Mitochondrial localization of telomerase as a determinant for hydrogen peroxide-induced mitochondrial DNA damage and apoptosis

Janine Hertzog Santos1,2,†, Joel N. Meyer1 and Bennett Van Houten1

1Laboratory of Molecular Genetics, National Institute of Environmental and Health Sciences (NIEHS), National Institutes of Health (NIH), 111 Alexander Drive, MD D3-01, Research Triangle Park, NC 27709, USA and 2Department of Pharmacology and Physiology, New Jersey Medical School of UMDNJ, Medical Sciences Building, H653, 185 South Orange Avenue, Newark, NJ 07103, USA

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We have previously shown that the protein subunit of telomerase, hTERT, has a bonafide N-terminal mitochondrial targeting sequence, and that ectopic hTERT expression in human cells correlated with increase in mtDNA damage after hydrogen peroxide treatment. In this study, we show, using a loxP hTERT construct, that this increase in mtDNA damage following hydrogen peroxide exposure is dependent on the presence of hTERT itself. Further experiments using a dominant negative hTERT mutant shows that telomerase must be catalytically active to mediate the increase in mtDNA damage. Etoposide, but not methylmethanesulfate, also promotes mtDNA lesions in cells expressing active hTERT, indicating genotoxic specificity in this response. Fibroblasts expressing hTERT not only show a ~2-fold increase in mtDNA damage after oxidative stress but also suffer a 10–30-fold increase in apoptotic cell death as assayed by Annexin-V staining, caspase-3 activation and PARP cleavage. Mutations to the N-terminal mitochondrial leader sequence causes a complete loss of mitochondrial targeting without affecting catalytic activity. Cells carrying this mutated hTERT not only have significantly reduced levels of mtDNA damage following hydrogen peroxide treatment, but strikingly also do not shown any loss of viability or cell growth. Thus, localization of hTERT to the mitochondria renders cells more susceptible to oxidative stress-induced mtDNA damage and subsequent cell death, whereas nuclear-targeted hTERT, in the absence of mitochondrial localization, is associated with diminished mtDNA damage, increased cell survival and protection against cellular senescence.

INTRODUCTION

Telomerase is a reverse transcriptase well recognized for its role in telomere biology. It is normally expressed during early stages of development. As development progresses, the expression of the catalytic subunit of telomerase, hTERT, is downregulated in most somatic tissues. Thus, the majority of normal cells in the adult do not show much telomerase activity, but non-differentiated somatic cells (such as hematopoietic, germ cells and the cryptic cells of the intestine) are telomerase-positive (1). Yet even in these cells, telomerase activity is insufficient to prevent telomere attrition with age. Telomerase, therefore, may have functions other than telomere elongation in highly proliferative normal adult somatic cells. This idea is further supported by recent findings suggesting TERT’s involvement in chromatin remodeling (2), DNA damage response (2–5) and regulating p53 target genes (6). In addition, we recently demonstrated that hTERT has a functional N-terminal mitochondrial leader sequence, and detected active telomerase in mitochondrial extracts from non-transformed human cells (7). These results were surprising, as no known function had been described for telomerase in the mitochondria, which contain a circular genome without telomeric structures.

Mitochondria have their own DNA, which in humans consists of a 16 569 bp circular molecule encoding two rRNAs, 22 tRNAs and 13 polypeptides involved in electron transport and ATP production (8). Numerous human disorders have been linked to mitochondrial dysfunction caused by alterations

*To whom correspondence should be addressed. Tel: +1 9739729729; Fax: +1 9739727950; Email: santosja@umdnj.edu

