–10).

Although the system is less well studied, a limited replicative life span also seems to characterize normal fibroblastic cultures from animal species (4, 11). However, a comparative approach to assess the replicative life spans of normal animal cells is complicated by the tendency of many nonhuman cultures to be spontaneously transformed into continuous cell lines (4, 11, 12). The chromosomal constitution is certainly most significant in this connection, because most continuous cell lines are not normally diploid. However, there are a few controversial cases, including the rat (13, 14) and the fat-tailed dunnart (2, 15) fibroblast cell lines, and some human lymphoblastoid cell lines (16). It should be noted though that Epstein–Barr virus transformation of lymphoblastoid cell lines is accompanied by chromosomal abnormalities, although there is a long lag time between the initiation of the transformation and the induction of these abnormalities (17). Most likely chromosome abnormalities eventually occur in all continuous cell lines (3, 4, 11, 12).

Central to the original concept of the limited *in vitro* life span of human cells was the demonstration of phase III, a period of apparent decrease in the rate of cell proliferation accompanied by morphological, biochemical, and cytological changes, which leads to the proliferative end of the culture (3, 7). In animal cell cultures such as mouse, rat, and rabbit, phase III has been demonstrated prior to or coincidentally with the establishment of many continuous cell lines (13, 14, 18–21). Thus phase III is the indicator of the replicative end of normal animal cells, because in the case of mouse and rabbit most of the cells after phase III had changed chromosomal constitutions (18, 19, 21).

In the present study, quantitative assessments of growth rates and chromosomal constitutions have been made in serial passages of a number of fibroblastic cell cultures from eight mammalian species. The results support the concept of a direct relationship between cellular life span and species longevity (1).

MATERIALS AND METHODS

Biopsies. Biopsy specimens were obtained from the lung, skin, testis, or the whole embryo of one or more male individuals belonging to eight mammalian species (Table 1). In all cases except for horses, the biopsies were performed after the death of the individuals. Horse biopsy specimens were obtained in connection with castration from the testicular capsule (tunica albuginea) and from the skin at the incision of the castration operation. The skin biopsy samples were principally composed of the superficial dermis. In the case of the mouse (Swiss strain), rat, and man, all material was taken from embryos, whereas the biopsies from the rat-kangaroo, mink, rabbit, bat, and horse were all performed on adult animals. Except for the horses, which were all 2–4 yr of age, the ages of the adult animals are not known.

Cell Cultivation. The biopsy specimens were immediately transferred to test tubes with 5 ml of tissue culture medium: Eagle's basal medium with Hanks' salts supplemented with 15%

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Abbreviations: PD, population doubling; PDL, population doubling level; MSL, maximal life span.

fetal bovine serum (BIO-CULT), streptomycin at 100 μg/ml, and penicillin at 100 international units/ml, pH 7.2. After washing in fresh medium, the biopsy materials were minced with scissors and plated in two to four milk-dilution bottles. The time needed to achieve confluent layers of fibroblastic cells varied with the tissues, individuals, and species. Confluent cells were detached from the glass by rinsing them quickly with isotonic sodium citrate solution before treatment with 0.25% trypsin (Difco) in Puck's saline A. After suspension in complete medium, the cells were transferred to new bottles. Routine subcultivations were carried out once or twice a week at split ratios 1:2, 1:3, or 1:4, depending on the rate of growth. This procedure ensured that the cells were predominantly in the exponential phase of growth during the entire cultivation period.

The number of PDs was calculated from the split ratios (S) by the formula PD = log(1/S)/log 2 (as modified from ref. 22), which for the split ratios of 1:2, 1:3, and 1:4 gives 1, 1.6, and 2 PD, respectively. The culture age was calculated as the PD level (PDL) starting from the first passage *in vitro*.

The cultures were carried in at least one of the two main batches of fetal bovine serum used; a few cultures were also carried in serum from other batches.

After initiation and cultivation to PDL 2–5, all cultures were frozen in complete medium supplemented with 10% dimethyl sulfoxide and stored in liquid nitrogen. Freezing to −70°C was done in liquid nitrogen vapor at an estimated rate of 1°C/min. Growth kinetics were studied in cultures initiated from biopsy specimens or frozen stocks (PDL 2–5). In the latter cases, confluent monolayers were obtained 1 or 2 days after the cells were thawed.

