Telomerase Can Extend the Proliferative Capacity of Human Myoblasts, but Does Not Lead to Their Immortalization

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Abstract

Normal cells in culture display a limited capacity to divide and reach a non-proliferative state called cellular senescence. Spontaneous escape from senescence resulting in an indefinite life span is an exceptionally rare event for normal human cells and viral oncoproteins have been shown to extend the replicative life span but not to immortalize them. Telomere shortening has been proposed as a mitotic clock that regulates cellular senescence. Telomerase is capable of synthesizing telomere repeats onto chromosome ends to block telomere shortening and to maintain human fibroblasts in proliferation beyond their usual life span. However, the consequence of telomerase expression on the life span of human myoblasts and on their differentiation is unknown. In this study, the telomerase gene and the puromycin resistance gene were introduced into human satellite cells, which are the natural muscle precursors (myoblasts) in the adult and therefore, a target for cell-mediated gene therapy. Satellite cells expressing telomerase were selected, and the effects of the expression of the telomerase gene on proliferation, telomere length, and differentiation were investigated. Our results show that the telomerase-expressing cells are able to differentiate and to form multinucleated myotubes expressing mature muscle markers and do not form tumors *in vivo*. We also demonstrated that the expression of hTERT can extend the replicative life of muscle cells although these failed to undergo immortalization.

Introduction

It has now been well established that phenotypically and karyotypically normal human somatic cells exhibit a limited capacity to proliferate. The process that limits the proliferative life span has been termed cellular or replicative senescence (1–4). This behavior of normal cells is in striking contrast to (*a*) cells that propagate the germ line and (*b*) the vast majority of tumor cells. This limit in proliferation can be bypassed in rodent cells, either by the forced expression of various viral oncoproteins (5, 6) or by spontaneous immortalization (7). These two mechanisms are at the origin of the numerous rodent cell lines defined by their immortalized state and a highly variable phenotype, depending on the immortalizing agent used and the initial state of the cells. However, escape from senescence and acquisition of an indefinite life span is an exceptionally rare event in normal human cells. Forced expression of viral oncoproteins, such as large T from SV40, can only extend the replicative life span of human cells, but does not lead to true immortalization (8–10), and a very large majority of the human cell lines have been derived directly from tumors. Because senescence imposes a stricter regulation of proliferation in human than in murine cells, several hypotheses have been raised in the past to explain this discrepancy. However, it is generally thought that more complex mechanisms have probably evolved in humans to limit the proliferative capacity and consequently to prevent tumor growth.

The limit in the proliferative capacity of human cells, which is much stricter in humans than in mice where spontaneous cell lines emerge with a much higher frequency, implies that mammalian cells have evolved a way to accurately count the number of cell divisions (11). Telomere shortening has been proposed as a good candidate in human cells for such a mitotic clock. Telomeres are long non-coding repeats of TTAGGG situated at the ends of the chromosomes. During DNA replication, some sequences are lost upstream of the last Okasaki fragment, as already predicted in 1971 (12, 13). Therefore, telomeric sequences shorten at each cell division, and it has been proposed that extensive shortening leads to the triggering of cellular senescence through a DNA damage signal inducing the p53 pathway (14, 15).

A two-stage model, M1 and M2, for the control of cellular growth has been proposed by Shay *et al.* (16). Mortality stage 1 or M1 represents the onset of senescence in normal cells. Viral oncoprotein such as large T from SV40, which can bind factors involved in the p53 pathway (17, 18), can override M1, leading to an extended life span. However, during this extended life span, the telomere sequences will continue to shorten, and cell proliferation will then be stopped in a stage initially defined as crisis, or mortality stage II (M2). Only a restoration and/or a stabilization of telomere length can allow cells to escape M2, whether this event occurs during the life span of the cells or at the end of it (19, 20).

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A complex including a DNA polymerase, originally discovered in *Tetrahymena* and called telomerase, is capable of elongating telomere repeats at the chromosome ends (21). Telomerase has been identified in mammals, both by its enzymatic activity and by the detection of its different components. It is a ribonucleo-protein enzyme complex composed of an RNA component that represents the template for telomeric DNA addition and a catalytic protein. In *Saccharomyces cerevisiae*, the EST 2 gene product, the catalytic subunit of telomerase, is essential for telomere maintenance *in vivo* (22). The enzymatic activity of telomerase has been detected in the majority of malignant tumors in humans and in the immortal cell lines derived from them (23–25). It is, however, absent from normal somatic cells except for germline cells (26), tissues with rapid turnover, and stem cells that express a low level of telomerase (23, 27–32). The human gene coding for the catalytic subunit (hTERT) has been cloned (28, 33, 34). The RNA template has been detected in a variety of human tissues (33), which indicates that it is rarely a limiting factor for telomerase activity. The introduction of the gene coding for hTERT into human fibroblast is sufficient to trigger telomerase activity, block telomere shortening, and to maintain the cells in proliferation beyond their usual life span (35, 36). Tumor-derived cell lines with telomerase activity usually show a limited, if any, possibility of differentiation. However, recent reports on bone marrow stromal cells (BMSC) show that hTERT expression in BMSC provokes an increase in their life span without hampering their osteogenic potential when shifted to osteogenic conditions (37, 38). Therefore, hTERT expression does not perturb the future determination of BMSC into osteogenic precursors. Human endothelial cells derived from neonatal foreskin also maintain their capacity to differentiate after being transduced by a hTERT construct and form microvasculature *in vivo* (39). However, there is no report yet concerning skeletal muscle precursors, which are already determined and can only produce skeletal muscle fibers with post-mitotic nuclei.