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in structure or function of mitochondrial and/or nuclear-encoded genes. For instance, mutations in the mitochondrial DNA polymerase γ have been associated with progressive external ophthalmoplegia and Alper’s syndrome in humans (9), and with accelerated aging in mice (10). Mutations in subunits 1, 4 and 6 of complex I NADH dehydrogenase, encoded by the mtDNA, lead to impaired electron transport and increased ROS production, and are involved in Parkinsonism, Alzheimer’s disease and in Leber’s hereditary optic neuropathy (11). Underlying these and other neurodegenerative diseases are increased ROS, altered mitochondrial function and cell death (12). The mitochondrial genome is highly susceptible to endogenous or exogenous oxidative stress. In fact, hydrogen peroxide (H₂O₂) has been shown to induce more lesions in the mtDNA than in the nuclear genome (13,14). Moreover, several recent studies have revealed that increased mtDNA repair enhances cell survival following oxidative stress (15), indicating the vulnerability of mtDNA to oxidative damage. Furthermore, loss of DNA repair capacity and subsequent mtDNA integrity can lead to cell death and human disease.

Here we used several strategies to elucidate the cellular consequences of the mitochondrial localization of telomerase and directly test the role of hTERT in oxidative stress-mediated mtDNA damage. We show using a catalytically inactive hTERT and a loxP hTERT construct that active telomerase is essential for mtDNA sensitization after H₂O₂ treatment. Moreover, we establish that this effect is genotoxicity dependent, as promotion of mtDNA damage was observed after etoposide (VP-16) but not methylmethanesulfonate (MMS) treatment. Finally, these effects are dependent on hTERT’s mitochondrial localization: site-directed mutagenesis abolishing mitochondrial targeting completely protected cells from H₂O₂-induced apoptosis and cell death. Taken together, our results demonstrate a biological role for mitochondrial telomerase in modulating the response of mammalian cells to genotoxin exposure.

RESULTS

mtDNA damage is dependent on the presence of catalytically active hTERT

We have previously shown that hTERT ectopic expression correlates with increased mtDNA damage after oxidative stress (7). To test the hypothesis that hTERT presence with its associated telomerase activity lead to increased mtDNA damage, we used normal human 82-6 fibroblasts (which lack endogenous hTERT) harboring a construct where the wild-type (WT) hTERT gene is under the loxP locus (pBloxTSH). By infecting cells with a vector encoding Cre recombinase (pLCRESH), hTERT is excised and cells previously telomerase positive become telomerase negative. This system has been characterized earlier (16), and has three main advantages as it: (i) allows monitoring the effect of telomerase after genotoxin treatment in the same cell context; (ii) eliminates possible effects of additional changes acquired in the genome by integration of the hTERT-containing vector; and (iii) potentially helps to discriminate between a direct role of hTERT versus the effect of long telomeres.

Parallel cultures of 82-6 cells expressing pBloxTSH were grown and half of the cultures were infected with pLCRESH. After an additional seven generations of cell growth, we assessed the presence (or loss) of hTERT mRNA and subsequent telomerase activity (Fig. 1A and B), and treatment of both cultures with H₂O₂ was performed. Lack of hTERT expression and associated telomerase activity significantly decreased the level of lesions induced by H₂O₂ when compared with cells expressing the WT protein (Fig. 1C) indicating that the presence of telomerase increases mtDNA damage. The enhancement in mtDNA damage cannot be attributed to differences in H₂O₂ decomposition, as H₂O₂ breakdown kinetics were indistinguishable. H₂O₂ had a half-life of about 30 min for both cells (Fig. 1D). This observation implies that the actual doses of H₂O₂ to which the cells are exposed throughout the treatment are considerably lower than the initial concentration (200 μM). In agreement with previous observations (7,13), no nuclear DNA (nDNA) lesions were detected by quantitative PCR (QPCR) in the above-mentioned cells after H₂O₂ treatments (data not shown).

