Telomerase Inhibition in RenCa, a Murine Tumor Cell Line with Short Telomeres, by Overexpression of a Dominant Negative mTERT Mutant, Reveals Fundamental Differences in Telomerase Regulation between Human and Murine Cells

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ABSTRACT

In contrast to human primary fibroblasts, mouse embryonic fibroblasts have telomerase activity, immortalize spontaneously in culture, and can be neoplastically transformed by oncogenic insult. Ectopic expression of the human telomerase catalytic subunit, human telomerase reverse transcriptase (hTERT), in human primary cells allows both spontaneous immortalization and neoplastic transformation by oncogenes. This suggests that telomerase activity, as well as the fact that mouse telomeres are longer than human telomeres, may explain some of the differences in cellular control between human and murine cells. Telomerase inhibition in immortal or transformed human cells using dominant negative hTERT mutants leads to telomere shortening and cell death. Here we study the effect of expression of a dominant negative mutant of the catalytic subunit of mouse telomerase, mTERT-DN, in a murine kidney tumor cell line, RenCa, whose telomeres are similar in length to human telomeres. After showing initial telomerase activity inhibition and telomere shortening, all clones expressing mTERT-DN reactivated telomerase and showed normal viability, in contrast with that described for human cells. This efficient telomerase reactivation coincided with a significant increase in the endogenous TERT mRNA levels in the presence of mTERT-DN expression. The results presented here reveal the existence of fundamental differences in telomerase regulation between mice and man.

INTRODUCTION

Telomerase is the cellular DNA polymerase involved in telomere replication (1). Telomeres are the ends of eukaryotic chromosomes and, in vertebrates, consist of tandem DNA repeats of the TTAGGG sequence and of associated proteins (2, 3). Telomeres are proposed to protect chromosome ends from degradation, recombination, and DNA repair activities. Loss of telomeric function, either by the loss of telomeric sequences or the mutation of telomere binding proteins, is associated with increased chromosomal instability and the loss of cell viability (4–10). Telomerase is a specialized reverse transcriptase that synthesizes telomeric repeats onto chromosome ends (11). The telomerase enzyme is composed of a catalytic protein subunit known as TERT (8, 12–18), an RNA molecule or Terc, which is used as template for the addition of new telomeric repeats (19–23), and associated proteins (24–29). The introduction of a constitutively expressed human telomerase catalytic subunit, hTERT, into human cells with a limited life span is sufficient to stabilize their telomeres and, in some cases, to extend their life span indefinitely without inducing changes associated with transformation (30–34), supporting the idea that telomere maintenance by telomerase is essential for immortal growth. Telomerase can also cooperate with oncogenes to transform primary human cells in culture, suggesting that telomere maintenance by telomerase contributes to malignant transformation (35). These findings led to the proposal that telomerase inhibition in human cancers would provoke telomere shortening, and compromise tumor growth with time. Indeed, telomerase inhibition in human tumor cell lines using dominant negative versions of hTERT resulted in telomere shortening and cell death (36, 37), suggesting that telomerase inhibition may be an effective way to halt tumor growth (38). RenCa is a murine renal carcinoma cell line (39) whose telomeres are similar in length to those of human immortal cell lines. Using this cell line, we have studied the effects of expressing a dominant negative Mt of mTERT in murine tumor cells. The results presented here reveal the existence of fundamental differences in telomerase regulation between mice and man.

MATERIALS AND METHODS

Plasmids, Cell Culture, and Transfection

The mouse RenCa renal carcinoma cell line, all pBabe- and pDN-transduced clones, as well as the tumor-derived cell lines were cultured in DMEM supplemented with 10% FCS. Site-directed mutagenesis of TERT residues Asp (861) and Asp (862) to Ala was carried out using the Sculptor in vitro mutagenesis system (Amersham Pharmacia Biotech, Stockholm, Sweden) using the full-length mouse TERT cDNA previously cloned into Bluescript SK as template. An EcoRI fragment containing the full-length Mt TERT cDNA and 3′-untranslated sequences was subcloned into a pBabe retroviral vector harboring a puromycin resistance gene.