Skeletal muscle is a relatively stable tissue, which in normal subjects has very little active regeneration or nuclear turnover. The nuclei responsible for actively transcribing the genes involved in muscle function and maintenance are post-mitotic (40). Their post-mitotic state is under the control of the expression of the retinoblastoma protein Rb. Whenever a muscle fiber is damaged, its replacement is ensured by a population of cells situated under the basal lamina of fibers called satellite cells (41). These cells are responsible for the pre- and post-natal growth of muscle, but are quiescent in normal adult muscle (42). Following damage, satellite cells will be activated, will proliferate, and differentiate to replace the damaged fibers (43). A small number of cells will escape the differentiation step and restore the pool of quiescent cells under the basal lamina. Human satellite cells can be isolated and cultured *in vitro*, and studies from our group have measured their limit in proliferative capacity as a function of donor age and have correlated this capacity with the evolution in the length of telomere sequences (44). Although there is little satellite cell proliferation involved during the normal functioning of adult skeletal muscle, the situation can be dramatically different in diseases involving extensive muscle degeneration, such as in muscular dystrophies. In biopsies from children suffering from muscular dystrophies, a dramatic erosion of telomeres, measured as telomere restriction fragments (TRFs), was observed even in early childhood (45). Our results imply that the regenerative capacity of dystrophic satellite cells is dramatically reduced, leading to abortive regeneration in the final stages of muscle dystrophies.

Because skeletal muscle has its own progenitors and is a very stable and highly irrigated tissue, it has been proposed as a target for cell-mediated gene therapy, concerning either structural muscle proteins or circulating factors (46–48). However, the limited proliferative capacity of the human satellite cells imposes a strict strategy, which must take this limit into account. Two approaches have been considered: (*a*) Using satellite cells from a donor distinct from the patient allows one to choose cells with an optimal regenerative capacity, but poses the problem of immune rejection; and (*b*) using the satellite cells from the patient will not be applicable if these cells have already exhausted all or part of their proliferative capacity, either in cases of muscle degenerative diseases or if the patient has reached an age where too few divisions are left to envisage an efficient participation to regeneration. This is particularly important to consider in view of the results obtained in experimental myoblast injection trials in mice, where the group of Beauchamp has shown (49) a massive cell death of the injected cells. An even greater proliferative capacity is therefore required to ensure the success of cell-mediated gene therapy trials. The extension of the proliferative capacity of human satellite cells has thus become a priority in cell-mediated gene therapy trials, and we describe here the possibility of using telomerase to increase this limit and confirm that this expression has no effect on muscle cell differentiation.

Results

Replicative Capacity and Telomere Shortening of Human Satellite Cells

During prolonged periods of culture, the population of satellite cells isolated from the quadriceps of a 5-day-old infant (cell strain CHQ5B) underwent a progressive decrease in proliferative potential. Fig. 1A shows the evolution in the number of cell divisions made by this population plotted as a function of time. In the initial phase after isolation, the cell population divided rapidly. During their *in vitro* life span, this rate of division progressively decreased until a plateau was reached when no more cell divisions were observed. In this final phase, the cells continued to have a metabolic activity and remained attached to the substrate. At the end of their proliferative life span, the cells were flatter and larger with a morphology similar to that described for senescent fibroblasts (data not shown). It should be noted that the rate of division, calculated at each cell passage, represents a mean for the whole population. In this study, as revealed by desmin expression, cell cultures were over 80% myogenic throughout their proliferative life span, but they were not synchronized in terms of cell division, and the results therefore represent an average. Nevertheless, this gives an accurate estimation of the *in vivo* situation, where heterogeneity in terms of proliferative capacity is also present.

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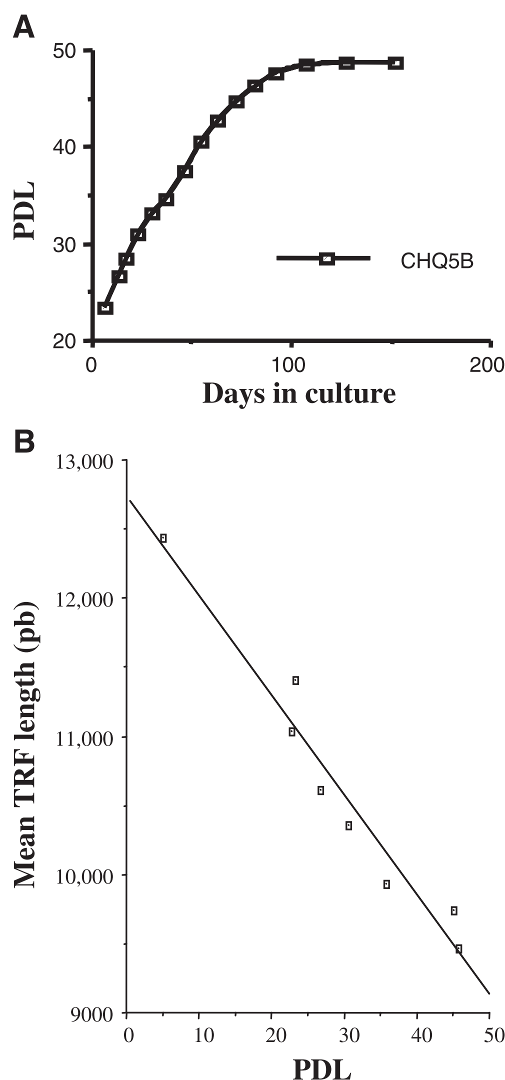

FIGURE 1. **A.** Life span curve (*i.e.*, number of divisions made as a function of time in culture) of normal human satellite cells isolated from a muscle biopsy of a 5-day-old donor (*CHQ5B*). **B.** Analysis of TRFs in CHQ5B. Graphic representation of the change in TRF length as a function of the divisions made in culture. *P*, Population; *D*, Doubling; *L*, Level.

DNA was prepared at various population doubling levels (PDLs), and the mean values of TRF length were analyzed and plotted (Fig. 1B). As expected, in normal human satellite cells, the TRFs shortened regularly and 93 bp of DNA were lost on average per cell division. In this set of experiments, the normal satellite cell population stopped dividing at 48 PDL with an average value for the mean TRFs of 8.5 kb. Clonal analysis was independently performed twice in parallel on the same cultures. The life spans of 45–50 clones were analyzed. The maximum life span observed for a clone was 60–62 divisions in each experiment (data not shown). Extrapolation of the curve presented in Fig. 1B indicated that the mean TRF length of these cells at isolation was of 12.7 kb. The results of this analysis were used as controls to compare with those obtained with cells transduced with the construct containing the hTERT gene.

