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Minireview

Duration of senescent cell survival in vitro as a characteristic of organism longevity, an additional to the proliferative potential of fibroblasts

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Abstract More than 40 years have passed since the original publication by Hayflick and Moorhead led to the concept of the 'Hayflick limit' of the maximum number of divisions which somatic cells undergo in vitro. This concept is still regarded as a fundamental characteristic of species longevity. Here we want to emphasize another characteristic of somatic cells, namely, the duration of their survival in vitro in the non-dividing state after cessation of proliferation. This is suggested on the basis of results of recent experiments with so-called Japanese accelerated senescent mice. Results of these experiments reveal a good correlation between the longevity of the mice, the number of duplications of their fibroblasts in vitro, and the survival time of these cells in the non-dividing state. In routine culture conditions, cell survival time may be very long, as much as a few years. However, when the cells are grown under conditions of oxidative stress, cellular longevity is markedly shortened. This new test may serve as an additional marker of organismic longevity. The comparative value of both tests, the classical 'Hayflick limit' and the new test, is discussed.

Key words: Senescence; Hayflick limit; Ageing; Survival; Senescence accelerated mouse; Telomerase

1. Introduction

Originally, cell senescence was considered to occur when cell division in vitro decreased and then ceased, accompanied by predictable and easily observable morphological changes specific for different cells [1]. Cells that completed their proliferative life span and reached a terminal post-mitotic state were referred to as being senescent. This definition was derived from the belief that the irreversible non-dividing state is related to ageing of the organism [2]. The question about probable relevance of cell senescence to organism ageing [3,4] occupied researchers for many years and is still the subject of intensive discussions. Reactions to the problem of the role played by cell senescence in human ageing have varied from enthusiasm to skepticism.

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More than a century ago, August Weismann postulated that normal somatic cells senesce and cease to divide, whereas germ and cancer cells do not [5]. He also suggested that cells from long-living animal species can undergo a greater number of divisions than cells derived from animals with a shorter life span [6]. However, Weismann's initial insight into cell ageing was promptly and thoroughly ignored. For many years, a dogma was accepted that animal cells in culture may proliferate indefinitely. This belief in the immortality of germ cells and all somatic cells was supported in the first half of the 20th century by the well-known scientists Alexis Carrel and Albert Eberling, who worked with cultured chicken cells [7].

It required remarkable courage for Leonard Hayflick and Paul Moorhead to suggest in 1961 [8] that cells have 'life spans' and that the indefinite somatic cell proliferative longevity reported by earlier researchers was due to technical errors in their work. In recent years, the 'Hayflick limit' has itself become a dogma of modern cell biology.

2. Dependence of the cell proliferative life span in vitro on organism longevity: experimental evidence pro and contra

It has been suggested that the behavior of cells in vitro reflects the ageing of the organism [9].

Four kinds of results favor such a proposal.

1. A limitation of the cell proliferative potential is seen not only in vitro but also in vivo. In contrast to the immortality of germ cell lines, a limited number of divisions are undergone by typical human somatic cells in vivo [10]. This restricted cell proliferation potential in vivo has been confirmed by two kinds of experiments, one in which serial transplantations of normal somatic cells were made to young hosts [11], and another in which bone marrow cells were transplanted to lethally irradiated mice [12].
2. A correlation exists between the life span of in vitro proliferated fibroblasts and donor longevity [13].
3. There is a decrease in fibroblast proliferation life span as a function of donor age [9,14–16].
4. A decrease in proliferative life span of fibroblasts obtained from patients with syndromes of premature ageing, such as Werner's [14,15,17–24].

In the 1990s, after experimental proof [25,26] of Alexei Olovnikov's fruitful marginotomy hypothesis [27], the key role played by telomeres and telomerase in cell proliferation control has been generally accepted. Proliferative potential began to be indubitably connected to telomere length.

