Human fibroblasts in vitro senesce with a donor-specific telomere length

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Received 17 January 2002; revised 15 February 2002; accepted 18 February 2002

First published online 7 March 2002

Edited by Barry Halliwell

Abstract In human fibroblasts, replicative senescence, telomere length and donor age are closely interrelated. We analyzed these relationships for fibroblast strains from 14 healthy human donors in the age range of 28–90 years. In vitro replicative capacity was correlated more closely with donor age than with telomere length ex vivo, especially for healthy donors. Telomere length at senescence was as variable as at cell explantation and increased with donor age. The data suggest a donor-specific, age-dependent regulation of the telomere length threshold that triggers senescence in human fibroblasts.

Key words: Fibroblast; Senescence; Hayflick limit; Telomere

1. Introduction

Since its first formal description [1], replicative senescence of untransformed human cells has been regarded as an important model of, and a possible contributing factor to, ageing (for review, see [2]). One major argument in favor of such a role was the negative correlation between donor age and replicative potential in vitro. While such a correlation was demonstrated for different cell types including, for instance, adrenocortical cells [3], a careful re-evaluation failed to confirm it for human fibroblasts from healthy donors [4]. However, telomere lengths were not recorded in this study.

There is good evidence that it is the shortening of telomeres with each round of DNA replication, which serves as 'replicometer' and signals irreversible cell cycle arrest, i.e. senescence, after reaching a certain threshold (for review, see [5]. Thus, the average length of all telomeres at a given population doubling level (PDL) has been shown to be a good predictor of the remaining replicative capacity of this culture [3,6,7]. However, the simple relation between telomere length and Hayflick limit becomes more complicated by a number of factors: (i) It is not clear whether the length threshold that signals senescence applies to the shortest telomere, a group of (short) telomeres or all telomeres in a cell. While some recent data indicate that maintenance of the shortest telomere(s) is most important for cell growth and viability [8,9], it has also been shown that very short telomeres can exist on some chromosomes long before senescence and that average telomere length correlates better with the remaining proliferative capacity than the length of any single telomere [7]. (ii) Probably, not only telomere length but also other factors, which influence the probability of unscheduled opening of the telomeric loop [10], might trigger a senescence signal. Those factors could be the available amounts of loop-stabilizing proteins TRF1 and TRF2 or the presence of telomeric strand breaks [11]. (iii) The threshold telomere length at senescence might not be constant between cell strains or even between experiments [12]. In fact, one study suggested a negative correlation between donor age and fibroblast telomere length at senescence [13]. (iv) The rate of telomere shortening varies between strains from different donors. Oxidative stress [12] and, especially, differences in the antioxidant defence capacity between strains [14] are the most important causes for this variation.

To better understand how donor age and telomere length at explantation and at senescence influence the Hayflick limit, we analyzed the multiple correlations between these parameters for fibroblast strains from human donors between 28 and 91 years of age. The Hayflick limit for fibroblasts derived from the skin of healthy donors correlated significantly with donor age, but not with telomere length ex vivo. Telomere length at senescence was highly variable and increased with donor age. The data suggest a donor-specific, age-dependent regulation of the telomere length threshold that triggers senescence in human fibroblasts.

2. Materials and methods

2.1. Cell culture

Fibroblast cultures were established from skin biopsies from the unexposed part of the left upper arm from 14 individuals (age range 28–91 years) after informed consent was obtained. All donors were non-demented and free of severe cardio- or cerebrovascular conditions. Cultures from 10 donors were grown until close to senescence. Cells were grown in Dulbecco's modified Eagle's medium plus 10% fetal calf serum under an atmosphere of 20% oxygen, 5% CO_2. In addition, six skin fibroblast cultures and one culture from fetal lung fibroblasts (MRC-5) were grown for 6 weeks under 40% normobaric hyperoxia in a three-gas incubator (Nuaire), returned to normoxia afterwards and grown under normoxia until senescence. Cells were always passaged before reaching confluence and counted at each passage in triplicate using a hematocytometer. To establish a proliferation record independent of the differences in the starting material, growth curves were extrapolated back to day 0 and the PDL at that day was set to 0. The Hayflick limit population doubling PD_{max} was obtained using the condition that the culture should perform less than one doubling in 4 weeks. To obtain error estimates from the cell counts, the growth curve was fitted with a Chapman equation

$$PD = y_0 + a(1-e^{-bt})^c$$

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Abbreviations: DCF, dihydrochlorofluorescein; PD, population doubling; PDL, population doubling level; PD_{max}, Hayflick limit; TRF, telomere restriction fragment length; TSR, telomere shortening rate

0014-5793/02/$22.00
PII: S0014-5793(02)02504-8

The equation was solved for the conditions as stated above to obtain PD_{max}, and the standard error of PD_{max} was calculated according to the Gaussian law of error propagation.

