Telomere maintenance by telomerase and by recombination can coexist in human cells

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Immortal human cells maintain their telomeres by two independent mechanisms, a prevalent one dependent on de novo synthesis of telomeric DNA by telomerase, and a rarer one based on telomere recombination [alternative lengthening of telomeres (ALT)]. Studies with yeast have indicated that expression of telomerase inhibits telomere recombination. In the present study, we have investigated whether expression of telomerase in cells that use ALT would similarly reveal dominance of telomere elongation by telomerase over telomere recombination. Telomerase-negative WI38 VA13/2RA ALT cells were reconstituted for telomerase activity through ectopic expression of the enzyme subunits, hTERT and hTR, and the presence and function of telomerase and ALT were monitored during long term cell growth by enzymatic assays, detection of the ALT-associated PML bodies (APBs) and analysis of telomere dynamics. Our results indicate that telomerase activity and APBs persisted in the cells over at least 90 population doublings. The activity of both pathways on telomeres was determined by analysis of telomere length versus time by gel electrophoresis and in situ hybridization. ALT cells are characterized by very heterogeneous telomeres with a much longer average size than the telomeres of telomerase-positive cells. Telomere dynamics in our cells were compatible with both ALT and telomerase being biologically active since the long telomeres typical of ALT were maintained, while short telomeres, thought to be the preferential substrate of telomerase, were elongated. These findings, indicating that human cells may be capable of concomitantly utilizing both mechanisms of telomere maintenance without effects on their growth and viability, have implications for cancer therapy.

INTRODUCTION

The integrity and function of telomeres, the nucleoprotein structures which cap eukaryotic chromosomes, are a critical factor in the lifespan of human cells. Due to incomplete replication of terminal DNA sequences by conventional DNA polymerases (1,2), cell proliferation is accompanied by telomere shortening (3), a process that can be counterbalanced by de novo synthesis of telomeric DNA catalyzed by telomerase (4,5). The lack of this enzyme in human somatic cells that are subject to senescence, and its presence in the germline, in stem cells and in the majority of in vitro immortalized and tumor cells, provided initial evidence suggesting that telomerase and telomere maintenance may be required for unlimited cell proliferation and for tumorigenesis (6,7). Strong support for this hypothesis has come from the observations that: (i) reconstitution of telomerase activity in somatic cells results in their immortalization (8,9); (ii) inhibition of the enzyme in immortal cells leads to their proliferative crisis (10,11); and (iii) combined expression of oncogenes and telomerase confers on cells a tumorigenic phenotype (12).

Human cells possess at least one additional mechanism for telomere maintenance, alternative lengthening of telomeres (ALT), that is independent of telomerase and relies on homologous recombination and copy switching (13–15). In ALT cells, telomeres are much longer and more heterogeneous than those of telomerase-positive cells (14) and undergo cycles of rapid shortening and elongation (13). ALT cells are also characterized by the presence of novel nuclear structures, the ALT-associated PML bodies (APBs), which contain the PML protein, the telomeric proteins TRF1 and TRF2, several proteins involved in DNA repair and recombination, and telomeric DNA (16–18). The colocalization of these molecules in APBs in viable cells is compatible with these structures being involved in telomere recombination (16). ALT is utilized much more rarely than telomerase, having been detected in ∼20% of immortalized cells and in a subset of tumors (14,19,20), and could be considered a backup mechanism that is activated when telomerase cannot be expressed. Nevertheless, since ALT cells are immortal, the existence of this mechanism is of relevance to potential cancer therapeutic approaches based on the reversal of cell immortality by inhibition of telomerase.

Fusion of normal cells with telomerase-positive or ALT cells results in loss of telomerase activity or ALT, respectively (21,22); in addition, ALT is suppressed in hybrids between ALT and telomerase-positive cells (22). Taken together, these observations suggest that normal cells harbor distinct repressors of the two pathways of telomere maintenance, and that repression of ALT in hybrid cells is not mediated by telomerase. Inhibition of telomere recombination by telomerase has however been documented in Saccharomyces cerevisiae.
another organism that normally uses telomerase to maintain telomeres but can switch to a recombinational mechanism when this enzyme is not expressed (23,24). Survivors of telomerase-negative yeast cells are defined as type I or type II depending upon the structure of their telomeres. Ectopic expression of telomerase inhibits recombination in both cell types although with strikingly different kinetics, inhibition being almost immediate in type I cells but requiring a substantial number of cell divisions in type II cells (23).