In the second approach, we tested whether catalytic activity is required for the mitochondrial effect. For these experiments, we used GM847 cells that do not express hTERT endogenously and that maintain long telomeres through a telomerase-independent pathway (17). Cells carrying a vector control, WT hTERT, or a dominant negative (DNhTERT) version of the protein were used. This latter construct has been shown earlier to be a catalytically inactive mutant that behaves as a dominant negative in the presence of endogenous hTERT (18). After confirming the presence or absence of hTERT message and of telomerase activity (Fig. 2A and B), cells were treated with H₂O₂ and mtDNA damage analyzed by QPCR. Figure 2C shows that DNhTERT cells have less mtDNA lesions than WT hTERT cells and, interestingly, that they also have ~50% less mtDNA damage than control cells (15 min treatment). No differences in kinetics of H₂O₂ decomposition between the cells were observed (Fig. 2D).

The effect of telomerase on the mtDNA is genotoxin-dependent

Next, we addressed whether mtDNA sensitization caused by mitochondrial hTERT was specific for H₂O₂ or a general phenomenon. We treated cells with the methylating agent MMS and with the topoisomerase II inhibitor VP-16, as both are known to damage nuclear and mitochondrial genomes (19,20). NHF and NHF hTERT cells were exposed to these drugs and DNA integrity analyzed by QPCR immediately after treatment. As shown in Figure 3A, MMS induced both nuclear and mtDNA damage. However, the presence of hTERT did not affect the number of lesions induced in either of these genomes. Conversely, there was a consistent and reproducible enhancement in mtDNA damage induced by VP-16 in NHF hTERT cells when compared with the parental strain, in which no lesions were detected (Fig. 3B). No nDNA damage was detected after VP-16 treatment (data not shown). This concentration of VP-16, while low, had been shown to induce
telomerase activity (21). Furthermore, this dose is biologically relevant, as 0.5–5 \( \mu \)M are actual doses to which cancer patients are exposed when undergoing chemotherapy (22).

Mitochondrial telomerase drives enhanced mtDNA damage and apoptosis

Human fibroblasts do not normally undergo apoptosis following an intermediate dose of \( \text{H}_2\text{O}_2 \) (50–250 \( \mu \)M) (23), but rather undergo cellular senescence (24). We previously found that hTERT expression leads to increased mtDNA damage after \( \text{H}_2\text{O}_2 \) exposure (7), and wanted to determine whether the presence of hTERT resulted in additional altered biological responses, such as increased apoptosis. Primary MRC-5 and NHF fibroblasts and respective hTERT derivatives were submitted to 60 min of \( \text{H}_2\text{O}_2 \) treatment, were allowed to recover for 24 h and then harvested to monitor apoptosis by three independent assays. As shown in Figure 4, more annexin-V-positive (A) and caspase-3 positive cells (B) were observed in treated hTERT than in the respective non-treated or parental strains. We also monitored cleaved PARP, a late marker of apoptosis, in the MRC-5 cells. As seen in Figure 4C, only treated MRC-5 hTERT cells show PARP cleavage. In addition, hTERT expression resulted in significant loss of cell viability 24 h after \( \text{H}_2\text{O}_2 \) treatment regardless of the cell type (\( P = 0.027 \), Fig. 4D).
Lack of mitochondrial localization of hTERT protects cells from cell death and mtDNA damage

We next evaluated whether the presence of hTERT in the mitochondria, and not just in the cell, is necessary for H$_2$O$_2$-induced cell death. Because mitochondrial matrix proteins are synthesized as precursors, which have a highly positively charged amino-terminal targeting signal (25), we disrupted targeting of hTERT to the mitochondria by deletion of the 20 amino acid-mitochondrial leader sequence and by site-directed mutagenesis of two residues in the N-terminus of the protein (Fig. 5A).

These proteins were first fused to EGFP to monitor subcellular localization and subsequently cloned into the pBabe retroviral vector to produce stable cell lines. While deletion of the first 20 amino acids of hTERT indeed abolished mitochondrial localization (data not shown), the stable cell lines started senescing (judged by cell morphology and β-galactosidase staining; data not shown) soon after selection, even though hTERT was being expressed (data not shown).