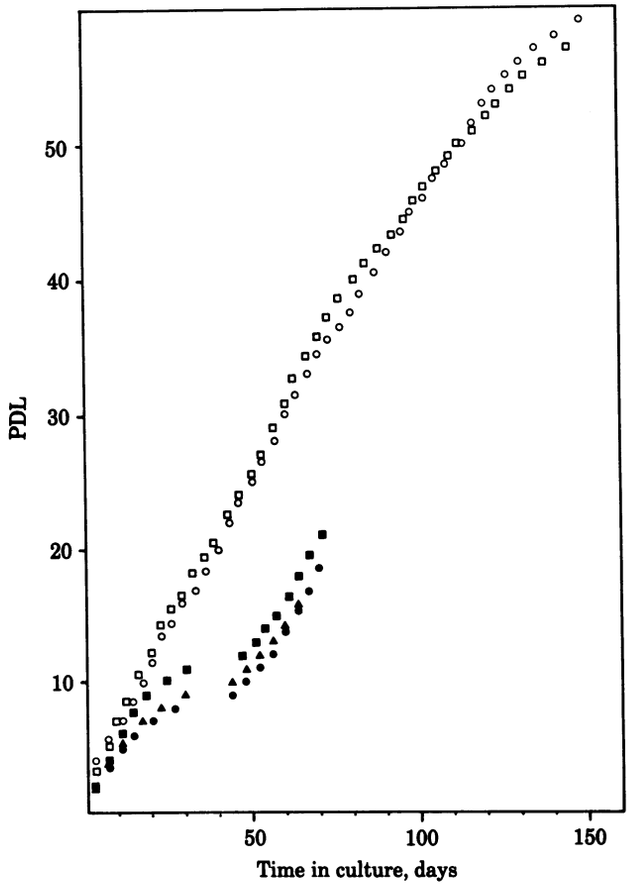

FIG. 1. Growth curves of two human (open symbols) and three mouse (filled symbols) cultures of embryonal fibroblasts.

Chromosome Preparations. Conventional air-dry preparations were made according to standard procedures at this laboratory (23).

RESULTS

Growth Crisis. The occurrence of a growth crisis was determined from growth kinetics plots as exemplified for mouse and human cultures in Fig. 1. Whereas all the mouse cultures passed through an apparent growth crisis at PDL 8–11 for about 2–3 wk before cell proliferation was resumed, phase III characteristically represented the end of the replicative potential of the human cultures. In the cases of mouse, rat, and bat, transformation into continuous cell lines occurred 2–3 wk after phase III in 5 out of 5, 3 out of 4, and 1 out of 3 cultures, respectively. Also, in the case of the horse, cell proliferation was resumed after the crisis in 2 out of 12 cultures, but these cases differed from those of the mouse, rat, and bat. Not only was the length of the crisis longer in these horse cultures, but also cell proliferation after the crisis was comparatively slow and continued only for about 10 PD before the culture reached its ultimate

Table 1. PDL at the time of phase III in the different cell cultures studied

Species	Cell culture*	PDL† at phase III: Lung	Skin	Testis	Whole embryo	Average
Mouse	ME 1				11, 9	10
Mus musculus	ME 2				9	9
	ME 3				8, 9	8.5
Rat	RES 1		13			13
Rattus	RES 2		16			16
norvegicus	RES 3		12, 10			11
Rat-kangaroo	PTL 2	13, 16				
Potorous tridactylus	PTS 2		10, 12			11
Mink	MVI	27, 23				25
Mustela vison						
Rabbit	RL	24, 21				22.5
Oryctolagus cuniculus						
Bat	VML	18, 20, 16				18
Vespertilio murinus						
Horse	ECT 1			36, 33		34.5
Equus caballus	ECT 2			36		36
	ECS 3		30			30
	ECS 4		39			39
	ECT 4			41		41
	ECS 5		17			17
	ECS 6		17			17
	ECS 8		23			23
	ECT 8			25		25
	ECT 9			25, 23		24
Man	HES 5		59, 67			63
Homo sapiens	HEL 2	57, 62				59.5

* The numerical designation of the culture refers to the individual of origin.