Telomerase Activity and Telomere Elongation in Human Satellite Cells

The viral constructs described in the "Materials and Methods" were used to infect the CHQ5B cell populations both at early stages in their proliferative life span (PDL 15 and 17.8) and just before senescence (PDL 46.4). The infected populations were immediately plated at clonal density in the presence of puromycin. Over 100 puromycin-resistant clones were isolated from each experiment, and the efficiency of infection reached 10%, as indicated by the fraction of puromycin-resistant clones. Interestingly, the doubling time of satellite cells was not significantly modified after infection (*e.g.*, 36 h in non-infected cells *versus* 38 h in cells infected at PDL 15), and the same observation was made with population infected at PDL 46.4. It should be noted that we observed no correlation between the level of telomerase activity, as evaluated by telomeric repeat amplification protocol (TRAP) assay, and the doubling time. Puromycin-resistant clones were expanded, and the telomerase activity was tested in these clones using the TRAP assay. As demonstrated in Fig. 2, telomerase activity was detected in all puromycin-resistant clones. This activity was absent in control clones (*i.e.*, either transduced with a vector containing only the puromycin resistance gene or not transfected). These results suggest that expression of hTERT, the catalytic subunit, is the limiting factor for telomerase activity in these cells.

To check whether the exogenous telomerase is active on a natural chromosome substrate, a functional assay of telomerase activity was performed in hTERT cells by measuring telomere elongation. An example of such an analysis is shown in Fig. 3, where it can be seen that in the population infected at 17.8 PDL, the telomeres were elongated (mean value: 12 kb) when compared with the non-infected population (mean value: 10.5 kb) at the same PDL (Fig. 3).

Expression of Exogenous Telomerase Does Not Impair Muscle Differentiation

Because differentiated muscle cells contain nuclei which are all post-mitotic and express numerous muscle-specific genes in

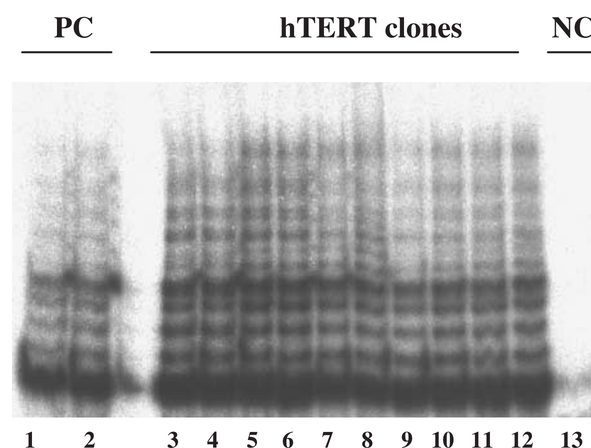

FIGURE 2. hTERT expression confers telomerase activity to infected CHQ5B cells. Clones were analyzed for telomerase activity by the TRAP assay. *Lanes 1 and 2,* positive control-H1299 cell extract. *Lanes 3–12*, clones isolated from hTERT CHQ5B populations. *Lane 13,* negative (untransfected) control.

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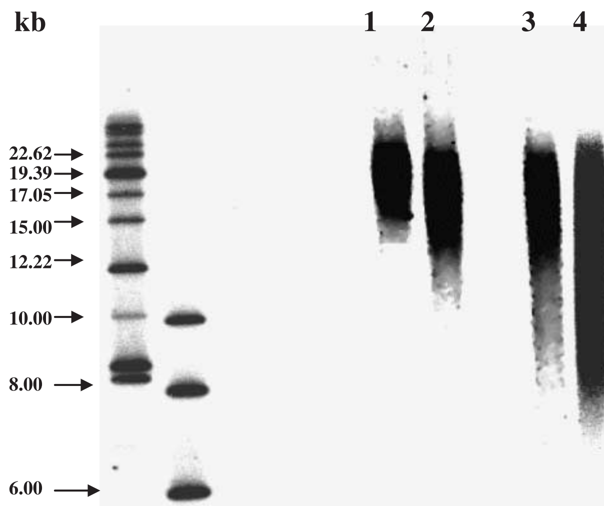

FIGURE 3. TRF analysis of human satellite cells (CHQ5B) infected by hTERT at 17.8 PDL (*lanes 1–3*) and uninfected population (*lane 4*). *Lane 1,* clone 10041 at 43 PDL (TRF = 19.2 kb); *lane 2,* clone 3015 at 45.8 PDL (TRF = 16 kb); *lane 3,* infected population analyzed at 24.5 PDL (TRF = 12 kb); *lane 4,* uninfected population at 26 PDL (TRF = 10.5 kb).

a concerted manner, we wanted to ensure that the expression of telomerase in satellite cells did not modify this program once differentiation was triggered. Clones of human satellite cells expressing hTERT were cultivated in conditions which induced differentiation. No significant changes were observed either in the final percentage of cell fusion which was measured in these cultures (mean value of 70% on both control and hTERT-expressing clones), or in the kinetics of fusion measured as the modification of the percentage of fusion with time, as compared to non-infected controls (data not shown). No significant difference in the size of the myotubes (*i.e.*, average number of nuclei per myotube) was observed. Both values were the same as those observed in uninfected satellite cell cultures. To verify whether the expression of telomerase induced qualitative changes in the myogenic program expressed by the hTERT clones, an immunocytochemical analysis was carried out on the differentiated clones using specific antibodies directed against the different myosin heavy chain (MHC) isoforms. MHC is a major component of the contractile apparatus and exists as a series of isoforms specific either for early stages of differentiation (*e.g.*, embryonic and foetal or slow and fast) expressed in the adult. Embryonic and neonatal (data not shown) as well as fast and slow isoforms were all co-expressed in these differentiated myotubes, and the pattern of expression (Fig. 4) was identical to that previously described in non-infected satellite cell populations (45).