The present study was undertaken to investigate the effects of reconstitution of telomerase activity upon telomere recombination in ALT cells. We found that biologically active telomerase and ALT can coexist during long term growth of clonal and polyclonal populations of ALT VA13 cells reconstituted for telomerase, with no evidence that telomerase expression affects the ALT pathway.

RESULTS AND DISCUSSION

Long term coexistence of telomerase and ALT in human cells

ALT cells are telomerase negative because they do not express the catalytic subunit of the enzyme, hTERT, or more rarely lack both hTERT and the telomerase template RNA, hTR (25–27). We and others have shown previously that in either case telomerase activity can be reconstituted in the cells by transfection of DNA encoding the enzyme subunit(s) (25,26). In a recent study, we have expressed wild-type and mutant telomerase in WI38 VA13/2RA (VA13) ALT cells, which lack both telomerase subunits, by transfection of wild-type hTERT and wild-type or mutant hTR (28). While expression of mutant telomerase resulted in cell death within very few cell divisions, reconstitution of the wild-type enzyme had no short term effect on cell growth and viability.

Here we have followed the long term growth of polyclonal and clonal populations of telomerase-positive VA13 ALT cells, monitoring for the presence and function of the wild-type enzyme and of ALT. Cells were grown for a total of 90 population doublings (PDs) following reconstitution of telomerase activity by transfection of hTR into a derivative of VA13 cells expressing hTERT (VA13/hTERT; 28). Determination of growth rate, based on the increase in cell number over 10 PDs, and of colony forming efficiency, indicated no significant differences between telomerase-positive ALT cells, at both early and late PDs, and parental VA13/hTERT cells (28 and data not shown). Telomerase activity was assayed by TRAP (29) at different PDs and was found to persist in all populations reconstituted for the enzyme (Fig. 1). Enzymatic activity in these populations ranged from a minimum of 30% to levels comparable to or higher than the activity of HeLa cells, but was always substantially higher than the activity of a second telomerase-dependent immortal cell line, HA1-IM cells (30,31), indicating that the reconstituted cells express amounts of telomerase that are sufficient for telomere maintenance.

As a marker of ALT, we used the colocalization of PML and TRF2 in APBs, which has been reported to be specific for cells that maintain telomeres by recombination (16). Cells were first incubated with PML and TRF2-specific antibodies, then with FITC- or Cy3-conjugated secondary antibodies, and APBs were detected by the merging of the fluorescent signals

Figure 1. (A) Telomerase activity in VA13/hTERT parental cells and derivative cell lines transfected with hTR. Whole-cell extracts (0.25 µg in each case) were assayed for telomerase activity by TRAP at the indicated PD levels, which were calculated from the time of hTR transfection for both polyclonal and clonal populations. As control, extracts from HeLa cells (0.25 µg) and HA1-IM cells (0.5 µg) were also assayed. An internal control for PCR amplification (IS) was included in each assay. (B) Telomerase activity shown in (A) was quantified as described in Materials and Methods. Activity in reconstituted VA13 cells and in HA1-IM cells is expressed relative to the activity in HeLa cells. Numbers under the bars indicate PDs.

(Fig. 2). At both early and late PDs, clonal and polyclonal populations of telomerase-positive ALT cells contained a substantial number of APBs and were, in this respect, comparable to the parental telomerase-negative ALT cell lines, VA13/hTERT and VA13. No APBs were detected in HeLa cells which are negative for ALT (data not shown). In both parental cells and their telomerase-positive derivatives, 50–60% of the cells were positive for APBs with no significant differences in this percentage between early and late PDs (Table 1). We also
determined the extent of PML and TRF2 co-localization by counting the number of APBs (Fig. 2) in a minimum of 30 cells from each population (Table 1). The average number of APBs was comparable among telomerase-positive and parental populations (12.5–16.6) and was constant with time in culture. Since suppression of ALT is accompanied by disappearance of

**Figure 2.** Analysis of APBs in ALT-positive cells. Cells were stained for the PML protein (green) and the telomeric TRF2 protein (red), and APBs were detected by the colocalization of the fluorescent signals (merge). Clonal and polyclonal populations of ALT cells expressing telomerase were analyzed at 24–30 PDs (early) and at 90 PDs (late) following transfection of hTR. Parental VA13/hTERT cells were analyzed at a single time point.

