Gerontologia *19:* 303–313 (1973)

Long-Term Culture of Human Adult Liver Cells: Morphological Changes Related to *in vitro* Senescence and Effect of Donor's Age on Growth Potential

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Abstract. The morphological aspects of ageing and growth capacity were studied in 100 lines of human liver cells. Under the culture conditions employed, liver cells appear to have a limited replicative life span. The morphological effects of ageing were similar to those described in the tissue culture of other organs. Studies on growth potential showed that there was a negative correlation between age of the donor tissue and age of the culture at the last passage, maximum number of doublings, number of doublings in phase II, and age of the culture at the end of phase II. The growth indices were different for epithelial-like and fibroblast-like cells.

Key Words
Cell culture
Human liver
Morphology
Growth potential

Introduction

In multicellular organisms cells undergo the normal process of ageing. It is now apparent that cells also age in tissue culture [10–15] and that an earlier study by Carrel [2], suggesting that such cells are ageless, is no longer tenable. That cells age *in vitro* is accepted by most investigators today [4–6, 23–25, 29, 33], but this concept still raises many problems yet to be elucidated. The ageing of animal cells in culture have been studied by many workers [3, 4, 6, 20–27, 29, 33].

This study was designed to define the morphological aspects of cultivated liver cells related to their *in vitro* senescence and to investigate correlations between a number of their growth indices and the donor's age.

Received: July 15, 1973.

The ageing of liver cells subcultures of differing morphological typology has been compared.

Material and Methods

Culture techniques. The culture technique previously described [1] has now yielded 600 primary cultures of normal or pathological adult human liver. 100 of these cultures were selected at random and underwent a first subculture to yield 100 cell lines, which formed the material of our study. They were maintained in tissue culture by a method previously described [17, 19].

Morphological studies. Live cells were examined by phase contrast microscopy using a Wild MG microscope. Fixed cells were examined after staining with the May-Grünwald-Giemsa stain. The cell type was characterized in 80 of 100 cell lines used in this study.

Growth characteristics studies. Growth curves were constructed by relating the number of subcultures obtained (ordinate) and the time interval between subculture and primary culture (abscissa). The following indices were calculated from such growth curves: (a) the age of the culture at the last passage (this criterion was used to provide an indirect approach of measuring the cell lines' total survival time ability; it was chosen rather than life span to avoid introducing interfering side effects resulting from inevitable variance in choosing the moment for discarding cultures) and (b) the doubling potential, i.e. the maximum number of doublings achieved by these cell lines at the end of phase III. These indices were determined for each of the 100 cell lines. In a further 68 lines, the end of phase II could be determined with sufficient precision to allow determination of the culture age at the end of phase II (c), the number of doublings achieved during phase II (d), and the mean doubling time of the cell population during phase II (e, derived from c and d).

Statistical methods. Correlation coefficients were calculated between the various growth indices estimations and the age of the donor material. Such calculations were possible for the culture age at last passage and doubling potential in 100 subcultures (cell type characterized in 80) as well as for 68 lines in phase II in which the end of phase II could be clearly identified (cell type characterized in 54).

Results

Morphology

Phase II Cultures

Two different cell types (A and B) were found during phase II.

A cells (fig. 1). These cells were spindle shaped, oriented irregularly, and had the characteristic morphology of fibroblasts. Occasionally the cells were binucleate. The shape of the nuclei was occasionally irregular but more commonly ovoid, with the long axis of the nucleus parallel to that of the cell. The number of nucleoli varied from 1 to 4; they were either punctiform or ramified in shape. Cellular organelles were confined to