Life Span of Clones Expressing hTERT: The PDL_{max} Is Not Related to the PDL at Infection

The human satellite cells used in this study had a maximum life span of 48 PDL. To determine if endogenous expression of telomerase would extend the life span of these cells, CHQ5B cells were infected either early (15–17.8 PDL) or late (46.4 PDL) in their proliferative life. Numerous clones were isolated

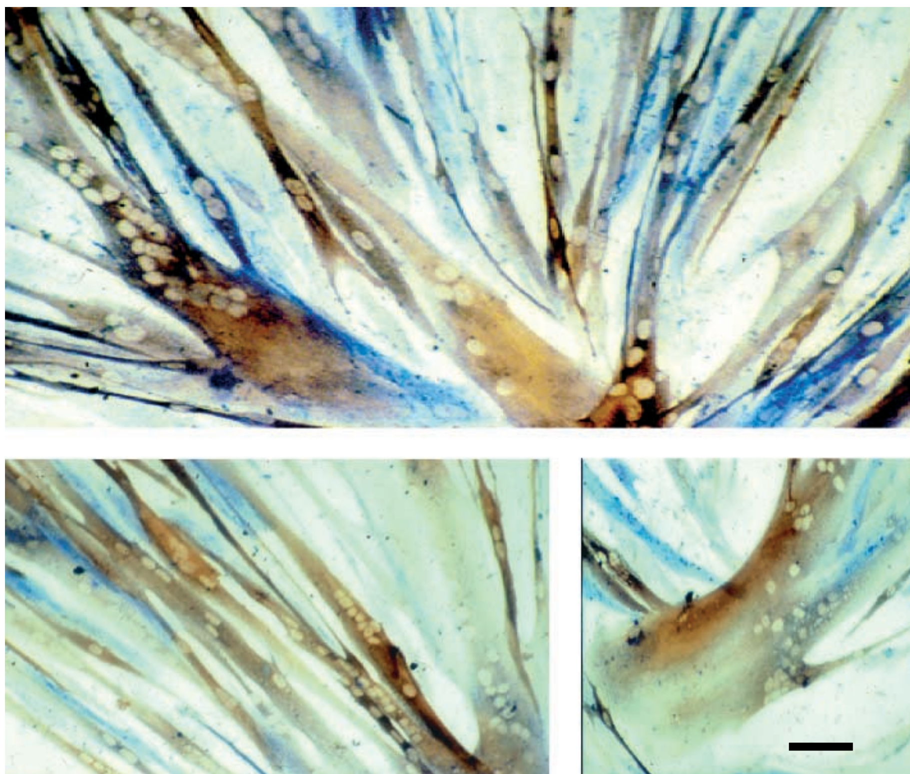

FIGURE 4. Double-immunolabeling of adult fast MHC, revealed by peroxidase (*brown*), and adult slow MHC, revealed by alkaline phosphatase (*blue*), on a differentiated culture of a clone isolated from CHQ5B expressing hTERT. *Scale bar,* 50 μm.

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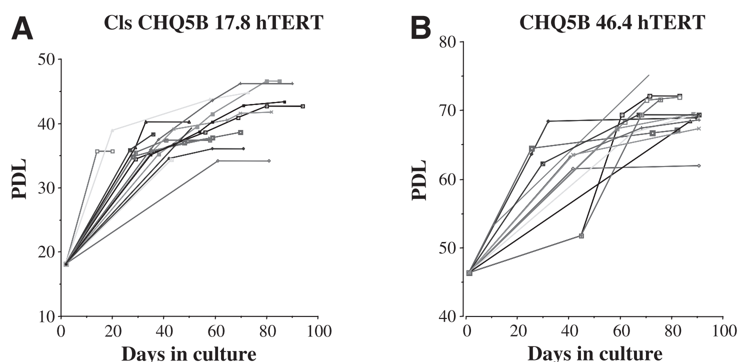

FIGURE 5. *In vitro* replicative potential of hTERT clones from populations of CHQ5B infected at different PDLs. Each graph shows the evolution of a single clone until it reached proliferative arrest. **A.** Expression of the number of PDLs accomplished by clones from CHQ5B infected at 17.8 PDL as a function of time. Note that one clone isolated from this population, after serial subcloning, reached finally 108 PDL before proliferative arrest. **B.** Expression of the number of PDL accomplished by the hTERT clones from CHQ5B infected at 46.4 PDL as a function of time in culture. Note that clone 3029 which presented an extended life span (maximum PDL reached: 108) is not represented in these graphs.

from populations infected either at 17.8 PDL or at 46 PDL and showed telomerase activity by the TRAP assay (not shown). The life span of 12 of these clones is illustrated for each population in Fig. 5.

As shown in this figure, clones expressing hTERT reached proliferative arrest after a variable number of divisions. The mean number of total divisions made by individual clones, that is, the sum of the divisions that were made before and after infection, when they reached proliferative arrest was 39.9 when cells were infected at PDL 17.8 and 69 when cells were infected at 46.4 PDL, that is, just prior normal senescence. It should be noted that this proliferative arrest was observed whether the cells were seeded at high (3600 cells/cm^2) or low (600 cells/cm^2) density. Therefore, there does not seem to be a correlation between the PDL reached when cells were infected and the PDL_{max} reached by the clones isolated from the infected population. However, clones isolated from the presenescent population showed clearly an extension of their life span. It should be noted that very rare clones emerged with a highly extended life span (estimated frequency: less than 1%). These clones are not represented in Fig. 5 (one such clone emerged from the population infected at 17.8), and their behavior is described in the next paragraph.