**Table 1.** Co-localization of TRF2 and PML (APBs)

<table>
<thead>
<tr>
<th>Cells</th>
<th>% of APB+ cells</th>
<th>APBs/positive nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early PDs</td>
<td>Late PDs</td>
</tr>
<tr>
<td>VA13</td>
<td>61.7 ± 3.6</td>
<td></td>
</tr>
<tr>
<td>VA13/hTERT</td>
<td>53.1</td>
<td></td>
</tr>
<tr>
<td>VA13/hTERT+hTR</td>
<td>Clone 4</td>
<td>61.1</td>
</tr>
<tr>
<td></td>
<td>Clone 5</td>
<td>53.8</td>
</tr>
<tr>
<td></td>
<td>Clone 8</td>
<td>55.5</td>
</tr>
<tr>
<td></td>
<td>Polyclonal</td>
<td>54.1</td>
</tr>
</tbody>
</table>

Cells were analyzed at PD 24–30 (early) and at PD 90 (late). The PD level of VA13 and VA13/hTERT cells was not determined (ND).

*A minimum of 100 cells were scored for each population.

#Thirty to fifty cells were scored for each population. Average numbers of APBs ± SD are shown.
APBs (32), these results are compatible with the ALT pathway remaining active in the cells after 90 PDs. Together with the observed persistence of telomerase activity over the same period of time, they also imply that human cells can tolerate the coexistence of two mechanisms for telomere maintenance.

Telomere dynamics in telomerase-positive ALT cells

In order to verify that both telomerase and ALT were active on telomeres, the length of the terminal restriction fragments (TRFs) of telomerase-positive ALT cell populations was measured at different PDs by gel electrophoresis. As shown in Figure 3A, VA13/hTERT cells and their telomerase-positive derivatives have the characteristic heterogeneous telomeres of ALT cells, ranging from very short (<5 kb) to very long (~48 kb). Within each telomerase-positive population, clonal or polyclonal, TRF length and heterogeneity remained essentially constant with time in culture. In clones 5 and 8 the distribution of telomere sizes at every PD was not significantly different from that of the parental cells. Similar results were obtained with clone 4 (data not shown). In the polyclonal population, on the other hand, there was a reduction in the amount of medium size and long TRFs even at the earliest PDs, resulting in a size distribution that was less skewed than in the parental cells. However, even in the polyclonal population, TRFs as long as 48 kb were present at all times. The persistence of the ALT TRF phenotype suggests that the ALT pathway remains active in the telomerase-positive populations.