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Along with data supporting the connection between proliferative potential and organismic longevity, there appeared a number of facts which fell outside the frame of this assumption.

It has been demonstrated that telomere length in a given cell phenotype may depend on cell localization in the body, and is therefore not directly dependent on the age of the organism. Thus, the telomere length of endothelial cells decreases as a function of donor age. However, there is a greater decline in telomere length with ageing in cells isolated from the iliac artery, in comparison with cells from the thoracic artery [28]. CD4+ T lymphocytes, CD8+ T lymphocytes, and B lymphocytes all show telomere shortening with age, although at different rates, and their mean telomere length also differs [29]. In the case of B cells, telomere lengths may actually increase during both tonsillar B cell differentiation and the formation of germinal centers in lymphatic nodes [30].

It has been demonstrated that various somatic cells have a defined replicative life span characteristic of the tissue from which they are derived. Thus, if we estimate cell senescence as the decline of the cycling fraction of cells per population doubling, human dermal fibroblasts and human peritoneal mesothelial cells show differences (0.89% versus 2.2% per population doubling, respectively) [31]. However, it is difficult to compare life spans of cells of different origin because they grow in different media and we cannot exclude the influence of media on their growth.

Attempts to revise the dependence of cell life span on donor age and connect the proliferative potential in vitro with diseases of the organism and the cell's liability to damage have been undertaken [32–34].

The proliferative potential of lymphocytes in Werner's premature ageing syndrome is unchanged whereas that of fibroblasts decreases. There is an explanation of this observation. In normal humans, lymphocytes retain the ability to regulate telomerase activity in response to stimuli of proliferation. If this occurs as well in Werner's patients, their lymphocytes should exhibit approximately normal proliferative life spans, a prediction that is supported by experimental observations [35].

There are serious difficulties in interpreting the interrelationship between proliferative life span and organism longevity among different animal species [36,37]. For instance, among primates, humans have the longest life spans but the shortest telomeres [38]. On the other hand, rodents have a short life span and the longest telomeres [39,40]. Telomeres are even longer in SCID mice [41]. A molecular mechanism has been proposed to explain how telomeres of different lengths may restrict cell proliferative potential [39].

In spite of all the difficulties and confusion, the assumption about the connection of cell senescence and organismic ageing has not been rejected. It is therefore reasonable to cite a short section from Hayflick's lecture delivered in 1998 at a conference on telomeres. "We suggested the Phase III Phenomenon (the period in which cell replication diminished and stopped) was the manifestation of ageing at the cellular level. Quite frankly, I expected that this idea would be disproved quickly, but I think it is fair to say that of the thousands of papers published in this field in the last 35 years, which I subsequently named cytogerontology, none have disproved this suggestion" [4].

3. Dependence of proliferative life span in vitro on organism longevity: theoretical considerations pro and contra

The most obvious mechanism in animals for maintenance of life is the ability to repair external and internal damage of tissues. In addition to tissue repair, there is also a continual replacement of most cells, as in skin epidermis and gut lining. This latter process involves the replacement of disposable cells and those lost during wear and tear. The maintenance and survival of the adult organism are completely dependent on a normal immune response, which, in turn, is fully dependent on cell proliferation. Consequently, adult body maintenance requires a well-organized planned and extraordinary expenditure of cells that is related to the longevity of the organism. This means that proliferative potential (pp) should be a function of the organism's longevity:

$$2^{pp} \approx \text{longevity}$$

On further reflection, it is clear that other factors modulate the original consideration described above and change its character. Four main factors are given here.