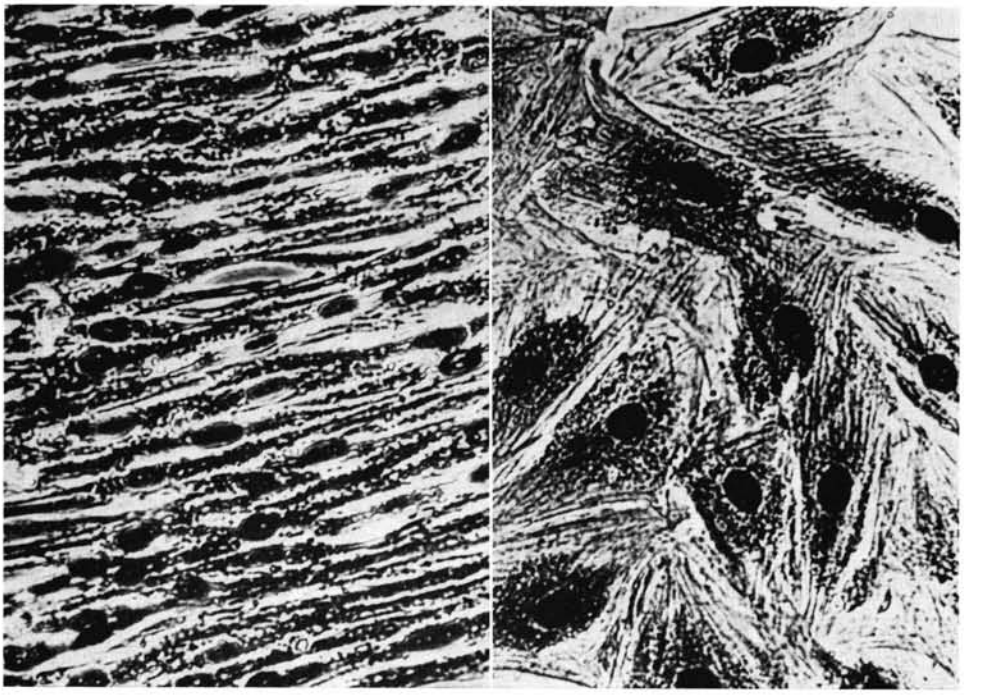

Fig. 1. Two different cell types found in human adult liver cell cultures during phase II. Left, a line of spindle-shaped A cells with the characteristic morphology of fibroblasts; right, a line of B cells which are loosely bound together in polygonal sheets.

a small area around the nucleus. When the cells became confluent, they had a characteristic appearance. The bottom of the tissue culture dish was covered with bundles of fibroblasts arranged in whorls in which the cells were orientated in one direction.

B cells. These cells resembled the type V cells that we have described previously in primary cultures [16, 17]. In contrast to fibroblasts, they were regularly orientated. Cytoplasmic projections were found, but they had no constant relationship to the cell. The cells were sometimes binucleate, and the nuclei were slightly elongated with a thin surrounding of cytoplasmic organelles. The number of nucleoli varied between 1 and 4. The cells were loosely bound together in polygonal sheets and never appeared in the form of bundles.