To evaluate telomere dynamics at the single cell level, parental and telomerase-positive populations were analyzed by Q-FISH (Fig. 4). In the parental cell line telomere signals were highly heterogeneous, varying from very strong to undetectable within the same metaphase (Fig. 4A). In addition, a large percentage of metaphases contained chromosome ends without visible telomeric signals (Table 2). In the telomerase-positive cell lines, relatively strong telomeres typical of the ALT phenotype were still present, but the percentage of metaphases with chromosome ends with undetectable telomeres and the total number of such ends were significantly reduced (Fig. 4B and C and Table 2). No decrease in the number of ends without telomeric signals was detected in a clonal population expressing the hTERT-HA mutant telomerase that is incapable of elongating telomeres (28,33), relative to its parental cell line (Table 2). These results strongly suggest that elongation of short telomeres in telomerase-positive ALT cells is due to the expression of the wild-type enzyme. For a more direct test of ALT function, we evaluated telomere stability by measuring the intensities of individual telomeres present on five easily identifiable marker chromosomes in clone 5 (Fig. 4D). Three of these chromosomes (Fig. 4Da–c) were frequently present at both early and late PDs, while the remaining two (Fig. 4Dd and e) were detected only at late PDs. With the exception of chromosome d, all of them had interstitial telomeric DNA signals; since such signals are expected to be stable over time, they were included in the analysis as control for the variability inherent to Q-FISH. As shown in Figure 5, at early PDs there were no major differences in variability between telomeric and interstitial signals. At late PDs, on the other hand, some telomeres exhibited a significant increase in variability indicative of substantial telomere size fluctuation from one cell to another [coefficient of variation for Fig. 5: a–p (32%), c–p (43%), d–p (47%) and d–q (64%)]. Telomere stability was also analyzed in a cell line expressing only telomerase (WI38+hTERT; 34) and in the parental telomerase-negative WI38 cells taking advantage of the presence of a chromatin heteromorphism which allows us to distinguish the two homologs of chromosome 9 (35). As also shown in Figure 5, no variability was detected on chromosome 9 telomeres in either cell type. These findings provide strong evidence in support of the persistence of ALT function in telomerase-positive VA13 cells. The variability of a given
Taken together, our results indicate that biologically active telomerase and ALT can coexist over time in human cells and cooperate in the maintenance of the cell telomeres. Similar conclusions have been reached with different telomerase-positive ALT cells by others (32,36). On the other hand, in a study published during revision of the present one, loss of very long telomeres and a decrease in the percentage of APBs positive cells were detected in VA13 cells expressing very high levels of telomerase (37), indicating that telomerase may be able to inhibit ALT. However, this effect was rare since it occurred in only two of nine clones analyzed. Because the majority of the clones retained the ALT phenotype, it is not clear whether telomere at one time point and not another in these cells may simply reflect the stochastic nature of the ALT process.

Figure 4. Detection of telomeres by in situ hybridization and identification of marker chromosomes. Metaphase spreads from parental VA13/hTERT cells (A), the polyclonal (B) and clone 5 telomerase-positive derivatives (C) were hybridized with a Cy3-labeled PNA telomere probe (red) and counterstained with DAPI (blue). Very strong telomeric signals are indicated by open arrowheads and missing telomeric signals by closed arrowheads. Marker chromosomes (D) present in clone 5 are indicated by letters and the interstitial telomeric DNA signals detected on chromosome a, and on chromosomes b, c and e (see inserts) are indicated by an arrow.

Table 2. Telomere analysis by FISH

<table>
<thead>
<tr>
<th>Cells</th>
<th>PD</th>
<th>Metaphases with chromosome ends with no signal (%)</th>
<th>Ends with no signala</th>
<th>Total</th>
<th>Mean/met. (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VA13/hTERT</td>
<td>32</td>
<td>9/33 (27)</td>
<td></td>
<td>14</td>
<td>1.5 (1–3)</td>
</tr>
<tr>
<td>VA13/hTERT+HTR</td>
<td>89</td>
<td>8/28 (29)</td>
<td></td>
<td>17</td>
<td>2.1 (1–4)</td>
</tr>
<tr>
<td>Clone 5</td>
<td>26</td>
<td>4/36 (11)</td>
<td></td>
<td>7</td>
<td>1.7 (1–3)</td>
</tr>
<tr>
<td>VA13/hTERT-HA</td>
<td>86</td>
<td>2/34 (6)</td>
<td></td>
<td>3</td>
<td>1.5 (1–2)</td>
</tr>
<tr>
<td>VA13/hTERT-HA+HTR</td>
<td>58</td>
<td>30/30 (100)</td>
<td></td>
<td>170</td>
<td>5.7 (1–10)</td>
</tr>
</tbody>
</table>

aOnly metaphases with chromosome ends with undetectable telomeres were considered.
The mean of 10–20 telomeres or interstitial signals with the corresponding SD. Analyzed at PD 36 (T1), 48 (T2), 58 (T1) and 75 (T2), respectively. Bars represent the mean of 10–20 telomeres or interstitial signals with the corresponding SD.