2.2. Telomere length

For preparation of highest quality genomic DNA, cells were embedded in 0.65% low-melting agarose plugs at a density of 10^7 cells/ml before deproteination by proteinase K treatment [15,16]. DNA was digested until completion with *Hin*fI (60 U/plug; Boehringer Mannheim, Germany) at 37°C. Plugs were analyzed in a 1.0% agarose gel by pulsed field gel electrophoresis (Bio-Rad, Hercules, CA, USA). Gels were blotted to Hybond N+-membranes and hybridized with the telomeric probe $(TTAGGG)_4$, conjugated directly to alkaline phosphatase (Promega, Madison, WI, USA). A chemiluminescence signal was recorded on film within the linear range and analyzed in an imaging densitometer (Bio-Rad). The average telomere length was calculated as weighted mean of the optical density as described [15]. To standardize measurements between different blots, two different markers (1 kb ladder, and λ*Hin*dIII) were used. In addition, at least five lanes per blot were used for repetition of measurements from previous blots. Telomere length was measured at at least six different PDL, and measurements were performed in triplicate. Telomere shortening rates (TSRs) per PD were calculated by linear regression. First telomere measurements were done at PDLs between 5 and 15, and last measurements were performed at less than 5 PDL from senescence (with 2 exceptions). Linear extrapolation was used to obtain corrected values for the telomere length at explantation, TRF_0, and at senescence, TRF_s. Standard errors of these estimates were obtained from the 5% confidence intervals of the regression lines.

2.3. Oxidative stress

Fibroblast cultures were grown for at least three weeks in parallel under either normoxia (control) or 40% normobaric hyperoxia. Cells were stained for 30 min at 37°C in the dark with 80 μM dihydrochlorofluorescein-diacetate as described [17]. The intensity of dihydrochlorofluorescein (DCF) staining is an indicator of the intracellular oxidative stress and was measured in a flow cytometer (Partec, Munster, Germany) using blue excitation.

2.4. Statistical evaluation

Linear regressions were calculated in SigmaPlot 2000. SPSS for Windows was used to calculate correlation matrices.

3. Results

While fibroblast growth rates in culture varied between donors, there was no correlation to donor age or health status. However, growth rates were constant in each case for at least the first 6 weeks in culture, so that numbers of proliferating cells at day 0 could safely be extrapolated by linear regression. These cell numbers at explantation were used to fix PDL0 at day 0, and all subsequent cell counts including the estimation of the Hayflick limit referred to it. Cell numbers at explantation varied by up to two orders of magnitude between biopsies, indicating that estimates of the Hayflick limit according to the standard procedure (i.e. fixing the starting PDL at the point when the first confluent dish is obtained) might bear errors of up to seven PD between strains.

Table 1
Cellular peroxide content, growth rate and TSR under normoxia and 40% hyperoxia in seven human fibroblast strains

	DCF fluorescence (%)	Growth rate (PD/wek)	TSR (bp/PD)
Normoxia	100	1.68 ± 0.12	40.9 ± 12.6
Hyperoxia	152 ± 15	0.48 ± 0.13	236.0 ± 77.6

DCF fluorescence under normoxia was set to 100%. Data are mean ± S.E.M. from seven fibroblast strains (MRC-5 and six donors). Measurements were performed in triplicate.