† All values are given as rounded off integers except in the case of the averages. More than one value per culture represents cultivations in different sera.

Table 2. Frequency of diploid cells during the *in vitro* cultivation of cells representing the eight species studied

Species	Cell line	% diploid cells at different PDLs*
Mouse	ME 2	88 — 76 ——↓ — 7 —— 0 —— 0
Rat	RES 1	96 — 95 —— 90 —↓— 92 —— 93
Rat-kangaroo	PTS 2	96 —— 88 —— 84 —↓
Mink	MVI	— 95 —— 93 —— 93 —— 82 —↓
Rabbit	RL	95 —— 91 —— 84 —↓
Bat	VM	96 —— 96 —— 90 —↓— 52 —— 49
Horse	ECS 4	—— 96 —— 81 —↓ (15)
Man	HES 5	—— 94 —— 76 —↓
	HES 5	—— 96 —— 81 —↓
	HEL 2	—— 91 —— 84 —↓
	HEL 2	—— 94 —— 81 —↓

* Arrows indicate the occurrence of phase III and lines the period of growth of the culture.

replicative end (unpublished data).

Based on the growth kinetics plots as shown for mouse and man in Fig. 1, the PDLs at the growth crisis for all the cultures are summarized in Table 1. Evidently the major source of the overall variation is related to the species origin of the cultures. Between the extremes, man and mouse, the difference was about 6- to 7-fold. With regard to different individuals, tissues of origin, and batches of serum used, the variation was usually ±20% of the species mean, except for the horse cell cultures, for which it was ±40%.

Chromosome Analysis. Chromosome numbers in 50 cells and the gross morphology of the chromosomes in 10 cells were determined in at least one culture from each species. Table 2 shows that the cells with diploid numbers continuously decrease in frequency during the cultivation period in all cases except the rat. In all cultures except those from the rat and bat, the diploid cells represented some 75–85% of the cells just prior to the growth crisis. In the case of the rat, the diploid cells represented some 90% of the cells at this stage of the culture, but they increased somewhat in the cell population after the growth crisis. In the case of the bat culture, the cell population after phase III was represented by about equal numbers of diploid cells ($2n = 38$) and cells with 37 chromosomes. In accordance with previous findings of mouse cultures (18, 19), the cell populations obtained after the crisis were dominated by hypotetraploid cells with 71–79 chromosomes ($2n = 40$). In the case of the horse culture, the cell population after the growth crisis was hypertriploid, with more than 75% of the cells having 97–104 chromosomes ($2n = 64$).

Fibroblast and Erythrocyte Life Spans and Species Longevity. In Table 3 the PDLs at the growth crisis (given in Table 1) are averaged and listed in the order of increasing maximal life span (MLS) of the species. The PDL at the growth crisis is nicely related to the species longevity. In fact, calculated on a logarithmic basis (log/log), the relationship between the PDL at the growth crisis and the MLS of the species follows a strict power law (Fig. 2). The linear regression coefficient of MLS on PDL is 2.07, and the correlation coefficient, 0.95, is highly significant ($P < 0.001$). A point to be considered here is that the human, mouse, and rat cultures are of embryonal origin, whereas those of the other species are from adult donors. However, most likely this difference is not crucial to the relationship found here.

Table 3. Mean PDL at phase III and the MLS of the eight species studied

Species	PDL at phase III Range	PDL at phase III Mean ± SEM	No. of cultures	Species MLS, yr	Ref.
Mouse	8–11	9.2 ± 0.5 (6.9)*	5	2	24
Rat	10–16	12.8 ± 1.3 (9.6)*	4	3.5	25
Rat-kangaroo	10–16	12.8 ± 1.3	4	7	2
Mink	23–27	25.0 ± 2.0	2	10	25
Rabbit	21–24	22.5 ± 1.5	2	13	25
Bat	16–20	18.0 ± 1.2	3	13.8†	26
Horse	17–41	28.8 ± 2.4	12	46	25
Man	57–67	61.3 ± 2.2(46.0)*	4	110	27

* In parentheses the means of embryonal cultures are reduced by 25%.
† Based on the average MLS for species in the subfamily Vespertilionae, to which the bat belongs (26).