1. As the organism becomes larger, the planned and extraordinary cell numbers should necessarily increase.
2. A limitation of the proliferative potential may (or may not) serve as a defense mechanism against cancer [42,43]. This mechanism is less important (if it is important at all) in short-living species [44]. Repression of telomerase in the somatic tissues of humans, and probably other long-lived mammals, appears to have evolved as a powerful protective barrier against cancer [45–47].
3. In the course of ontogenesis, a repression of telomerase activity takes place in somatic cells. In fact, only from that moment we can speak about the limitation of cell proliferation. However, the tissue stem cells retain some reduced level of telomerase activity in the adult body. The concentration of such stem cells may differ in different species. The stem cell division rate may also be different as well as the regulation of their telomerase activity [44].
   The role of telomerase in stem cells was first identified within the hematopoietic system [48]. Telomerase is induced in hematopoietic stem cells by cytokine stimulation and is down-regulated again with proliferation and differentiation [49]. The presence of stem cells capable of expressing telomerase upon stimulation results in a slower overall rate of ageing in the immune system [50].
   The telomerase activity can be reactivated not only in stem cells. Some cells of the immune system and even fibroblasts and endothelial cells in the course of regeneration reactivate telomerase [51].
4. The technology used routinely to determine proliferative life span is itself open to criticism. Due to great technical difficulties in watching a single cell grow, the proliferation potential is usually measured as the number of population doublings. One problem is that the final value of proliferative potential is to a large extent based on a single most long-lived cell. This value may vary significantly, depending largely on selection and the size of the biopsy.

All of the considerations enumerated above complicate a thorough understanding of the interrelationship between cell proliferation potential and organism longevity. This hampers the use of the proliferative life span test in studying the nature and mechanism of ageing. This limitation is a stimulus for

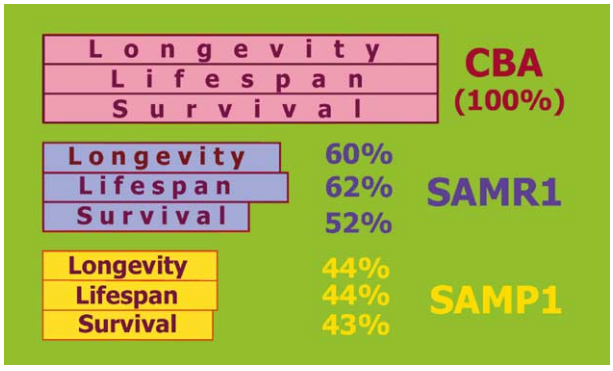

Fig. 1. Correlation of mouse longevity with proliferative potentials of the fibroblasts and survival time of senescent non-dividing cells.

examining other cellular characteristics that may serve as additional tests of ageing.

4. The role of post-mitotic cells in body maintenance

Any multicellular organism consists of dividing and non-dividing cells. Both their ability to divide and their ability for long-term life without division should be very important factors that determine organism longevity. Ageing is the result of natural selection [52–54] and all systems created by natural selection should be equally reliable, like the parts of a plane, to maintain the organism's life. If longevity of an organism depends on the proliferative potential and the survival time of non-dividing cells, these two parameters should correspond to each other.

In recent experiments [40] on the cells of so-called Japanese senescent accelerated mice, quite a good correlation between the mouse's longevity, the embryo fibroblast life spans in vitro and the time of senesced non-dividing fibroblast survival in culture was found (Fig. 1). A correlation between the organism's longevity and erythrocyte survival time was reported previously [55].

It is interesting to mention that both organism longevity and survival time of senescent non-dividing fibroblasts is greater in humans as compared with mice. These data suggest that the time of senescent fibroblast survival in vitro in the non-dividing state may be regarded as a different in vitro test of the organism's lifetime in vivo, in addition to Hayflick's characteristics.

A test for evaluation of cell survival in stationary cultures was suggested some time ago [56–58]. However, this test is rather difficult to interpret as the culture conditions during the experiment may undergo variable unpredictable changes.

A serious difficulty in using our suggested test may be connected with the long time of cell survival in culture of human cells, as long as years [59]. This problem may be overcome by growing cells under conditions of oxidative stress. It has been shown that the addition of hydrogen peroxide to the culture medium reduces the survival time, thus making it more practical to employ the test in investigations of organism ageing [60].