To determine whether the final proliferative arrest observed in clones was due to a disturbance induced in the telomere homeostasis by the exogenous expression of telomerase, analysis of TRFs was carried out. The telomere lengths of the different hTERT clones were determined at the oldest available PDL when the cells had just stopped dividing. As an example, the mean TRF lengths of the clones isolated from the population infected at 17.8, regardless of the PDL they reached, was 14.4 kb. It should be noted that the hTERT clones stopped dividing with telomeres that were relatively long (the shortest value was 10.4 kb) when compared to uninfected cells which usually stop dividing with a mean TRF length of 8.5 kb (see above).

The Majority of Clones Reached Proliferative Arrest Through Either Senescence or Spontaneous Differentiation

The previous results show that expression of exogenous telomerase is able to increase the length of the telomeres. This arrest in cell division was completely independent of the age of the population at the time of infection.

Morphological analysis of hTERT clones when they reached proliferative arrest revealed two distinct phenotypes. About 60% of the clones exhibited a typical senescent phenotype where the cells had become very large and flat, no longer divided (Fig. 6A), and expressed the senescence marker β-galactosidase (data not shown). The remaining 40% underwent spontaneous

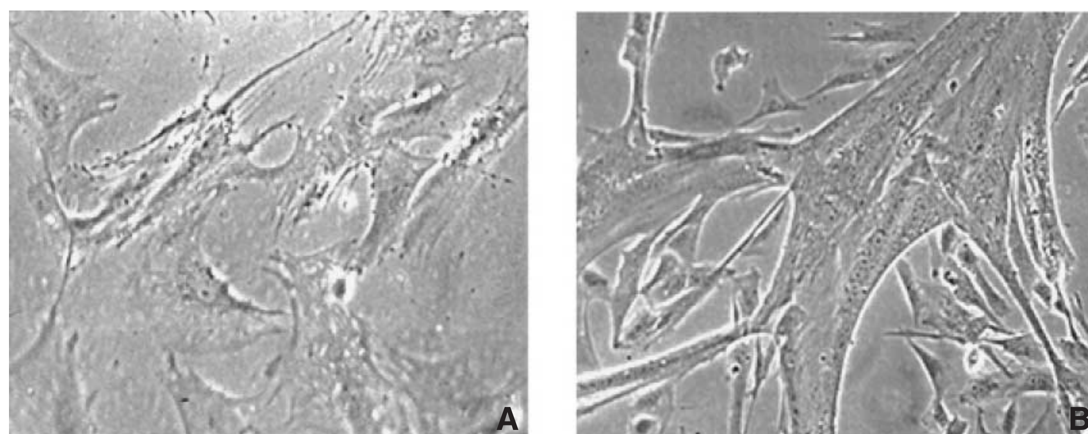

FIGURE 6. Morphology of hTERT-expressing cells when they reach proliferative arrest. **A.** Sixty percent of transfected clones showed a senescent phenotype. **B.** Forty percent differentiated spontaneously.

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differentiation (Fig. 6B) to form multinucleated myotubes even though the cells were maintained in 20% FCS, and the medium was changed regularly (twice weekly). Interestingly, clones that stopped proliferating, regardless of which pathway was triggered, still presented telomerase activity detectable by the TRAP assay, as shown in Fig. 7. The decision for a clone to use one or the other of the pathways involved in the proliferative arrest does not seem to be due to a modification in telomere homeostasis since the mean value of the TRFs is not significantly different in both situations (15.4 kb for the senescent clones and 13.4 kb for the differentiated clones). Taken together, these observations suggest that the rapid elongation of telomeres resulted in the triggering of a signal for growth arrest, which either drives the satellite cells toward premature senescence or toward differentiation.

Clones With Extended Proliferative Life Span

Clones that could proliferate well beyond the mean expected life span of the uninfected cells emerged with a very low frequency (less than 1%). To determine if this was due to a different type of regulation of telomere length by the exogenous telomerase, the TRF lengths were measured throughout the proliferative life span of one of these clones, and the results are presented in Table 1. The telomeres of this clone (clone 3029) were initially of 10 kb; they then decreased in size until they reached a value of around 6 kb, at which point they stabilized. Hence, it can be concluded that clones which had an extended life span also presented shorter telomeres, and therefore, a different mechanism of regulation for telomere homeostasis

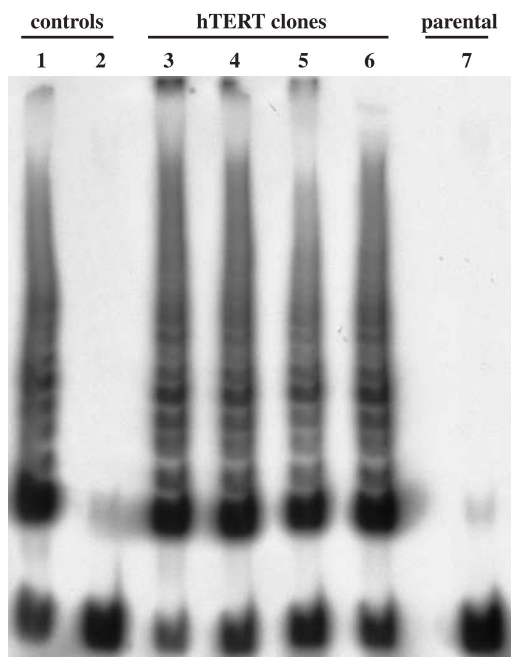

FIGURE 7. TRAP assay on clones expressing hTERT and that stopped proliferating for 3 weeks. *Lane 1,* positive control for hTERT activity (tumoral cell line H1299); *lane 2,* negative PCR control; *lanes 3–6,* hTERT clones that reached proliferative arrest; *lane 7,* parental non-infected cells.