It had been shown previously that mutations within the N-terminus of hTERT could give rise to mutant proteins with low telomerase activity that no longer immortalize cells (26). These data are interesting in that they suggest a threshold of telomerase activity for cell immortalization, but they are uninformative regarding hTERT function in the mitochondria.
So as to avoid the confounding effects associated with a senescent population, only the cell lines harboring the N-terminal substitutions were studied in more detail. Introduction of negative charges at the N-terminus of proteins impedes transport into the mitochondria (27); thus arginine (R) at positions three and six were substituted for glutamic acid (E) residues (R3E/R6E-hTERT). Subcellular localization showed fluorescence of R3E/R6E-hTERT-EGFP exclusively in the nucleus, and no co-localization with the mitochondrial probe Mitotracker red was observed (Fig. 5B). This result shows that this mutant hTERT does not enter the mitochondria, and also demonstrates that simple over-expression is not sufficient for mitochondrial localization of hTERT.

NHF and MRC-5 primary fibroblasts were infected with the pBabe vector carrying either WT or R3E/R6E hTERT to create stable cell lines. The mutant protein not only retained catalytic activity (Fig. 5C) but allowed cells to replicate for more than 100 population doublings (data not shown), suggesting that it maintained its telomere function. These mutant cells that have hTERT only in the nucleus allowed us to test whether hTERT has to be in the mitochondria to mediate mtDNA damage. Not only did cells carrying the mutant protein show less mtDNA damage than WT-expressing cells but, surprisingly, the integrity of the mitochondrial genome (as assayed by QPCR), after 15 min of treatment in both of the R3E/R6E-hTERT-expressing cell lines, was greater than non-hTERT parental cells (Fig. 6A, compare open bars to black bars). Thus, the absence of hTERT in the mitochondria leads to less mtDNA damage, and its presence only in the nucleus is protective.

We speculated that the cellular compartment to which hTERT was localized, and the associated mtDNA sensitization, would be predictive of an apoptotic response. To test this hypothesis, we monitored the levels of apoptosis in cells expressing the mutant protein. R3E/R6E-hTERT-expressing cells and their respective controls (cells infected with either empty vector or WT hTERT) were exposed to H2O2, and analyzed 24 h after the treatments. The same trend of apoptosis was observed in the annexin-V and caspase-3 assays; representative caspase-3 activation data are presented. Low levels of apoptosis were observed in cells that were untreated or that expressed the R3E/R6E-hTERT protein (Fig. 6B). In contrast, NHF and MRC-5 cells carrying WT hTERT (Fig. 6B) displayed on average a 10–30-fold increase in apoptosis over their non-treated controls. In addition to the increased cell death, a significant number of floating cells were detected 24 h after treatment, in both WT hTERT cells. Floating cells were not observed in non-treated or mutant-expressing cells (data not shown). The introduction of R3E/R6E hTERT led to increased viability when compared with WT hTERT-expressing NHF and MRC cells after H2O2 treatment. In fact, cells carrying the mutant hTERT protein were almost confluent 24 h after H2O2 treatment (data not shown), indicating that they had gone through at least one round of cell division following the treatment. Cell viability was also significantly reduced in WT hTERT-expressing cells as compared with non-treated controls (Fig. 6B). Differences in H2O2 detoxification were ruled out as a possible explanation for these large differences in cell survival (data not shown). Collectively, these results demonstrate that hTERT must be in the mitochondria to promote mtDNA damage and apoptosis after H2O2 treatment, and furthermore point to a protective effect of telomerase when the enzyme is only localized to the nucleus.