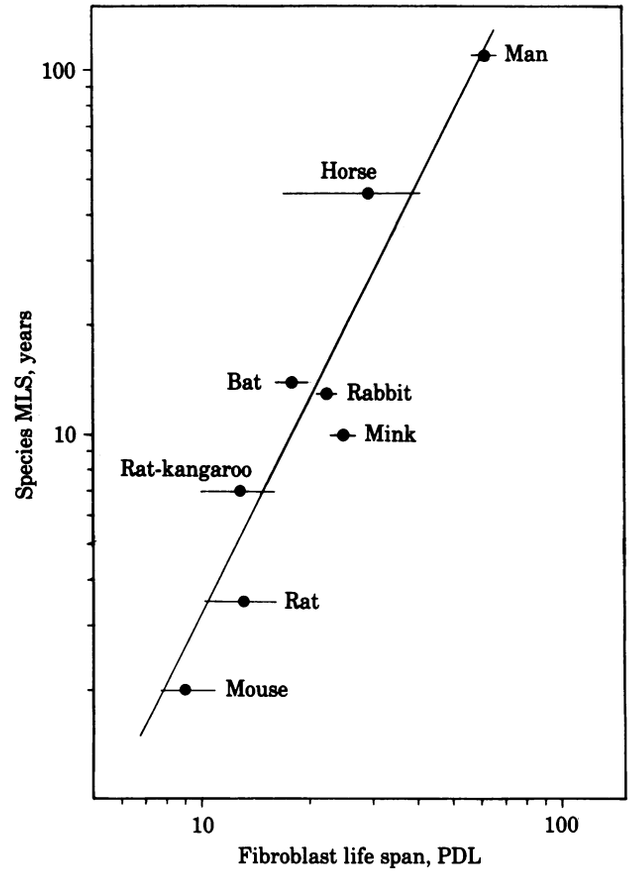

FIG. 2. Linear correlation (log/log) between species MLS (yr), and their fibroblast life spans *in vitro* (PDL) based on the data given in Table 3. The linear regression of $\log_{10}$MLS on $\log_{10}$PDL is given by the equation $\log_{10}\text{MLS} = 2.07 \log_{10}\text{PDL} - 1.58$. The correlation coefficient is 0.95.

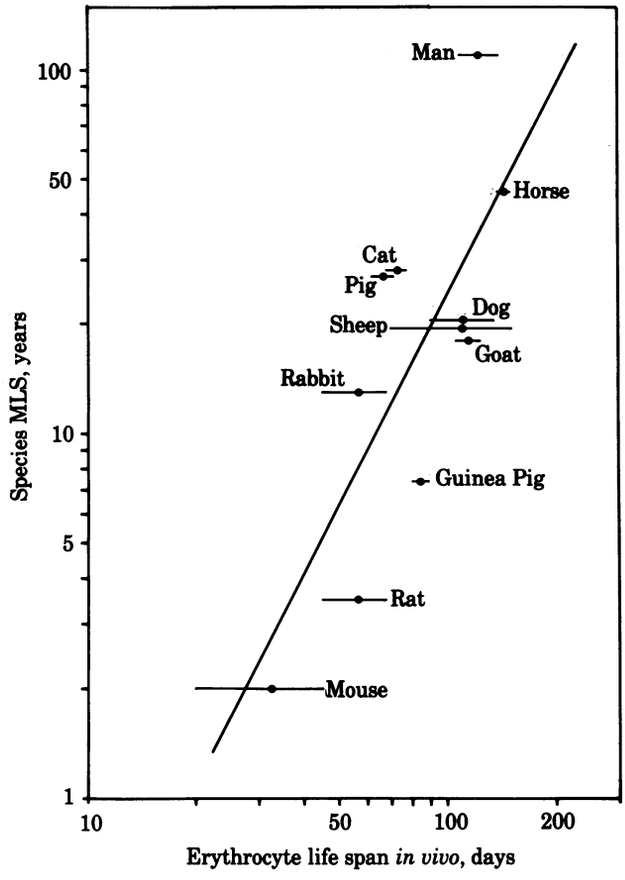

FIG. 3. Linear correlation (log/log) between species MLS (yr) and their erythrocyte life span *in vivo*, T (days), based on recorded data (25). The linear regression of $\log_{10}$MLS on $\log_{10}T$ is given by the equation $\log_{10}\text{MLS} = 1.96 \log_{10}T - 2.54$. The correlation coefficient is 0.77.