**Figure 5.** Quantitation of intensity of individual telomeres. Mean pixel values corresponding to individual telomeres and to interstitial telomeric DNA signals, when present, were measured. Results are expressed as relative fluorescence intensity for direct comparison between metaphases and cell types. Telomerase-positive VA13 clone 5 cells were analyzed at PD 28 (early) and 86 (late) for chromosomes a–c, or only at PD 86 for chromosomes d and e. The telomeres of the two homologs of chromosome 9 (a and b) in wild-type and hTERT-positive WI38 cells were analyzed at PD 36 (T1), 48 (T2), 58 (T1) and 75 (T2), respectively. Bars represent the mean of 10–20 telomeres or interstitial signals with the corresponding SD.

telomerase was solely responsible for the inhibition of ALT in the affected clones or whether additional events contributed to this process.

Overall, the findings with human ALT cells differ from those with telomerase-negative yeast survivors in which reconstitution of enzyme activity consistently inhibits telomere maintenance by recombination (23). That this effect, at least in type II yeast cells, becomes detectable only after a very long period of growth could suggest that in human cells inhibition of ALT may require more than 90 PDs. Although we cannot formally exclude this possibility, several observations argue against it. Telomerase-negative normal human cells lose telomeric DNA at a rate at least 10–20-fold faster than yeast cells (50–100 bp versus 3–5 bp per generation; 3,23) but there is evidence of a much more rapid rate of loss upon suppression of ALT (22,37). Indeed, in telomerase-positive × ALT hybrids, the inhibition of ALT was clearly detectable as early as 20 PDs due to the dramatic reduction in telomere length (22,32). In contrast to this, we detected no significant differences in TRF length and size distribution and in APBs between clonal populations of telomerase reconstituted cells and parental cells. Moreover, in all telomerase-positive populations, these parameters remained constant over 90 PDs. Coexistence of ALT and telomerase for an even longer period of growth (about 150 PDs) has been reported recently (32).

The ability of human cells to utilize concomitantly two mechanisms of telomere maintenance has important implications for anti-telomerase based cancer therapy, since inhibition of telomerase in such cells would not abrogate their immortal phenotype. Activation of ALT in tumor cells is a much rarer event than activation of telomerase (20), and may normally occur under selective pressure when expression of the enzyme is prevented (38,39). However, it is conceivable that tumor cells, due to their hyper-mutability, may physiologically activate both pathways for telomere maintenance, likely by loss of the respective suppressors. Interestingly, tumor samples with a phenotype compatible with this hypothesis, i.e. telomerase activity and telomeres longer than those of normal tissues, have been described in the literature (6,20).

**MATERIALS AND METHODS**

**Cells and transfection**

WI38 VA13/2RA human cells, here referred to as VA13, are derived from SV40-transformed WI38 lung fibroblasts. They were obtained from ATCC and were grown in α-MEM with 10% heat-inactivated fetal calf serum (FCS). Stable cell lines expressing wild-type hTERT or the hTERT-HA mutant, VA13/hTERT and VA13/hTERT-HA, were obtained by transfection of pCI-neo-hTERT or pCI-neo-hTERT-HA, encoding the hTERT cDNAs under the control of HCMV immediate early gene promoter and the neomycin resistance gene (33), using Lipofectamine Plus (Gibco BRL) and G418 selection, as reported previously (28). VA13/hTERT and VA13/hTERT-HA cells were reconstituted for telomerase activity by transfection with pLXSP-hTR, encoding the wild-type hTR gene and the puromycin resistance marker (28). Transfected cells were selected in 0.5 µg/ml puromycin; polyclonal populations were harvested after 9 days, and individual clones isolated after 3–4 weeks. WI38 and derivative WI38+hTERT were obtained from ATCC and J.Campisi (Berkeley National Laboratory; 34), respectively and grown in Eagle MEM with 10% non-inactivated FCS. HeLa and HA1-IM cells (30) were grown in α-MEM with 10% heat-inactivated FCS.