Retroviral constructs were introduced by standard calcium phosphate transfection into Phoenix packaging cells, and the viral supernatant was used to transduce RenCa cells as described (5). Transduced clones were selected with 0.5 μg/ml puromycin.

To estimate the accumulated PDs at the time of clone isolation (considered to be the time at which the isolated clone reached confluence in a 100-cm dish), we assumed that the cell colonies that were picked were composed of ~10³ cells (10 PDs). Then the subsequent number of accumulated PDs until confluence was reached in a 100-cm dish was calculated using the formula

$$\Delta \text{PDL} = \log(n_f - n_i)/\log2$$

where $n_f$ is the initial number of cells and $n_i$ is the final number of cells. Most clones reached confluence at 16–24 PDs.

4 E. González-Suárez and M. A. Blasco, unpublished results.
Inhibition of telomerase activity by expression of a dominant negative Mt of mTERT. S-100 extracts were prepared from pBabe-transduced (pBabe1, pBabe3, pBabe4, pBabe5, pBabe6, and pBabe7), pDN-transduced (pDN1, pDN2, pDN3, pDN5, pDN6, pDN7, pDN9, and pDN12), and control RenCa cells and assayed for telomerase activity using the TRAP assay (see “Material and Methods”). Extracts were pretreated (+) or not (−) with RNase. In the case of clones pDN2 and pDN12, RNase-treated extracts showed background levels of telomerase activity, which were never detected in the untreated lanes. Number of cells or protein concentration/reaction is indicated.

**Proliferation Assay**

Cell proliferation was analyzed using the Cell Proliferation ELISA/ bromodeoxyuridine (Roche Diagnostics, Mannheim, Germany) and a [3H] incorporation assay. Cells (3 × 10^5) were plated in round-bottomed, 96-well plates. After 24-h [3H]thyminide pulse, cells were harvested onto glass fiber strips using an LKB Wallac 1295-001 Cell Harvester. [3H]thymidine incorporation of each cell lysate was determined in a Rackbeta Liquid Scintillation Counter (ICN, Costa Mesa, CA). Each sample was processed in triplicate.

**Telomerase Activity Assay**

Telomerase activity was measured using a modified version of the TRAP assay (5). In some cases, an IC for PCR efficiency was included in the TRAP assay (TRAPEze kit, Oncor). Alternatively, the Telomerase PCR ELISA Kit (Roche Diagnostics) was used to detect PCR products, according to the manufacturer’s instructions.

**Telomere Length Analysis**

**Southern Blot TRF Analysis.** Genomic DNA was extracted with a DNA Extraction Kit (Qiagen, Hilden, Germany). Genomic DNA (10 μg) was digested overnight with 10 units each of HinfI and Rsal (MBI Fermentas, Vilnius, Lithuania), loaded onto a 0.7% agarose gel, and transferred to a nylon membrane (Amersham). DNA fragments were hybridized to a digoxigenin-labeled telomere probe (TTAGGG)₄. Telomeric sequences were detected by chloro-substituted 4t-dioxetane, chemiluminescent substrate for alkaline phosphatase-star chemiluminescence (Roche Diagnostics).

**Flow-FISH.** Telomere length was also measured by Flow-FISH as described (40), for which 10⁵ cells were used in each case. To normalize Flow-FISH data, two mouse leukemia cell lines (LY-R and LY-S) of known telomere lengths (9 and 40 kb, respectively; Ref. 41), were used as ICs. The telomere fluorescence was measured of at least 2 × 10⁵ cells gated at G₀/G₁, cell cycle stage using a Coulter Flow EPICS XL cytometer with the SYSTEM 2 software (Coulter, Miami, FL). The mean telomeric fluorescence was calculated after subtracting the background from the probe alone.

**Genetic Instability**

Between 80–100 metaphases of each clone were scored for telomere fusions, chromatid breaks, and chromosome fragments by superimposing the telomere image on the 4′,6-diamidino-2-phenylindole chromosome image in the TFL-telo program. Metaphase chromosomes were prepared by treatment of the cells with 0.1 μg/ml Colcemid for 4 h, followed by hypotonic lysis in 0.06 mmol KCl and fixation with methanol/acetic acid. After preparing metaphase slides, FISH was performed as described (10).