**DISCUSSION**

This study demonstrated that localization of hTERT to the mitochondria causes increased mtDNA damage and apoptotic cell death in human fibroblasts following oxidative stress. We established that increased mtDNA sensitivity is dependent both upon functionally active telomerase and on the type of genotoxic agent used. In addition, we showed that the 2–3-fold increase in susceptibility to mtDNA damage elicited by mitochondrial telomerase after H2O2 treatment is associated with an altered response of fibroblasts to oxidative stress.
stress from stress-induced premature senescence to apoptotic death (Fig. 4). Furthermore, we showed by site-directed mutagenesis of the mitochondrial leader sequence that nuclear targeting in the absence of mitochondrial localization of hTERT (Fig. 5) is remarkably protective against cell death (Fig. 6). Finally, by showing that nuclear- and mitochondrial-localized hTERT have opposite effects after H2O2 treatment, our findings help explain conflicting results in the literature.

**Mitochondrial hTERT induces apoptosis after oxidative stress**

The apoptotic response of the WT hTERT fibroblasts observed in the present study is surprising, as it has been observed that:

(i) human diploid fibroblasts treated with moderate levels (up to 300 μM) of H2O2 do not undergo apoptosis or necrosis, but rather arrest growth for a prolonged period, developing features of stress-induced replicative senescence (23,24), and

(ii) telomerase expression is believed to protect against cell death (3). It has even been shown that high levels of hTERT expression in apparently non-stressed cells can lead to cellular senescence (28).

The nature of hTERT resistance against genotoxin-induced apoptosis is still elusive. In fact, no specific mechanism that could explain hTERT’s protection, besides telomere stabilization, has been demonstrated (3,29–33). However, even its action at the telomeres cannot account for all telomerase protection as it has been reported that the inactive dominant negative hTERT still confers resistance against apoptosis (29). In addition, in the context of equal telomeric length hTERT was shown to have no effect (4) or increase rather than decrease sensitivity of human cells to H2O2 (33), which is in agreement with our data. Here we have shown that hTERT when present both in the nucleus and mitochondria increases apoptosis (Figs 4 and 6). However, when it is restricted to the nuclear compartment (by substitution of amino acid charges that abolish its mitochondrial localization), it completely abrogates H2O2-induced apoptotic death (Fig. 6). These data are in accordance with previous work showing that hTERT restricted to the nucleus had an anti-apoptotic effect. Haendeler et al. observed that hTERT was shuffled from the nucleus to the cytoplasm (under conditions of exogenous and/or endogenous oxidative stress) through phosphorylation of tyrosine 707 (Y707) (34). By over-expressing a mutant hTERT (Y707F), which is resistant to ROS-induced nuclear export, they observed a decrease in apoptosis when compared with cells expressing WT hTERT. The authors further proved that nuclear localization of hTERT was important for its anti-apoptotic capacity by sub-cloning WT hTERT into a nuclear shooter vector, which leads to forced expression of the protein in the nucleus. Over-expression of the nuclear targeted hTERT (hTERTnuc) exerted an enhanced apoptosis-suppressive effect that was comparable to that seen with the mutant hTERT(Y707F). Collectively, these data demonstrate that nuclear-only hTERT contributes to the anti-apoptotic function of telomerase. The authors attributed this effect to hTERT’s telomere protection. However, in light of our experiments, a complementary explanation is that lack of hTERT in the mitochondria likely also contributes to its anti-apoptotic activity. It would be interesting to monitor a cell line where hTERT is only targeted to the mitochondria. These experiments are currently underway in our laboratory.

It is worth noting that some researchers add N-terminal tags to hTERT (26,35–39) because the addition of epitope tags to its C-terminus has been shown to abolish telomerase’s in vivo telomere-extension function (40). However, N-terminal tags (most of which are negatively charged) are likely to block mitochondrial targeting and thus any mitochondrial mediated effect of the protein. Hence, caution is necessary in interpreting the results obtained with this approach that, while valuable, is not fully congruent with the biological role of the unaltered protein.

Thus, the identification in this study of the different biological response of cells to oxidative stress depending on hTERT subcellular localization helps explain, at least in part, the protection or lack-there-of against cell death conferred by telomerase.