**Cell growth and viability**

Telomerase-positive ALT cells and control ALT cells were routinely subcultured at a 1:4 split ratio as they reached confluence. For the telomerase-positive populations, PDs were calculated from the time of hTR transfection and are comparable among all populations. Evaluation of initial PDs for the polyclonal population was based on control transfections with a plasmid encoding the green fluorescent protein (GFP) and the number of GFP-positive viable cells present after 48 h and recovered at the end of selection (polyclonal population; 28), and for the clones on the number of cell divisions required for expansion of one transfected cell to a confluent 60 mm plate. Growth rates were determined at early and late PD levels after isolation of the telomerase-positive populations by seeding 5 × 10^4 in duplicate 60 mm plates and counting the cells versus time for 10 PDs. As cultures reached confluence, cells were reseeded as above. Colony forming efficiency was evaluated at early and late PDs by seeding 10^3 cells/100 mm plates in triplicate; colonies were stained with crystal violet after 14 days and counted.

**Telomerase assay**

Whole-cell extracts were prepared by detergent lysis and assayed by the PCR-based telomere amplification protocol (TRAP; 29) using amounts of protein within the linear range of the assay (0.25 µg for VA13, telomerase-positive derivatives and HeLa cells, and 0.5 µg for HA1-IM cells). Telomerase activity was quantified by integrating the radioactive signal from the telomerase ladder in each lane and normalizing it to the radioactive signal of the corresponding internal standard.
Immunofluorescence

Cells were grown in 35 mm dishes for 2 days, fixed in 1% formaldehyde for 10 min, permeabilized in 0.25% Triton X-100 and blocked in phosphate-buffered saline (PBS) containing 0.1% BSA for 1 h. For co-localization of PML and TRF2, cells were incubated overnight at 4°C with a goat polyclonal antibody against PML (N19, Santa Cruz; 2 µg/ml) and a rabbit polyclonal antibody against TRF2 (1:150 dilution; 17). Primary antibodies were detected with FITC-conjugated donkey anti-goat IgG and Cy3-conjugated donkey anti-rabbit IgG (Jackson Immuno Research), respectively, at 1:100 dilution for 1 h at room temperature. Nuclei were stained with 1 µg/ml Hoechst 33258 and cells were analyzed by confocal microscopy. For each experiment, at least 100 cells were scored to calculate the percentage of cells in which PML and TRF2 colocalize; cells were considered positive if they contained at least one APB. Thirty to fifty cells were scored to determine the total number of TRF2+PML+ foci (APBs) and quantify the extent of colocalization.

TRF analysis

Genomic DNA was extracted using standard procedure, digested with HindIII and Rsal and separated by pulsed-field gel electrophoresis (PFGE) (14). Following in gel DNA denaturation of the metaphase, relative fluorescence intensities of individual telomeres were calculated by normalizing their pseudo-color images were used to colocalize chromosomes and telomere signals. Original black and white Cy3 images were saved for quantitative analysis using the Iplab Spectrum (Vysis) [settings: gain = 3 (red), 1 (blue); binning = 4].

Q-FISH

Metaphase chromosome spreads were prepared from cells treated with colcemid (0.1 µg/ml, 1 h; Eurobio), then hypotonic KCl and fixed in methanol:acetic acid (3:1 v:v). Fixed cells were dropped onto clean slides and let to dry overnight prior to hybridization with a telomeric specific (CCCTAA)11-Cy3 PNA probe (PerSeptive Biosystems) as described (40), and staining with DAPI. Fluorescent signals were visualized under a UV microscope (Axioplan2, Zeiss) equipped with a Cy3 filter wheel, and were captured with a CCD camera (Photometrics-Sensys) using the Smart-Capture software (Vysis) [settings: gain = 3 (red), 1 (blue); binning = 4] and a fixed exposure time of 2 s. A flat field template was used to correct for unevenness in field illumination. Merged DAPI-Cy3 pseudo-color images were used to colocalize chromosomes and telomere signals. Original black and white Cy3 images were saved for quantitative analysis using the Iplab Spectrum P software (Skalanetics). Overall telomere fluorescence was estimated by calculating the mean pixel value using an automatic segmentation protocol provided by the software. When pixel values were calculated for specific telomeres, a manual segmentation protocol was used. Relative fluorescence intensities for individual telomeres were calculated by normalizing their mean pixel value to the overall telomere mean pixel value of the metaphase.

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