**In Vivo Tumor Formation Experiments**

Cells (10⁵) were injected s.c. in two leg sites of BALB/c nude immunodeficient mice (Harlan, Barcelona, Spain). Tumor size was determined every second day from day 12 postinoculation.

**Quantitative Detection of Wt mTERT and Mt mTERT-DN Transcript Levels Using the Light-Cycler PCR**

Total RNA was extracted by RNAzol (WAK Chemie, Germany) and the reverse transcriptase reaction performed using 200 ng of RNA in a one-step RT-PCR. The RT-PCR reactions were done using the Light Cycler Instrument (Roche) and the LightCycler-RNA Amplification Kit SYBR Green I (Roche). The RT-PCR reactions were carried out using sets of primers specific for either the endogenous Wt (Wt primers) or the Mt mTERT-DN sequence (Mt primers; specific for the Mt sequence). The primers used to detect the endogenous Wt TERT were: wt-mTERTup (5′-TTT TGT TGA CTT TCT TCT-3′) and mtTERTlow (5′-TCT GGG CAT AAC CTG AGT-3′). As an IC, β-actin-specific primers UBA-U144 (5′-GTG GGG CGC CCC AGG CAC CA-3′) and LBA-660 (5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′) were used to calculate the relative concentration. For measurement of relative concentrations, a melting curve analysis of the amplified products was performed. The melting curve allows identification of the specific products as well as peak area calculations. To calculate the relative abundance of specific mTERT products, the peak area of the mTERT products was corrected by that of IC products.

**RESULTS AND DISCUSSION**

**mTERT Aspartic Acid Mt Acts as a Dominant Negative of Mouse Telomerase.** In budding yeast and in man, telomerase activity can be abolished by replacement of any of the three conserved aspartic acid residues with alanine and serine to create a dominant negative allele (14, 15). The first of these three aspartic acids lies within a highly conserved region of the human TERT (Fig. 1). Inhibition of telomerase activity by expression of a dominant negative Mt of mTERT. S-100 extracts were prepared from pBabe-transduced (pBabe1, pBabe3, pBabe4, pBabe5, pBabe6, and pBabe7), pDN-transduced (pDN1, pDN2, pDN3, pDN5, pDN6, pDN7, pDN9, and pDN12), and control RenCa cells and assayed for telomerase activity using the TRAP assay (see “Material and Methods”). Extracts were pretreated (+) or not (−) with RNase. In the case of clones pDN2 and pDN12, RNase-treated extracts showed background levels of telomerase activity, which were never detected in the untreated lanes. Number of cells or protein concentration/reaction is indicated.
Telomerase inhibition in a mouse tumor cell line

Flow-FISH analysis was used for quantitative measurement of telomere length. Flow-FISH allows estimation of the length of TTAGGG repeats as the fluorescence intensity of telomeres in interphase nuclei previously hybridized with a fluorescent PNA-telomeric probe (see “Materials and Methods”). Fig. 2B shows the telomeric fluorescence of a pBabe-transduced clone (pBabe7) at different PDs, the telomere fluorescence of the parental RenCa cell line, as well as that of a control cell line (LY-S cells; see “Materials and Methods”). Cytogenetic analysis of metaphases from pBabe3 and pDN1 clones at two different PDs (early and late PD) showed lower telomere fluorescence than the pBabe3 clone, in agreement with telomere shortening in these cells (Fig. 2B). Intriguingly, after the initial shortening, telomere length was maintained or slightly increased with increasing PDs in some of the pDN clones (pDN7, pDN10, and pDN23; Fig. 2A), whereas other clones showed further telomere shortening (pDN1 and pDN2; Fig. 2B; see next section for discussion). Fig. 2C shows representative images of metaphases from pBabe3 and pDN1 clones at two different PDs (early and late PDs) previously hybridized with a fluorescent PNA-probe against telomeres. Cytogenetic analysis of metaphases from pBabe3 and pDN1 clones revealed that chromosomal instability with increasing PDs was similar in both clones (not shown).