Table 1. Mean TRF Length of Clone 3029 Isolated From CHQ5B Infected at PDL 17.8

PDL	41	42.5	47.2	57.4	67	69	73.2	74.6	79.7
TRF (kb)	10.3	10	9	9.4	8.3	6.2	6.7	6.3	6.7

Note: One subclone isolated from this clone eventually reached proliferative arrest at PDL 108, but no further change was observed in the TRF lengths.

must have been induced by the exogenous telomerase in these clones. Clone 3029 did, however, finally reached proliferative arrest. It should be noted that subclones isolated from clone 3029 reached a PDL_{max} of 108 and still retained the ability to differentiate in the absence of serum. Analysis of the karyotype of one of these subclones, examined randomly with other samples, showed a normal chromosomal distribution with no differences with the control samples (data not shown).

Telomerase-Infected Cells Did Not Form Tumors in Vivo

Culture conditions can have a major effect on the life span of human cells, and the results observed *in vitro* do not necessarily predict the behavior of the same cells *in vivo*. To investigate the capacity of these myogenic, telomerase-positive cells to survive and proliferate *in vivo*, and to determine their potential tumorigenicity, cells from clone 3029 were injected into the tibialis anterior (TA) muscle of $RAG2^{-/-}/\gamma c^{-}$ mice 3 days after the induction of muscle damage by injections of cardiotoxin.

Injected muscles were removed 2 months after injection and analyzed histologically to detect any tumor growth. Human cells were detected by antibodies specific for human lamin, a protein localized in the nucleus, and for human spectrin, a protein specific to muscle fibers (data not shown). While no nuclei positive for human lamin were detected in the contralateral muscles, only a few (10–50) nuclei were detected in the TA injected with hTERT-expressing cells. These numbers were too low to allow any statistical analysis and much lower than what we detected after injection of the same human strain at an early PDL (50). The frequency of myotubes expressing human spectrin was extremely low; however, no tumors (0 in the five animals) were ever observed. This result implies that the limited proliferation that we observed *in vitro* is also maintained after injection of these cells *in vivo*. We conclude that human satellite cells expressing telomerase present a limited proliferative life span *in vitro* as well as *in vivo* and are not tumorigenic.

Discussion

Although human satellite cells are responsible for muscle repair, they have a limited proliferative capacity and stop dividing in culture after a finite number of divisions in a state called senescence or Hayflick's limit (also known as mortality stage 1 or M1). We have shown previously that normal adult skeletal muscle satellite cells *in vivo* do not reach M1, even at old ages (44). However, this is not the case in muscular dystrophies when repeated cycles of degeneration occur (45). In these situations, the proliferative capacity is exhausted rapidly, leading to abortive regeneration and fibrosis. The limited proliferation of human cells is probably partly

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responsible for the failure of the early clinical trials using cell-mediated gene therapy for muscular dystrophies. We have shown (44) that telomeres shorten regularly during normal satellite cell divisions (93 bp on average in the conditions described in this report), and that telomere length is therefore a good indicator of previous proliferation of human satellite cells. We estimate that the mean TRF length of the control population used in this report was 13 kb at isolation, and that they shorten *in vitro* until the cells reached proliferative arrest when the mean TRF length had decreased to 8.5 kb. It is worth noting that the mean TRF length of the satellite cells (as measured after isolation *in vitro*) is within the same range as for other young human tissues (10–20 kb). However, muscle cells stop dividing with relatively long telomeres when compared to other cell types (*e.g.*, fibroblasts stop at 7 kb) (51–55). This discrepancy between skeletal muscle and other cell types could be due to the generation of a signal, which triggers proliferative arrest for different values of TRF depending on the type of tissue. Alternatively, it could be due to a proliferative arrest that occurs before the classical M1, a situation observed for other cell types. Human mammary epithelial cells, for example, have been described as senescing in two stages: an initial arrest (M0) that occurs after 20 PDL and a subsequent M1 arrest where the few cells which spontaneously bypassed M0 also stopped (56). However, it has been recently demonstrated that this premature proliferative arrest known as M0 reflects inadequate culture conditions (57).

In previous studies, it has been shown that hTERT can be used to extend the proliferative life span of human cells (35). In the present study, hTERT was introduced into human satellite cells and the expression of this exogenous telomerase on the program of myogenic differentiation was analyzed in parallel with the homeostasis of telomere length. The introduction of hTERT resulted in a high level of telomerase activity in all of the tested clones. Telomerase activity was accompanied, in a large majority of clones, by an elongation of the telomeres. When these clones were cultivated in conditions which induce differentiation, no difference was observed either in the percentage of cell fusion or in the number of nuclei per myotube as compared to clones that were either not infected or infected with a vector containing only the antibiotic resistance gene. We have previously shown that expression of T antigen from SV40 provoked the absence of expression of the adult myosin isoforms (58). This is not the case with hTERT, since all of the clones that differentiated formed large myotubes which co-expressed both fast and slow adult isoforms in their myotubes. Therefore, we can conclude that expression of hTERT does not modify, *per se*, either cell fusion or differentiation of human satellite cells.

The majority of clones that received the hTERT construct and expressed telomerase activity did not show any change in their growth rate after infection, suggesting that telomerase has apparently no impact on the cell cycle. Moreover, there was no correlation between the growth rate and the level of telomerase activity, as evaluated by the TRAP assay in different clones, and which varied from one clone to another (this variation can be due either to multiple sites of integration, or to various activities of the construct in single sites). Because integration of retroviruses is random, and growth rate did not vary considerably from one clone to another, a relationship between telomerase activity and growth rate is therefore unlikely. All the clones that received the construct and expressed telomerase activity as measured in TRAP assays did eventually reach proliferative arrest, although after variable numbers of divisions. Our results show that telomerase activity is not sufficient in these standard culture conditions to overcome senescence (M1) in human satellite cells, because clones that stopped dividing still presented telomerase activity. It should be noted that there is a down-regulation of telomerase activity in immortalized fibroblasts at confluence when they stop dividing by contact inhibition (57). Counter *et al.* (19) have demonstrated that human embryonic kidney cells virally transformed to overcome M1 and subsequently transfected by telomerase became immortalized. Therefore, the combination of two events, that is, bypassing M1 by transformation with viral oncogenes and the stabilization of telomere length by telomerase to bypass M2, is able to immortalize some human cell types. Other types of cells which initially showed resistance to the immortalization by telomerase have been shown to be arrested in M0, also called stasis or stress-induced premature senescence (SIPS), have finally been immortalized by manipulating culture conditions (59). Other cell types such as fibroblasts only need one event (the expression of hTERT). It has been reported recently that SV40 in combination with telomerase can immortalize human muscle cells (60). Unfortunately, our group demonstrated previously that the expression of T antigen both delays and reduces cellular fusion but more importantly, it interferes with the expression of an adult myogenic program (58). In addition, the oncogenic properties of T antigen represent an obstacle for its use in therapeutic clinical trials. However, the success of immortalization of human myoblasts by a combination of telomerase and large T antigen indicates that myoblasts present most probably a control of proliferation, which is distinct and more complex than fibroblasts.