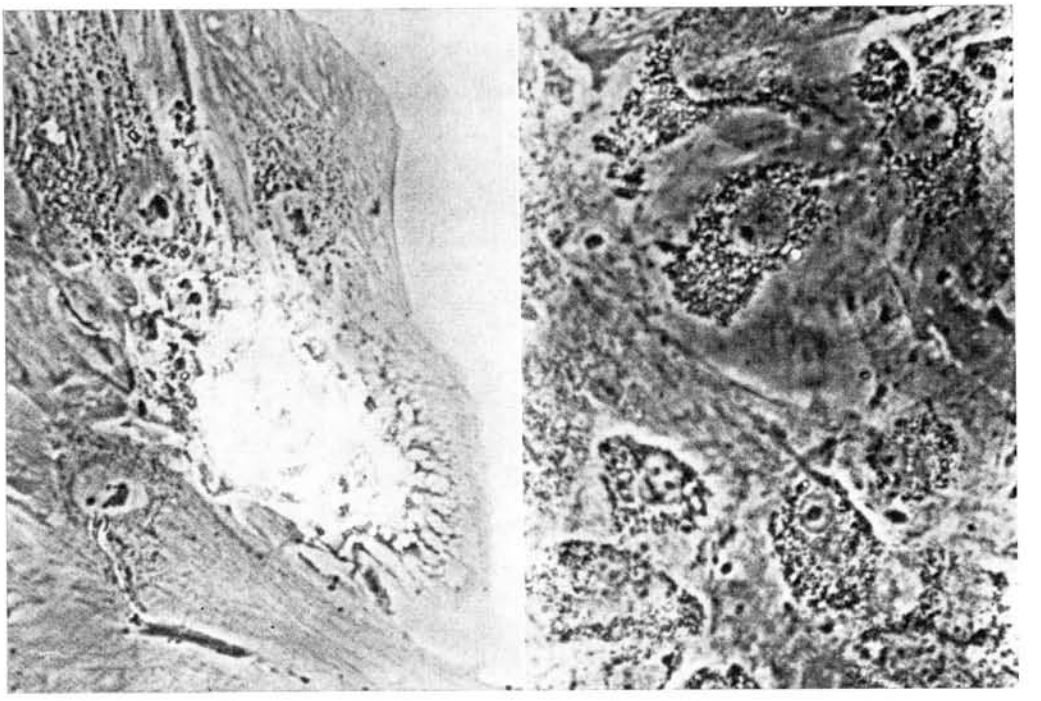

Fig. 2. Human adult liver cell lines, in phase III. Left, cells with scattered cytoplasmic granules; right, a clump of cells.

Both types of cells were sometimes found together, but the predominance of one or another cell in some subcultures allowed us to classify them as follows: line A, predominance of first cell type (A); line B, predominance of second cell type (B); line A-B, mixed cell population (A and B).

Phase III Cultures (fig. 2)

Phase III cultures were quite different under light and electron microscopy from those in phase II. Phase III cultures were characterized by the presence of cytoplasmic debris which surrounded the cells and persisted despite frequent washings and changes of the culture medium. Accumulation of this cytoplasmic debris signified the onset of the degenerative processes which accompany ageing. These degenerative changes in the cell lines were also marked by appearance of other features which occurred almost exclusively in phase III. Such features were as follows:

Table I. Growth index values in the three groups of human adult liver cell lines[1]

Growth index		A lines	A–B lines	B lines
Age of the culture at the last passage	m	164,5	178.17	161.2
	n	11	35	34
	SEM	12.7	9.3	13.4
Doubling potential	m	24.5	17.4	11.5
	n	11	35	34
	SEM	4.0	0.9	0.8
Age of culture at the end of phase II	m	112	117.3	94.4
	n	10	27	19
	SEM	13.1	9.3	6.1
Number of doublings reached at the end of phase II	m	20.4	14.8	9.7
	n	10	27	19
	SEM	3.9	1.1	0.8
Mean doubling time during phase II	m	4.6	6.6	8.7
	n	10	27	19
	SEM	0.4	0.2	0.5

[1] Abbreviations: m = mean value for each group of cell lines, n = number of determinations, SEM = standard error of the mean.

Formation of large sheets of large granular polyhedral cells. These cells were similar in their form and arrangement to epithelial cells and were characterized by the presence of scattered cytoplasmic granules. The cells were larger, mainly because of an increased amount of cytoplasm which had fine extrusions representing filipodes [7].

Re-arrangement of cells. The fibroblasts were arranged in a circular fashion with the cells radiating from a central point, overlapping and even overlaying each other. Contact inhibition did not occur in such cultures. In addition to these features, clumps of cells became irregularly arranged in the culture dish. This pattern is sometimes detected early in cell lines of type A: it often occurred before the end of phase II or at the beginning of phase III. In cells of lines B, however, irregularly distributed clumps of cells were only found towards the end of phase III.