**Mechanism for mitochondrial hTERT involvement in apoptosis**

The results presented here suggest that mitochondria hTERT affects the integrity of the mtDNA, as its presence exacerbates damage induced both by H2O2 and VP-16. More importantly, we show that when hTERT expression is restricted to the
nucleus (by substitution of amino acid charges that abolish its mitochondrial localization) it reduces H$_2$O$_2$-induced mtDNA damage by a factor of three (Fig. 6). We have previously demonstrated that levels of mtDNA damage after H$_2$O$_2$ treatment correlate with the degree of telomerase catalytic activity (7). Although the levels of telomerase activity are indeed lower in the R3E/R6EhTERT mutant than WT, cells bearing this mutant protein still have less mtDNA lesions than telomerase-negative parental strains (Fig. 6). Thus, we conclude that reduced mtDNA damage observed in cells carrying R3E/R6EhTERT is in fact due to lack of mitochondrial localization. It is noteworthy that the presence of either nuclear-only hTERT or of the catalytically inactive protein (Figs 2 and 6) results in a level of mtDNA lesions reduced even beyond that of no-hTERT control cells, suggesting a yet unknown indirect effect of hTERT upon the mitochondria.

It has been shown previously that the integrity of the mtDNA is intimately related to cell viability: increased mtDNA repair enhances cell survival while uncoupling of repair increases cell death following oxidative stress (for review see 15). It is interesting that WT hTERT-expressing cells have more mtDNA damage than parental and mutant-expressing cells, which translates to decreased cell viability. We and others have shown that damage to the mtDNA leads to the production of ROS, which in turn further damages the organelle. The outcome of this vicious cycle of

Figure 5. Site-directed mutagenesis of the mitochondrial leader sequence of hTERT abolishes its mitochondrial localization while retaining telomerase activity. (A) N-terminal sequence of hTERT showing in bold amino acids of the mitochondrial leader sequence; the respective amino acid substitutions are shown in red. (B) Subcellular localization of EGFP alone (first row); WT hTERT-EGFP fusion protein (second row) and R3E/R6E hTERT-EGFP (third row). The extent of co-localization of red and green signals was further investigated using mathematical computation through the Zeiss LSM Image Browser software (data not shown). (C) Telomerase activity was assayed from 500 ng of total cellular extracts. Lanes 1, 3 and 5 show NHF pBabe, NHF hTERT and NHF R3E/R6E hTERT, respectively; lanes 7, 9 and 11 are MRC-5 pBabe, MRC-5 hTERT and the MRC-5 R3E/R6E hTERT derivative; lanes 2, 4, 6, 8, 10 and 12 contain the same order of samples heat-inactivated. IC stands for the internal control of the assay.
Lack of mitochondrial localization of hTERT abolishes apoptosis and protects against H2O2-induced mtDNA damage. (A) Parental strains and cells expressing WT hTERT and the R3E/R6E hTERT derivative were treated with 200 μM of H2O2 for 60 min. DNA damage analysis was performed with QPCR immediately after exposure of NHF (left graphs) and MRC-5 (right graph) cells to H2O2. Level of lesions shown are above background levels. P-values comparing NHF R3E/R6E hTERT pBabe mutant and WT hTERT-expressing NHF cells were 0.003 (15 min) and 2.82 × 10^{-2} (60 min). The values for NHF R3E/R6E hTERT when compared with the parental NHF pBabe strain were 0.008 (15 min) and 0.001 (60 min). P-values calculated based on results of MRC-5 R3E/R6E hTERT pBabe mutant when compared with MRC-5 hTERT cells were 8.4 × 10^{-2} (15 min) and 2 × 10^{-4} (60 min) and 1.47 × 10^{-2} and 0.5 for 15 and 60 min treatments, respectively, when comparing with MRC-5 pBabe parental. (B) Cells expressing WT hTERT and the R3E/R6E hTERT derivative were treated with 200 μM of H2O2 for 60 min and allowed to recover in conditioned medium for 24 h. Cells were then harvested and assayed for caspase-3 positive cells based on fluorescence of AFC alone. The percentage of positive cells was compared between treated cells and their respective non-treated controls. Results are representative of four biological experiments. Mean fold difference (over non-treated controls) was, respectively, 33.4 ± 0.7 and 0.05 ± 0.07 for treated NHF hTERT and NHF R3E/R6E hTERT. MRC-5 hTERT treated cells showed an average of caspase-3 positive cells that was 17.1 ± 9.6-fold above non-treated control levels, whereas in MRC-5 R3E/R6E hTERT these values were 0.01 ± 0.01. Green represents normal cells, yellow shows caspase-3 positive cells and, in red, dead cells as judged by propidium iodide staining. (C) Cells expressing WT hTERT (nuclear and mitochondrial localization) show loss of viability 24 h after H2O2 treatment, whereas cells expressing R3E/R6E hTERT (nuclear-only localization) do not. H2O2 treatment, subcellular localization of hTERT and cell type all affected cell viability (P = 0.002, 0.009 and 0.02, respectively, for main effects), but the effect of H2O2 treatment depended on the localization of hTERT (P = 0.0013, treatment by hTERT localization interaction). By post hoc analysis, the H2O2 treatment affected cell viability in cells expressing WT hTERT (P = 0.0015) but not in cells expressing R3E/R6E hTERT (P = 0.8309).
nucleic-acid/protein damage and ROS production is ultimately cell death (10,13,14). It is a formal possibility that the cells used in the present study died because of damage to the nuclear genome. However, we have been unable to detect nDNA damage in cells treated with H2O2 using QPCR, while MMS-induced nuclear damage was identified (data not shown and Fig. 3). Thus, it appears to be more likely that the effects of hTERT on the mtDNA initiate a cascade of mitochondrial demise, leading, eventually, to apoptosis.