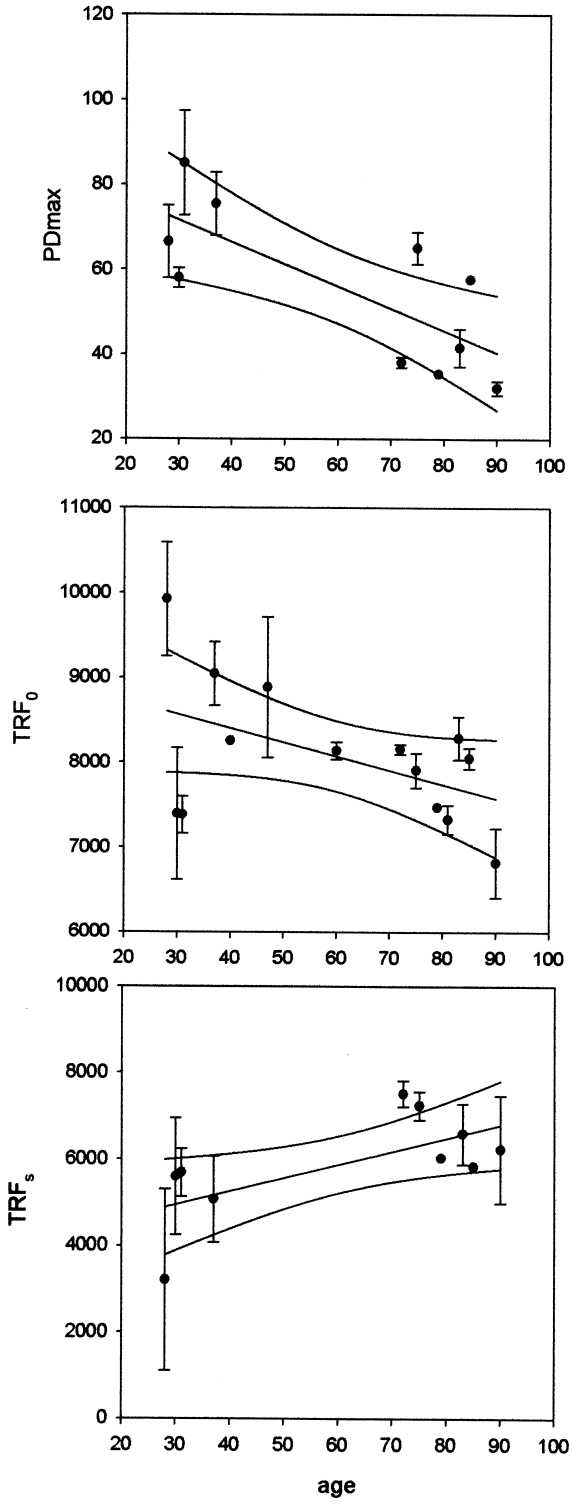

Fig. 1. Donor age dependencies (age in years) of (top) Hayflick limit (PD_{max}), (middle) telomere length at explantation (TRF_0), and (bottom) telomere length at senescence (TRF_s). Data are mean ± S.E.M. Linear regressions including 5% confidence intervals are indicated, and regression slopes *m* and significance levels *P* are given.

Our data show a significant dependency of the Hayflick limit for cultured human fibroblasts from donor age, with a decrease of about 0.5 PD per year of age (Fig. 1, top). The length of fibroblast telomeres ex vivo tends to decrease with

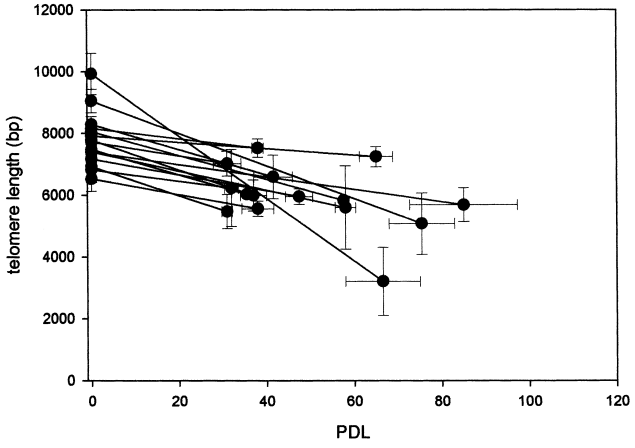

Fig. 2. Telomere length vs. PDL for 15 fibroblast strains from different donors (including five strains derived from donors suffering from stroke or dementia) grown until senescence under normoxia. Standard errors are indicated.

age by 17 bp/year (Fig. 1, middle), however, this decrease is statistically not significant.

Fibroblast strains from different donors senesced not with the same average telomere length. Rather, telomere length at senescence increased significantly with age by 31 bp/year (Fig. 1, bottom). Accordingly, the variation of telomere length between strains at senescence was as large as at explantation (Fig. 2). Rates of telomere shortening varied as well between strains (Fig. 2) but this variation was not age-correlated.