Death of Subcultures

The cultures exhibited the features described in phase III for many weeks after multiplication stopped. Age-related degenerative changes

Table II. Correlations between growth characteristics of human adult liver cell lines and the chronological age of donors

Growth index		All lines combined	A lines	A–B lines	B lines
Age of the culture at the last passage	correlation coefficient	−0.20	−0.51	−0.20	−0.15
	number of experiments	100	11	35	29
	p	<0.05	n. s.	n. s.	n. s.
Doubling potential	correlation coefficient	−0.49	−0.77	−0.46	−0.56
	number of experiments	100	11	35	29
	p	<0.001	<0.01	<0.01	<0.01
Age of culture at the end of phase II	correlation coefficient	−0.40	−0.57	−0.47	−0.06
	number of experiments	68	10	27	17
	p	<0.001	n. s.	<0.05	n. s.
Number of doublings reached at the end of phase II	correlation coefficient	−0.56	−0.80	−0.57	−0.30
	number of experiments	68	10	27	17
	p	<0.001	<0.01	<0.01	n. s.
Mean doubling time during phase II	correlation coefficient	+0.13			
	number of experiments	68			
	p	n. s.			

n. s. = No significant difference.

continued and the death of the cells occurred spontaneously. The number of cells in the population decreased progressively. The main morphological changes resulted from the diminished adhesiveness of the cell membranes to the supporting structures, and the cells rearranged themselves irregularly. For a while, however, the cells had the ability to adhere to the plastic of the tissue culture dishes by means of their thin cytoplasmic projections. As a result, the morphology of the cells changed. Finally the cells lost contact with the walls of the dish, floated to the surface of the culture medium, and died.

Growth Characteristics

Estimations of the various growth indices of cells found in phase II were related to the age of the subject from whom the original liver was obtained. The results are tabulated in tables I and II and are shown in figure 3.

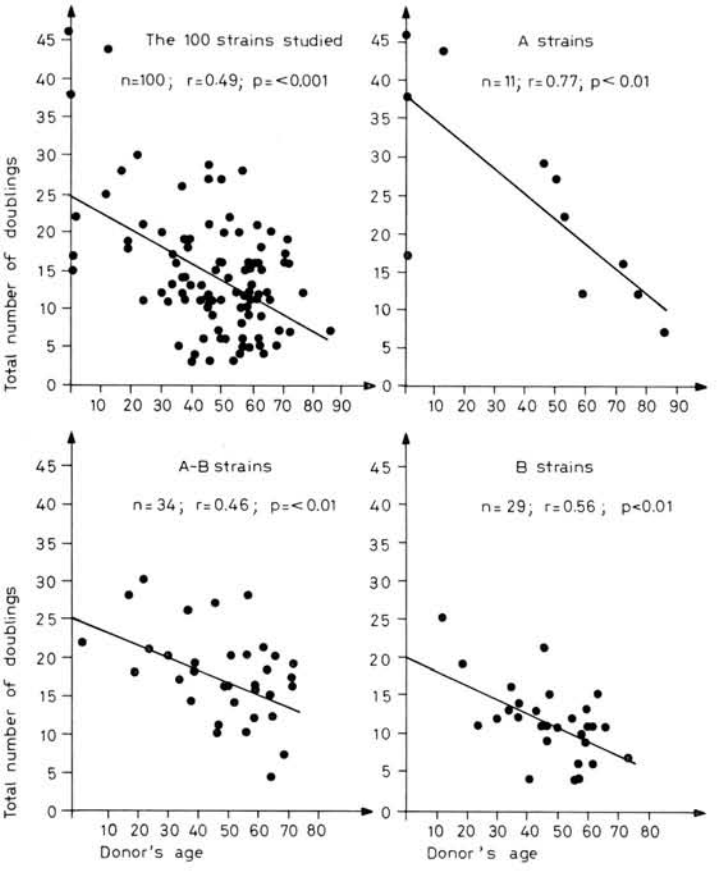

Fig. 3. Correlations between doubling potential and age of donors.

Age of cultures at the last passage. This index varied between 47 and 372 days for different cultures, and there were no significant differences between the main three groups of cell lines. When all the subcultures were considered, there was a weak but significant correlation between the age of the culture at last passage and the age of the donor ($r = -0.20$; $p<0.05$). There was no significant correlation between this parameter and donor age when each of the three cell groups were considered individually.