All together, these data indicate that telomerase inhibition via expression of mtTERT-DN results in initial telomere shortening in the pDN-transduced clones, as previously described for human cells (36, 37); however, some pDN clones showed telomere maintenance or elongation at later PD after clone isolation (see below).
Telomerase activity was analyzed using both the conventional TRAP assay (see Fig. 3A for representative examples) and the telomerase PCR ELISA Kit (see Fig. 3B for representative examples). A control pBabe4 clone showed similar telomerase activity levels throughout the different PDs analyzed, as determined both by TRAP and the PCR ELISA Kit (Fig. 3, A and B). Strikingly, all pDN clones analyzed reactivated telomerase activity to different levels with increasing PDs after clone isolation (Figs. 3A and 3B show representative examples). Clone pDN2, in particular, showed very low telomerase activity at isolation (PD24), although activity had already recovered to normal levels at PD32 and was maintained thereafter (Fig. 3A). Curiously, telomerase up-regulation in pdn clones was not always sufficient to prevent telomere shortening with increasing PDs (i.e., clones pDN1 and pDN2; Fig. 2B). This can be explained by insufficient telomerase reactivation to prevent telomere shortening in these particular clones.

Quantification of Wt mTERT and mTERT-DN Transcripts with Increasing PDs in pBabe and pDN-transduced Clones. The efficient reactivation of telomerase activity in the pDN-transduced clones cultured under puromycin selection suggests that it was independent of the presence of the pDN construct. We thus developed an RT-PCR-based method to quantify endogenous mTERT and mTERT-DN mRNA levels. Total RNA from the different clones was used for a one-step RT-PCR using the Light Cycler (see “Materials and Methods”), using sets of primers that were specific for either the mTERT-DN (Mt primers) or the endogenous TERT (Wt primers; see “Materials and Methods”). As shown in Fig. 4A, at 65°C and using the Mt primers, the mTERT-DN transcripts were detected in a pDN clone (pDN2 at PD24 and PD47) and in a pDN2-derived tumor cell line (pDN2 T) but not in a pBabe clone (pBabe3 at PD45), in a pBabe3-derived tumor cell line (pBabe3 T), or in the RenCa cells, indicating that the Mt primers are specific for the mTERT-DN sequence and do not recognize the endogenous mTERT (see Fig. 4A). The Wt primers at 65°C did not detect any products (see below for RT-PCR at lower temperatures). For measurement of relative concentrations, a melting curve analysis of the amplified products was performed. The melting curve allows identification of the specific products as well as peak area calculations. Fig. 4A shows the quantification of the Mt mTERT-DN mRNA levels as peak area units, which have been previously corrected by the IC signal (see “Materials and Methods”). As shown in Fig. 4A, at 65°C and using the Mt primers, the mTERT-DN transcripts were detected in a pDN clone (pDN2 at PD24 and PD47) and in a pDN2-derived tumor cell line (pDN2 T) but not in a pBabe clone (pBabe3 at PD45), in a pBabe3-derived tumor cell line (pBabe3 T), or in the RenCa cells, indicating that the Mt primers are specific for the mTERT-DN sequence and do not recognize the endogenous mTERT (see Fig. 4A).
RenCa cell line (Fig. 4C; see results at 52°C and 58°C). Strikingly, the endogenous mTERT transcript levels were always higher (2-fold) in the pDN-transduced clones than in the pBabe-transduced clones or in the RenCa cells at all different temperatures tested (see Fig. 4C for representative examples; this difference was statistically significant as determined by Student’s t test; P < 0.01), suggesting that endogenous mTERT expression is up-regulated in the pDN clones as compared with the pBabe clones. The endogenous mTERT mRNA up-regulation in the pDN clones preceded telomerase reactivation (i.e., clone pDN2 showed increased mTERT mRNA levels at PD24 when telomerase activity was still low; Fig. 4C).