To examine further the regulation of telomere length at senescence, 7 fibroblast strains were also grown for 6 weeks under 40% chronic normobaric hyperoxia, and then cultivated further on under normoxia until senescence in parallel to control cultures constantly held under normoxia. Hyperoxia induces oxidative stress as indicated by an increase in DCF fluorescence. It slows down growth and accelerates telomere shortening (Table 1), in accordance with earlier data [12,14,18]. After resumption of basal conditions, cell growth and TSRs paralleled that in controls. Consequently, cultures held under hyperoxia senesce 5–10 PD earlier (Fig. 3A). However, due to the opposite effects of oxidative stress on growth rate and TSR, hyperoxic and normoxic cultures senesce with the same donor-specific telomere length. This was true irrespective of whether the strain senesced with long or with short telomeres (Fig. 3B). The notion that each strain senesces with its own telomere length is not only true with respect to the average telomere length. As Fig. 3B clearly shows, the length of the shortest telomeres discernible in the Southern blot is strain-specific as well.

Table 2
Pearson correlation matrix for the Hayflick limit PD_{max}, donor age, TSR, telomere length at explantation, TRF_0, and telomere length at senescence, TRF_s, in 10 fibroblast strains derived from healthy donors

	PD_{max}	Age	TSR	TRF_0	TRF_s
PD_{max}	1.000	**−0.748**	0.200	0.363	**−0.465**
Age		1.000	−0.419	−0.414	**0.670**
TSR			1.000	**0.820**	**−0.866**
TRF_0				1.000	**−0.591**
TRF_s					1.000

All parameters were measured under normoxia. Coefficients indicating significant correlations are printed in bold.