Regardless of being a cause or consequence of the observed apoptosis, mtDNA damage is clearly exacerbated by the presence of mitochondrial hTERT after H2O2 and VP-16 treatments. It is not yet understood how hTERT could be affecting the integrity of the mitochondrial genome. However, the genotoxic dependence of the hTERT effects (Fig. 3) suggests several hypotheses. As the types of damage as well as the processing of the lesions induced by these three genotoxic agents are fundamentally different (8,20,41–44) it is likely that hTERT is altering the amount or kind of damage induced in the mtDNA, or it is interfering with mtDNA repair, or both.

Biological implications

Telomerase is expressed during development, in highly proliferative somatic cells and in the majority of cancers. In human development, telomerase activity has been detected from the blastocyst stage up to completion of organogenesis, when it is systematically turned off in different tissues (1). To date, the only role ascribed to telomerase in development and in proliferative adult cells is the maintenance of telomeres. If telomerase interferes with lesion processing (creation and/or removal) in the mtDNA, it is possible that mitochondrial hTERT has a ‘pruning’ role by pushing undifferentiated cells, such as fibroblasts during development or hematopoietic cells in the adult, with damaged mtDNA (either due to replication errors or oxidative stress) towards the apoptotic pathway.

MATERIALS AND METHODS

hTERT constructs

WT and hTERT mutants used in this study were constructed by PCR amplification of hTERT from the hTERT-pLXIN vector (provided by Dr J. Carl Barrett, NCI). Two N-terminal mutants were created: a truncated version of the gene lacking the first 20 amino acids, and a charge-altered protein in which arginine was substituted in positions three and six for glutamic acid (R3/E6). The above constructs were introduced into the retroviral vector pBabe (provided by Dr Christopher Counter, Duke University) using the restriction enzymes EcoRI and SalI. After cloning, all vectors were sequenced. Mutant proteins were cloned into pEGFP-N1 (Clontech), which was cut with EcoRI and KpnI. WT hTERT-EGFP was described previously (7).