All together, these results indicate that loss expression of the pDN constructs is not the cause of the elevation in telomerase activity with increasing PDs in the pDN-transduced clones. The efficient reactivation of telomerase activity in all pDN clones coincides, however, with a significant up-regulation of the endogenous mTERT mRNA levels, suggesting that this is one of the possible mechanisms by which telomerase activity is reactivated in these clones. These results have important implications regarding the use of murine cells to screen for telomerase inhibitors.

Effect of mTERT-DN Expression on Cell Viability. During the process of clone isolation or shortly thereafter, some of the pDN clones contained cells with abnormal morphology (flat and enlarged cells showing a senescence-like phenotype). Fig. 5A shows representative images of these senescent-like cells in clones pDN2 and pDN7, as well as a control pBabe4 clone with normal cell morphology. In addition, 32% of the pDN clones did not survive culture after clone isolation, suggesting the loss of viability in these clones. To study this, we performed [3 H]thymidine incorporation assays in pBabe and pDN-transduced clones at the time of isolation. pDN clones showed, on average, a 30% decrease in proliferative capacity compared with simultaneously isolated pBabe clones (Fig. 5B). It is interesting to note that those pDN clones that survived the isolation process showed no loss of viability at later PDs. This is in marked contrast to previous descriptions of human clones expressing dominant negative hTERT, which showed a dramatic loss of viability (36, 37).

Tumor Formation in Vivo by mTERT-DN-transduced RenCa Cells. To study the effect of murine telomerase inhibition on tumor formation in vivo, early PD pBabe (pBabe1, pBabe4, and pBabe7) and pDN-transduced clones (pDN2, 3, 6, and 7) were injected s.c. into...
immunodeficient mice, and tumor formation was monitored over a 32-day period (Fig. 6; see “Materials and Methods”). No differences in tumor latency or tumor size were observed between pBabe- and pDN-injected mice. The lack of inhibitory effect of the dominant negative mTERT mutation on tumor growth in mice is likely to be the consequence of telomerase reactivation in the mTERT-DN-transduced clones (see above). Telomere length and telomerase activity were measured in several cell lines derived from pDN-induced tumors: pDN1T1 and pDN1T2 were derived from two independent tumors produced by the pDN1 clone; pDN5T1 was derived from a tumor produced by the pDN5 clone; pDN7T1 was derived from a tumor produced by the pDN7 clone; and pBabe4T1 was derived from a tumor produced by the pBabe4 clone. Telomerase activity was detected in all pBabe and pDN-derived tumor lines (see Fig. 3B), in agreement with reactivation of telomerase activity in the injected pDN cells in vivo. On the other hand, the pDN7-T1 tumor cell line showed a TRF length similar to that of the parental pDN7 clone, also suggesting that telomeres have been maintained in this tumor in vivo (Fig. 2A) and confirming the presence of telomerase activity in the pDN-induced tumors despite the expression of the mTERT-DN construct as shown in Fig. 4.

Conclusions. Here we show that ectopic expression of a dominant negative Mt of murine TERT in the RenCa renal carcinoma cell line, which has telomeres similar in length to those of human cells, results in initial telomerase inactivation and telomere shortening. In contrast to inhibition of human telomerase using hTERT dominant negative mutants, however, telomerase activity was efficiently reactivated in the RenCa clones expressing the dominant negative mTERT. Furthermore, we observed no loss of viability or impact on tumor formation by these cells in long-term assays. All together, these results indicate that telomerase inactivation in RenCa tumor cells by mTERT dominant negative expression does not prevent tumor formation attributable to a rapid reactivation of telomerase activity, even in the presence of the Mt mTERT-DN mRNA. Using quantitative Light-Cycler RT-PCR, we have also established that this telomerase activity reactivation is mediated by an up-regulation of the endogenous mTERT mRNA levels. It is likely that the pressure to maintain telomeres in these cells may provoke telomerase up-regulation, which overcomes the presence of the Mt mTERT-DN transcript. A plausible mechanism for this efficient telomerase up-regulation may be amplification of the endogenous mTERT gene, as recently described for the hTERT gene in human tumors (42). In summary, the results presented here contrast with those published for telomerase inhibition in human
tumor cell lines and suggest the existence of important differences in telomerase regulation between human and murine cells.

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