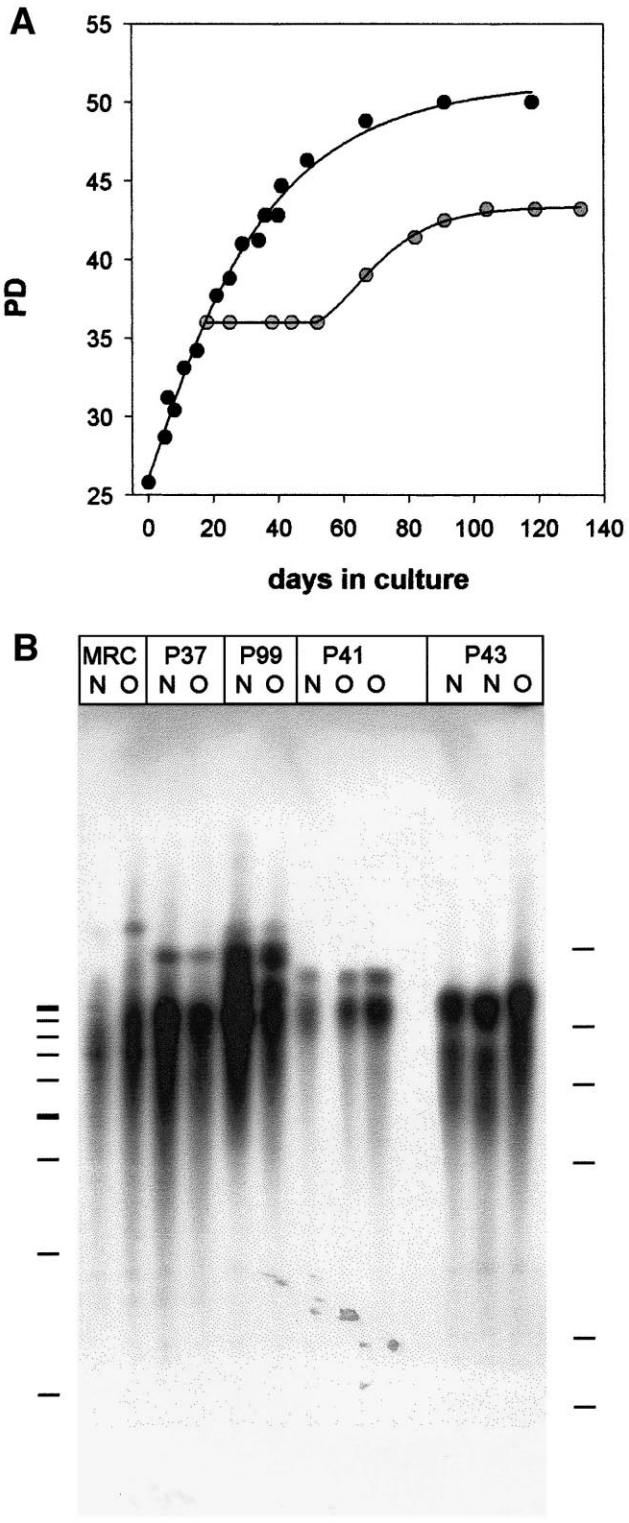

Fig. 3. Cell growth and telomere length under normoxia and hyperoxia. A: Typical growth curve. Cells were grown until senescence under either normoxia (control, filled circles) or for 6 weeks under hyperoxia (open circles) followed by further growth under normoxia (gray circles). B: Telomere length at senescence after growth under normoxia (N) or under normoxia/6 weeks hyperoxia/normoxia (O). Skin fibroblast cultures from four different donors and one embryonic lung fibroblast strain (MRC-5) are shown.

To examine further the relative roles of donor age, TSR, telomere length ex vivo and at senescence for the Hayflick limit, we calculated the correlation matrix for these parameters (Table 2). In accordance with the results shown in Fig. 1, donor age was found as the strongest predictor of the Hayflick limit, followed by telomere length at senescence, while the correlation to telomere length at explantation was not significant at the 5% level. While TSR did correlate strongly to telomere length, it did not at all to the Hayflick limit.

4. Discussion

According to our data, donor age is the strongest predictor of the replicative capacity of human fibroblasts. While this is in favor of the concept of an age-dependent decrease of replicative potential, it is not in accordance with results of a recent large study [4], which could not detect a significant negative correlation between replicative potential and donor age for healthy adult donors. Although sample size was small in our study, we believe that taking into account the cell number at explantation for the assignment of the PDL greatly improves the reliability of our results.

Studying fibroblast strains from 31 donors of unreported health status, Allsopp et al. [6] found the strongest correlation between replicative capacity and ex vivo telomere length and suggested telomere loss during proliferation in vivo as the major predictor of the lower in vitro replicative capacity of fibroblasts from old donors. We cannot confirm this. Examining fibroblast strains from healthy donors, and estimating telomere length at explantation, we did not find significant correlations to either replicative capacity or donor age (Table 2). However, if fibroblasts from five elderly patients with stroke or probable vascular dementia were included (age range 64–91 years, median 84 years), correlations between telomere length at explantation and either Hayflick limit or donor age became significant ([14] and data not shown). We have shown earlier that lymphocyte telomeres from patients with vascular dementia were significantly shorter than those in age-matched healthy controls, and that there was a strong intraindividual correlation between telomere lengths in lymphocytes and fibroblasts [14]. Shorter telomeres were also found in lymphocytes from atherosclerotic patients in a likewise small study [19]. Abundant in vitro data suggest that shorter telomeres in cells from diseased donors might not simply be the result of faster cell cycling, but might be due to oxidative stress-induced higher rates of telomere shortening per cell division [14].

We found that neither the average telomere length, nor the length of the shortest telomeres at senescence was constant between strains. On the contrary, telomere length varied between strains at senescence at least as much as at explantation. Moreover, our data suggest that donor age determines the threshold telomere length that signals senescence (Table 2). Differences between telomere lengths at senescence were also found by comparing fibroblast strains from two infant and two adult donors [13]. The threshold at which (average or shortest) telomere length signals senescence is set differently for each donor. This threshold is not shifted by a period of increased oxidative stress, which accelerates telomere shortening and shortens the replicative lifespan (Fig. 3A,B and [18]). The same might not be true under chronically diminished oxidative stress since chronic antioxidant treatment of MRC-5 fibroblasts elongated the replicative lifespan, but slowed telomere shortening even further, so that treated cells senesced with longer telomeres [12].

Our data suggest that the age-dependency of the Hayflick limit can partially be explained by an increase of the telomere length at senescence with donor age (Fig. 1, bottom). A cause for this age-dependent shift has not been established yet. However, it has been suggested that insufficient stability of the telomeric loop [10], resulting in unscheduled loop opening and exposure of the G-rich telomeric single-stranded overhang to the nucleoplasm, triggers replicative senescence in telomerase-negative cells [11]. Telomeric single-stranded G-rich DNA is a strong inducer of a p53-dependent senescence-like cell cycle arrest [20]. At least one loop-stabilizing protein, i.e. TRF2 [21], increases in abundance as human fibroblasts reach senescence [13]. It is not known yet whether the expression of such proteins is regulated in an age-dependent fashion.

Acknowledgements: Work was supported by grants from the Verum Foundation, Germany, and the MRC (UK).

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