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Mild hyperoxia limits hTR levels, telomerase activity, and telomere length maintenance in hTERT-transduced bone marrow endothelial cells

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ABSTRACT

Reactivation of telomerase in endothelial cells (ECs) may be an effective approach to the treatment of vascular disorders associated with telomere attrition and EC senescence. However, overexpression of human telomerase reverse transcriptase (hTERT) does not prevent net telomere loss in ECs grown in standard culture medium with exposure to atmospheric oxygen (21% O_2). Since these culture conditions are hyperoxic relative to normal tissue *in vivo*, where oxygen tension is estimated to be 1%–6%, we examined the effects of reduced exposure to oxidative stress (OS) on telomere length maintenance in hTERT-transduced bone marrow endothelial (BMhTERT) cells. Propagation of BMhTERT cells in the free radical scavenger, *tert*-butylhydroxylamine (tBN), and/or in 5% O_2 increased telomerase enzyme activity and facilitated telomere length maintenance. The enhancement of telomerase activity correlated with higher levels of the telomerase RNA component (hTR). We also investigated the role of the telomere binding protein, TRF1, in telomere length regulation under alternate OS conditions. Inhibition of TRF1 function had no effect on telomere length in BMhTERT cells grown under standard culture conditions. However, alleviation of OS by growth in tBN plus 5% O_2, elevated hTR levels, enhanced telomerase enzyme activity, and enabled progressive telomere lengthening. The direct impact of hTR levels on telomerase-mediated telomere lengthening was demonstrated by overexpression of hTR. BMhTERT cells transduced with hTR exhibited very high telomerase enzyme activity and underwent dramatic telomere lengthening under standard culture conditions. Overall, these results demonstrate that hTR levels are reduced by mild hyperoxia and limit telomerase-mediated telomere lengthening in hTERT-transduced ECs.

1. Introduction

Substantial evidence indicates that the replicative exhaustion of endothelial cells (ECs) and the functional alterations that occur during senescence contribute to the development of age-associated vascular disorders, such as atherosclerosis and chronic ischemic heart disease [1–6]. As in other normal somatic cells, the onset of replicative senescence in cultured human ECs and in vascular cells *in vivo* is attributed (at least in part) to the gradual erosion of telomeres [4,7–10]. Telomere shortening occurs with consecutive cell division as a consequence of the inability of DNA polymerases to replicate the 5′ terminus of linear DNA (reviewed in Hug and Lingner [11]). Progressive telomere shortening has been demonstrated in serially passaged cultures of ECs and in arterial ECs *in vivo* in association with increasing age [7,8,12,13].

Abbreviations: AU, arbitrary units; BMECs, bone marrow endothelial cells; di-LDL, diacetylated low-density lipoprotein; ECs, endothelial cells; EV, empty vector; FCS, fetal calf serum; hTERT, human telomerase reverse transcriptase; hTR, human telomerase RNA component; NAC, *N*-acetylcysteine; OS, oxidative stress; PD, population doubling; PSG, penicillin, streptomycin, and glutamine; Q-TRAP, quantitative telomeric repeat amplification protocol; ROS, reactive oxygen species; tBN, *tert*-butylhydroxylamine; TRF, terminal restriction fragment

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Telomere shortening and the onset of senescence are accelerated in primary human fibroblasts and ECs exposed to oxidizing agents [14–16]. Indeed, *in vitro* culture in standard medium and the supraphysiological levels of oxygen present in the atmosphere (~21%) generate sufficient oxidative stress (OS) to accelerate telomere erosion in normal fibroblasts [17–19]. Accumulating evidence suggests that OS contributes to age-dependent telomere shortening in ECs, as well as the excessive telomere attrition observed in ECs at sites of hemodynamic stress and/or from patients at risk of coronary artery disease [7,8,16,20,21]. Telomere loss observed under oxidative conditions is in part due to the exquisite sensitivity of G-rich telomeric DNA to hydroxylation and single-strand breaks [19,22,23].

In contrast to the telomere shortening observed in ECs and other normal somatic cells, telomeres are maintained or elongated in 80%–90% of cancer cells through the catalytic activity of telomerase [24]. Telomerase activity is very low or undetectable in most somatic cells, with the exception of stem and progenitor cells, including endothelial progenitor cells, which express telomerase at moderate levels. Telomerase activity is downregulated during differentiation [25]. Using a highly sensitive PCR-based assay, very low levels of telomerase activity (relative to tumor cells) have been detected in early-passage mature ECs; however, this was shown to be rapidly extinguished upon propagation [12,26,27].