In this study, we have clearly demonstrated that the majority of the clones which expressed hTERT reached proliferative arrest by two very distinct mechanisms. Clones expressing hTERT either stopped dividing by spontaneous differentiation, even when they were cultivated in the presence of 20% FCS, or they entered a non-dividing state that closely resembles senescence. It should be noted that this behavior is clonal, and that all of the cells from one clone will escape from the cell cycle using the same pathway. There did not seem to be a preferential pathway for this proliferative arrest, because 40% of the clones entered spontaneous differentiation while 60% stopped dividing without differentiating. Moreover, the choice of the pathway does not seem to be influenced by telomere length (average values of TRF length are not significantly different: 15 kb for differentiating clones and 13.8 kb for the senescent ones). It should be noted that these values are higher than values observed in the non-infected satellite cell population, showing clearly that telomere shortening is certainly not the mechanism that triggered growth arrest. Long telomeres could be responsible for the proliferative arrest by being incompatible with extensive cell proliferation of the

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muscle satellite cells. Telomeres that are too long could finally perturb T loop formation and/or stable access to the DNA of TRF binding proteins that stabilize telomeres, although it is clear that this kind of mechanism must be a progressive one.

It is interesting to note that only male germinal cells have long telomeres (61), whereas cells expressing telomerase activity whether they are normal (*e.g.*, activated lymphocytes) or isolated from tumors do not have long telomeres. Cell lines isolated from tumors, which represent the ultimate state of immortalization, usually have short stable telomeres, which remain constant in length even after repeated cell divisions. It is probably the equilibrium of telomere homeostasis which is crucial for continuous cell proliferation, as confirmed by the observation that the introduction of a sequence antisense to the template of the telomerase into HeLa cells rapidly leads to telomere shortening and the triggering of senescence (33). The group of Zhu (20) proposed several years ago a capping role for hTERT in human cells, similar to its role in yeast. This hypothesis could explain the extended life span of the myoblast clone with relatively short telomeres, but does not explain why clones with longer telomeres do not have an extended life span. An eventual role for hTERT in capping should still be present in these clones. Another alternative could involve the stability of the T loop at the end of the chromosomes, but this hypothesis needs further support.

Several hypotheses can be proposed to explain this phenomenon. For instance, this dual behavior could reflect the presence of two different populations of myoblasts present in the ratio of 60% and 40%. However, these two populations would have to have the same growth kinetics to maintain these proportions; otherwise, one population would be eliminated through serial passages. Although our former analyses of human populations of myoblasts did not show any heterogeneity among satellite cells, this hypothesis cannot be completely ruled out. Alternatively, satellite cells could be in two different states of cell cycle regulation, with the same restrictions as the ones expressed above. Satellite cells would then be able to exit the cell cycle by a yet unknown anti-tumor mechanism, and could even go randomly in either direction, differentiation or senescence. It should be noted that there are common factors, such as Rb, p21, or p53 involved in both pathways. There is evidence that Rb could be involved in an anti-proliferative mechanism because its presence and phosphorylation by cyclin-dependent kinase is required for progression through the cell cycle. Rb is also involved in the establishment of the irreversible non-proliferative state of differentiated myonuclei (62). Moreover, T antigen is known to bind Rb, probably inactivating its function (62). This mechanism could explain the additive effects of T antigen and telomerase (see above) that can lead to the immortalization of human muscle cells. However, the same result can be obtained, at least at rather early PDL, by manipulating culture conditions (59), thus emphasizing the importance of the cellular response to its environment in the control of the cell cycle. Although human myoblasts were not included among the cell types for which culture on feeder layers allowed immortalization by expression of the hTERT gene (58), the same authors have tested various culture conditions on the same strain of human myoblasts as the ones described in this manuscript, and have not observed immortalization of these cells.[1] It should also be noted that the efficiency of hTERT myoblasts to participate in muscle regeneration in immunodeficient mice was extremely poor, although they were in physiological conditions.

In this study, we observed only a few clones that could proliferate well beyond the mean life span, which had been determined in the non-infected cells. Detailed analyses revealed that the clones which had an extended mitotic activity also had telomere homeostasis which was strikingly different when compared to all other clones. This is demonstrated by clone 3029 which had relatively short telomeres. In contrast, the majority of the clones isolated in the same experiment had relatively long telomeres once they had entered the extended portion of their life span. The cells then went through a period during which TRF length decreased, although they still expressed telomerase activity. Finally, the mean telomere length stabilized around 6 kb. It is interesting to note that this value is much lower than those measured in all the other clones of this experiment and that most human immortal cell lines isolated from tumors generally exhibit telomerase activity and short telomeres (3–5 kb). In addition, cells expressing telomerase, but presenting long telomeres, never showed any sign of tumorigenicity *in vivo*, as already suggested by Seigneurin-Venin *et al.* (63). This supports the concept that telomere length needs to be stabilized for immortalization of the cell to be successful. However, clone 3029 eventually stopped dividing and became senescent. Therefore, although it seems that stabilization of telomere length is important for extension of life span, it is not sufficient to result in the immortalization of human muscle satellite cells.