Cells and cell culture

Primary MRC-5 and NHF fibroblasts and the respective hTERT-pLXIN-derivatives were described previously (7). The pBabe retroviral vector containing hTERT proteins was transfected into the packaging cell line PA317 to produce viruses that were used to infect both NHF and MRC-5 primary fibroblasts. Control cells were infected with the empty vector. After infection, cells were grown in selection medium containing 2 μg/ml of puromycin for 2 weeks. Resistant colonies were pooled and tested for telomerase activity as well as immortalization (>100 population doublings). Cells were routinely grown and sub-cultured as described previously (13), with exception of pBabe-containing cells that were grown under puromycin selection. HeLa cells used for transient transfections were maintained in MEM medium (Gibco) supplemented with 10% FBS and penicillin/streptomycin. pBloxTSH as well as the retroviral pLCRESH vector carrying cre recombinase were a gift from Dr Judith Campisi (Lawrence Berkeley Laboratories) and were described in reference 30. Dr Robert Weinberg (MIT) provided cell lines GM847, GM847 hTERT and GM847 DNhTERT described in Stewart et al. (17).

Genotoxic treatments

H2O2 (Sigma) treatments were performed as described earlier (13). MMS and VP-16 were also obtained from Sigma, and treatments were performed in non-supplemented medium for 60 and 180 min, respectively. Cells were washed twice and harvested immediately after treatments for DNA analysis.

DNA isolation and DNA damage analysis by QPCR

High molecular weight DNA was extracted and QPCR performed and analyzed as described previously (45,46). Large fragments of both nuclear and mitochondrial genomes were amplified; the primers sequence can be found elsewhere (46). A small (139 bp) fragment of the mtDNA was also amplified, and was used to monitor mitochondrial copy number and to normalize results obtained with the large fragment [for more details see Santos et al. (46)].

Telomeric repeat amplification protocol

Total protein extracts of 500 ng were assayed for telomerase activity using the TRAPEze kit from Chemicon as instructed by the manufacturer and modified as in Santos et al. (7).

Apoptosis and cell death/viability assays

Annexin-V and Caspase-3 activity were followed by flow cytometry using kits from Santa Cruz Biotechnology. NHF, MRC-5 cells and their respective hTERT derivatives were exposed to 200 μM of H2O2 for 1 h and allowed to recover for 24 h, when cells were harvested and the protocols were followed as recommended by the manufacturer. Caspase-3 assay is based on release of highly fluorescent AFC generated by caspase-3 cleavage of DEVD-AFC. Results shown are representative of four biological experiments. PARP cleavage was detected by western blots (details below). Cell death was judged by PI uptake while cell viability was calculated according to the fraction of cells that excluded PI, subtracting the percentage of cells that were either annexin-V-positive or caspase-3-positive. Data were analyzed by a three-factor
ANOVA, with H₂O₂ treatment, presence/absence of hTERT, cell type as independent variables and percent viable cells as the dependent variable, n = 7 or 8 per group. Subsequent post hoc analysis of the effect of treatment on WT hTERT- or R3E/R6E hTERT-expressing cells was performed across cell types (one-factor ANOVA).

Western blots

Whole cells extracts were obtained from parental and hTERT derivatives 24 h after H₂O₂ exposures, and protein concentrations estimated with the Bradford assay. A total of 30 μg of protein were loaded and PARP cleavage detected using an antibody from Pharmingen. A positive control consisting of cells treated with staurosporine, supplied by the manufacturer, was also included (data not shown). Actin antibody (Chemicon) was used to monitor protein loading.

Confocal microscopy

Transient transfections were performed as described previously (7). A laser scanning confocal microscope (LSM 510 mounted on Axiovert 200 M microscope, Carl Zeiss) was used to obtain fluorescence images. The objective lens used was the PlanApo 63×/1.4 oil DIC, and the pinhole was set to achieve a z-resolution of 1.0 μm.

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Conflict of Interest statement. none.

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