Reduction of the PDLs of the embryonal cultures by a factor of 25% (Table 3), which corresponds to the average difference found in human cultures of embryonal and adult (30–40 yr of age) origin (8), does not have much effect on the regression coefficient, which in this case is 1.99. The correlation also is as strong as before, the coefficient being 0.95.

Based on the published data of the *in vivo* life span of circulating mammalian erythrocytes (25), a similar comparison with the species MLS is presented in Fig. 3. The MLS is here calculated as a function of the erythrocyte life span merely to facilitate the comparison with the fibroblast case. Because different techniques have been employed to determine these life spans, the calculations are based on the mean of the lowest and highest value recorded for each species. The regression coefficient of the life span (days) on MLS is 1.96, and the correlation coefficient is 0.77, which is clearly significant ($P < 0.01$).

DISCUSSION

A direct relationship was found between the longevity of eight mammalian species and the occurrence of phase III in their cultured fibroblasts. The major implication of this is that this potential is primarily determined by an intrinsic cellular program (3, 7).

Unfortunately, direct comparisons of the replicative potentials of normal cells in culture obtained in different studies are hampered by the fact that they may vary considerably due to differences in tissues of origin (11, 28) and cell types (29) as well as to the specific culture conditions employed, including the nutrient medium, serum, and cultivation procedures (30–34). Also, intraspecific genetic differences may play a role as suggested from the fact that fibroblast cultures from humans who age prematurely, Werner syndrome and progeria, may undergo fewer PDs if compared to normal cells (8, 35, 36). However, it should be noted that the timing of the growth crisis and chromosomal changes obtained here are within the range of the variation previously reported for the well-studied species such as man (4, 6), mouse (18–20), rat (13, 14), and rabbit (21).

Stanley *et al.* (2) could not find a relationship between species longevity and the replicative potential of its normal cells. However it is difficult to interpret this study, because the time of onset of transformation was arbitrarily determined by the time of appearance of heteroploidy without simultaneous quantitative assessments of growth rates in serial passages. Thus, as examplified by the case of rabbit, Stanley *et al.* reported a normal life-span of 70 PD, whereas Martin and Ogburn (21) observed phase III at about PDL 20, the same level as demonstrated here, after which a pseudodiploid cell population resumed cell proliferation, showing only subtle chromosomal alterations.

That the life span of circulating erythrocytes appears to be related to the species longevity in the same way as that of cultured fibroblasts is remarkable not only because they represent another cell type, but also because they are postmitotic and experience *in vivo* conditions. A square-root relationship to the species longevity of cellular life spans, as suggested here for erythrocytes and cultured fibroblasts, also may apply to the *in vitro* survival of lymphocytes as recorded by Ling and Kay (37). Although these data are incomplete, they still indicate that the 25% survival of lymphocytes is 1.9 ± 0.4 days for the four most short-lived species, with an average MLS of 4.2 ± 1.1 yr, compared to the 6.8 ± 1.9 days for the four most long-lived ones, with an average MLS of 44.5 ± 22.1 yr. The survival ratio between these groups of cultured lymphocytes, 3.6, is about the same as the square root of the MLS ratio, 3.3, between these groups of species. Thus, despite the present paucity of data that bear on comparative cellular life spans, the results discussed here on fibroblasts, erythrocytes, and perhaps also lymphocytes are suggestive of the existence of a common functional basis for the regulation of the life span operating both *in vivo* and *in vitro*.

In the case of erythrocytes, convincing experiments demonstrate that the mechanism directly involved in the elimination of senescent erythrocytes from the body is apparently the selective attachment of immunoglobulin G to the cell surface of old cells, which functions as a marker for phagocytosis of these cells (38). The different life spans of erythrocytes from various species is thus likely to be dependent on the rate by which the cell membrane or surface is changed to allow such immunoglobulin G attachment. Thus, if there is a common basis for the limited life spans of erythrocytes and fibroblasts, as suggested by the present results, this would also imply a primary role of the cell membrane or surface in the regulation of the fibroblast life span.

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