We hypothesize that there are mechanisms involved in the regulation of the cell cycle which are specific for myoblasts. These mechanisms are dominant over the mitotic clock and could involve factors regulating the exit of cell cycle in both senescence and muscle differentiation.

Materials and Methods

Cell Culture

The satellite cells used in this study (CHQ5B) were originally isolated, in accordance with the French legislation on Bio-ethics, from the quadriceps of a 5-day-old infant (44) who died suddenly due to cardiac malformations and presented no signs of neuromuscular disorders. The cells were cultivated in a growth medium consisting of Ham's F-10 (Life Technologies, Inc., Cergy, France) supplemented with 50 μg/ml of gentamycin (Biomedia, Bousses, France) and 20% FCS (Biomedia). Cell populations were trypsinized when they reached half confluence. The number of population doublings (expressed as PDL) at every passage was calculated according to the formula log N/log 2, where N is the number of cells at the time of passage divided by the number of cells initially attached after seeding. The cultures were considered to be senescent when they failed to divide during 3 weeks in proliferative conditions.

Differentiation was induced at subconfluence by replacing the growth medium with DMEM (Life Technologies) supplemented with 50 μg/ml of gentamycin, 100 μg/ml transferin (Sigma Chemical Co.) and 10 μg/ml insulin (Sigma, St Louis, MO) (64).

[1] W. Wright, personal communication.

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Infection Procedure

The sequence coding for the catalytic subunit of human telomerase (hTERT) was cloned into the retroviral vector pBABE-puro under the control of the MPSV-LTR sequences. A control construct, which contained only the puro-resistance gene, was used.

Human satellite cell populations were infected with virus containing either the hTERT or the control construct at three different PDLs: two populations had undergone only a few cell divisions (15 and 17.8 PDL) and one population had been amplified and was near senescence (46.4 PDL). Infection was carried out by incubating the cells with 0.22 μm-filtered supernatants of producing cells in the presence of 4 μg/ml of polybrene (Sigma). The infecting medium was left in contact with the cells for at least 10 h, and the whole procedure was repeated each day for three consecutive days. The infected cells, that is, populations or cells seeded at clonal density, were then submitted to selection in the presence of puromycin (1 μg/ml for 7 days). The efficiency of infection was estimated as the number of surviving clones as compared to the non-selected populations. Over 100 clones were isolated from each infected population, and 20–30 clones were randomly chosen and further characterized.

Immunocytochemistry

The myogenic purity of satellite cell populations and clones was calculated using desmin as a marker for myogenic determination (65). Cells in growth conditions were rinsed three times with PBS and fixed for 10 min with 95% ethanol. Desmin expression was revealed using the antibody D33 (DAKO, Trappes, France, dilution 1/50), revealed by a biotin-streptavidin complex (DAKO) as previously described (44, 45). The myogenic program expressed by the cultures after 5 days of differentiation was revealed using either single labelling or double labelling with specific antibodies directed against the embryonic, foetal, and adult fast and slow MHCs, as previously described (45).

Analysis of Telomere Length

The cells were washed in PBS, trypsinized, and centrifuged. Cells were collected as pellets containing 2×10^6 cells and were frozen at $-20°C$ until further procedures; DNA was purified as described previously (44).

DNA samples obtained from cell culture were digested with the restriction enzyme *Hin*fI overnight at 37°C to generate TRFs that contained the TTAGGG tandem repeat and a constant subtelomeric fragment. Five micrograms of digested genomic DNA were resolved by electrophoresis in a 0.7% agarose gel for 14 h at 120 V. The gel was then dried for 10 h, denatured in the presence of NaOH 0.5 M/1.5 M NaCl for 15 min, then neutralized in NaCl 1.5 M/Tris-HCl 0.5 M for 15 min. The TRFs were detected by hybridization to a $^{32}P-(TTAGGG)_4$ probe as described by Allsopp *et al.* (51–66). Different exposure times were used so that the samples had equal signal intensities within the linear response of the X-ray film. The signal responses were analyzed by a computer-assisted system derived from NIH Image 1.62.

Detection of Telomerase Activity

Telomerase activity was detected using the TRAP assay according to the manufacturer's instructions as previously described (67). PCR reaction products were separated on 12.5% non-denaturing acrylamide gels. After fixation [50% ethanol, 0.5 M NaCl, and 40 mM sodium acetate (pH 4.2)], the gels were directly exposed to an X-ray film (Kodak Biomax, Paris, France) with an intensifying screen (Biomax, Kodak).

Transplant of Telomerase-Expressing Cells Into Immunodeficient Mice

All surgical procedures were performed under general anesthesia with Hypnorm and Hypnovel in $RAG2^{-/-}/\gamma c^-$ as described previously (50, 68, 69). Skeletal muscle regeneration was induced in the mouse 3 days before injection of cells by a single i.m. injection of cardiotoxin dissolved in PBS (final concentration = 10 mM Latoxan, Rosans, France). Five animals were injected in each set of experiments (hTERT-expressing cells or non-infected control cells), which thus corresponds to five experiments with injection of hTERT-expressing cells and control cells in each experiment.

Cells expressing telomerase, as well as non-infected cells used as controls, were trypsinized and collected by centrifugation at 1500 rpm for 5 min. Cell pellets were resuspended in F-10 medium such that 5×10^5 cells could be transplanted per injection in a total volume of 15 μl. The right TA muscle was exposed along its entire length and cells were injected at three different sites along the muscle, while contralateral TAs were not injected to be used as negative controls. Animals were sacrificed at 1 week and 2 months and muscles were analyzed for the presence of tumors. Muscles were frozen in cooled isopentane and transverse sections (5 μm) were stained with antibodies recognizing specifically human lamin (Novocastra, Newcastle, UK), a nuclear protein, which did not cross-react with the mouse, or human spectrin, a muscle-specific protein.

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