5. Comparison of two different cellular tests to describe organism longevity

The eventual outcome of cell senescence is cell death (Fig. 2). It can be seen that each of the previously discussed tests is pertinent to a different aspect of cell life, active proliferation and the non-dividing state. Both tests therefore reflect the process of cell senescence.

Results of the comparison of both tests are given in Table 1.

Each test has its own advantages and disadvantages, but the second test that we are directing attention to in this review, duration of senescent fibroblast survival, has fewer disadvantages than the first test, proliferative potential of fibroblasts.

Some of these characteristics require a more detailed discussion. There are some available data [61,62] making it possible to attribute fibroblasts to typical differentiated cells such as hepatocytes, leukocytes, neurons, or myocytes. A peculiarity of fibroblasts is that damage can be a factor inducing their differentiation [62]. Furthermore, fibroblast differentiation with subsequent survival in a non-proliferative state is more independent of various external factors than that of many other differentiated cells. For example, cardiomyocytes crucially depend on the coronary arteries for their survival. They undergo little direct tissue ageing [63], and their survival time is probably determined by the senescence of endothelial cells lining the arterial walls. Neuron survival depends on both endothelial and glial cells.

Regarding fibroblasts, their survival in vivo is mainly self-determined. Fibroblasts participate in tissue repair and live mainly in irregular conditions. The length of their life in the non-dividing state is determined by intrinsic abilities to carry out repair functions after damage and survive under unfavorable conditions. Therefore, an organism from a long-lived species would be expected to have post-mitotic fibroblasts that are likewise long-lasting. It may thus be concluded that fibroblast survival time in vitro is a good reflection of organism longevity. It does not depend either on the organism's body size, on the anti-cancer defense mechanisms, or on the tissue stem cell distribution and telomerase regulation (Table 1).

Ordinarily, when investigators examine the longevity of proliferating fibroblasts in vitro, they do not take into account the fact that the cells are growing in an oxygen concentration

Table 1
Comparison of the two characteristics of cell senescence

Proliferative potential of fibroblasts	Duration of senescent fibroblast survival
Should be dependent on longevity Should be dependent on stress (in vitro culture conditions)	
Should be dependent on animal size	No apparent dependence on animal size
Can be restricted or not due to anti-cancer defense	No apparent dependence on anti-cancer defense
Should be dependent on tissue stem cell distribution and telomerase regulation	No apparent dependence on tissue stem cell distribution and telomerase regulation

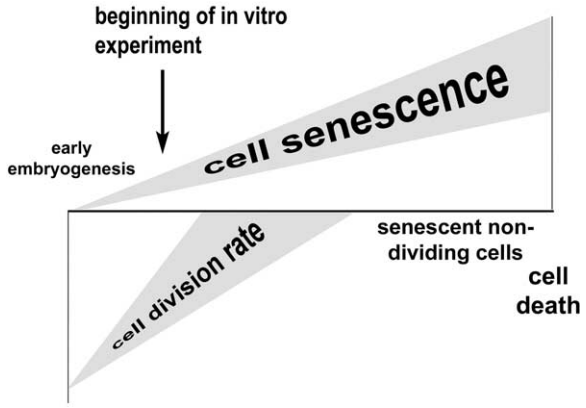

Fig. 2. Relationships of cell senescence, cell proliferation, and survival of senescent non-dividing cells.

several times greater than in vivo. This means that cultivated fibroblasts are actually growing under conditions of oxidative stress. When the oxygen concentration in culture is reduced, there is a resultant increase in cell life [64,65]. As was mentioned above, the duration of survival of senesced cells is also dependent on the oxidative stress [60]. This fact to a certain degree serves to unite the fibroblast life span and the survival tests.

We therefore conclude that the parallel use of both cellular tests discussed in this paper enlarges the possibilities of studying the longevity of organisms as well as illuminating the nature of ageing.

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