Doubling potential. The doubling potential was always greater than 5 and less than 45 in each culture. In A cell lines the average number of maximum of doublings achieved was 25, for B cell lines the average num-

ber was 11, and for mixed cell lines the number was 17. Statistical analysis showed that the doubling potential was inversely related to the donor's age (whole group $r = -0.49$; $p<0.001$; individual cell lines $p<0.01$).

Age of culture at end of phase II. The age at this point varied between 47 and 261 days (mean 100 days). The mean age of the cultures was lower for B cell lines compared with A cell lines. The age of the cultures for mixed cell lines (A-B) ranged intermediately between that of the ages of the A and B cultures.

There was a negative correlation between donor age and the time at which phase III appeared. Within the A and B groups, however, this correlation did not achieve statistical significance. In the case of A lines, this may have been a result of the small sample ($r = -0.57$), although this explanation is not tenable in the case of B cell lines ($r = -0.06$).

Number of doublings reached at end of phase II. The number of doublings achieved during phase II varied between 4 and 34, depending on the cell type. For type A cultures, the mean number of duplications was 20, for type B cultures 10, and for mixed cultures (A-B) 15.

When the cells lines were considered as a whole, the number of doublings in phase II correlated inversely with donor age ($r = -0.56$; $p<0.001$). This correlation was also found in type A cultures ($r = -0.80$; $p<0.01$) and in A-B cultures ($r = -0.57$; $p<0.01$) but not in B cell lines ($r = -0.30$).

Mean doubling time in phase II. The mean doubling time was 4–5 days for type A cultures, 6–7 days for A-B cultures and 8–9 days for type B cultures. There was no correlation between the mean doubling time and donor age.

Discussion

This study shows that the human liver cell, under tissue culture conditions has a limited replicative life span similar to that of cells of other origins [3, 5, 9, 14, 22, 23, 27, 30, 34]. In addition, ageing is morphologically similar to that of cells of other organs in tissue culture [9, 14, 25, 27, 33] and this applies particularly to the increase in cell size in phase III [25, 30], the presence of cell debris, and the frequent overlaying of the cells [3, 9, 14].

This work supports the theory that ageing of cells in tissue culture is related to senescence in the whole animal: in spite of the same culture conditions used for all strains, there was an inverse relationship between

donor age and age of cultures at last passage, doubling potential, number of doublings during phase II, and age of culture at the end of phase II. The degree of statistical significance was also higher for growth indices determined at the end of phase II (the period of active proliferation) than for those corresponding to phase III. Of the five indices that were measured, only one, doubling time during phase II, did not correlate with the age of the donor liver.

In any event, this one index does not provide a method of estimating the age of human adult liver cells in tissue culture. The other findings support those of some investigators which suggest that ageing *in vitro* is an intrinsic programmed function of the cells [6, 9, 23, 30]. However, ageing *in vitro* can be affected by altering environmental conditions [31]. Thus the life span of a culture can be prolonged by enrichment of the medium [32], conditioning of the medium [28], and addition of drugs such as hydrocortisone to the tissue medium [4, 34].

This work has also shown that cell lines of human liver cells behave differently, depending on their main cell type. Only the life span was similar in the three main groups, reflecting a longer phase III for type B cultures. Type A cultures had a higher multiplication potential than type B cultures. This finding corroborates the suggestion that the multiplication potential is inversely related to differentiation of the cell and provides a method of distinguishing between the two cell types. The cell types can also be distinguished by biochemical and morphological criteria [7, 8, 17–19].

In conclusion, we have shown that there is a negative correlation between the various growth indices and the age of the donor material in tissue culture of human liver cells. The correlation, which is marked when the various subcultures are considered as a whole, also applies to each separated group of cell lines; yet, because the numbers are small, the degree of significance is not as high. Correlations were notably weaker for B cell cultures than for A cell cultures, presumably because the greater differentiation of the former cell reduced their ability to reflect the age of the donor material.

Acknowledgements

The authors wish to thank N. DUBRULLE N. PASDELOUP (INSERM) and M. SAUZIERE for their technical assistance. This investigation was supported by the Institut national de la Santé et de la Recherche médicale (INSERM).

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