Telomerase is a ribonuclear protein that includes a reverse transcriptase (hTERT) as a catalytic core, an RNA component (hTR) that serves as a substrate for synthesis of telomeric repeats and the RNA binding and modifying protein, dyskerin [28]. Telomerase-mediated telomere lengthening is regulated by the secondary structure of the telomere, which is folded into a t-loop conformation that conceals the single-stranded 3′ end [29] (reviewed in de Lange [30]). The t-loop structure is maintained by a complex of telomere-binding proteins, which includes TRF1 [31,32]. TRF1 binds as a homodimer to double-stranded telomeric DNA through its Myb binding domain and tethers other telomere binding proteins to the shelterin complex [32,33]. TRF1 has been shown to function as a negative regulator of telomere length in tumor cells, presumably by securing the telomere in a secondary structure that is inaccessible to telomerase [34].

Overexpression of hTERT reconstitutes telomerase activity and confers normal human ECs with an extended replicative life span and properties of phenotypically young cells [12,26,35–37]. The activation of telomerase has therefore been proposed as a potential means for rejuvenating ECs for therapeutic application in age-associated vascular disorders [36,37]. However, unlike normal fibroblasts and epithelial cells that exhibit telomere length maintenance or extension following reconstitution of telomerase [38–40], our group and another have shown that, under standard culture conditions, hTERT transduction and reconstitution of telomerase did not prevent net telomere loss in normal human ECs derived from bone marrow, dermis, and umbilical vein [12,26]. Clonal analyses of hTERT-transduced bone marrow ECs (BMECs) revealed dramatic fluctuations in telomere length, with transient periods of telomere loss or net telomere loss as the cells proliferated beyond senescence. Telomeres were eventually stabilized at a very short mean length (~3 kbp) in most immortalized BMEC cultures [41].

The current investigations aimed to determine whether inefficient telomere length maintenance in hTERT-transduced BMECs (BMhTERT cells) was a consequence of exposure to OS. The results show that chronic exposure to mild OS, inflicted by propagation under standard culture conditions and atmospheric oxygen (21%), dampened telomerase enzyme activity and impeded telomere length maintenance despite the constitutive overexpression of hTERT. Furthermore, it was shown that telomerase enzyme activity and telomere maintenance were restricted by limiting amounts of hTR when the cells were cultured under these mildly hyperoxic conditions.

2. Materials and methods

2.1. Cell culture

The establishment of BMhTERT cell lines was previously described [12,41]. BMhTERT(2A2) and BMhTERT(2A10) are hTERT-transduced BMEC clones that were established by limiting dilution of the BMhTERT-1 mass culture [41]. BMhTERT-2 was established as an independently transduced mass culture [12]. BMECs immortalized with hTERT were previously shown to express EC markers, including VEGFR-1, VEGFR-2, CD31, and von Willibrand factor VIII. They also have capacity for uptake of diacetylated low-density lipoprotein (di-LDL) [12].

Parental BMECs, BMhTERT-2, BMhTERT(2A2), and BMhTERT (2A10) cells were grown in EC growth medium, which was composed of M199 base medium (Invitrogen, Carlsbad, CA) supplemented with 20% fetal calf serum (FCS; ThermoTrace, Noble Park, Australia), 5% human serum (Sigma, St Louis, MO), heparin (Pharmacia, Rydalmere, Australia), fibroblast growth factor β (Sigma), EC growth factor (Roche Mannheim, Germany), and penicillin, streptomycin, and glutamine (PSG; Invitrogen). To assess the effects of alleviating OS, the medium was supplemented with 0.1 mM of the free radical scavenger *tert*-butylhydroxylamine (tBN; Sigma) [42] and/or the cells were cultured in 5% O_2 in a PRO OX model 110 C chamber fitted with a PRO OX oxygen controller (BioSpherix, Lacona, NY). PDs were calculated using the formula: PD = [log (expansion)/log 2], where expansion was the number of cells harvested divided by the initial number of cells seeded. Normal parental BMECs senesced at approximately 35 PDs under control culture conditions [12].

2.2. Detection of reactive oxygen species (ROS)

BMhTERT-2 cells (1×10^5) were incubated in 3 μM of DHR123 (Sigma) for 20 min at 37 °C and then washed twice in phosphate-buffered saline (PBS; Invitrogen). The cells were harvested with trypsin–EDTA (Invitrogen) and subsequently washed twice in cold PBS before analysis on a flow cytometer (FACSCabilur; BD Biosciences, San Jose, CA) using Cell Quest Software (BD Biosciences). The fluorescence of oxidized DHR123 was measured with an excitation wavelength of 514 nm and emission wavelength of 680 nm. DH123 fluorescence was corrected for autofluorescence by subtracting the geometric mean fluorescence intensity of unstained cells from the geometric mean fluorescence intensity of the stained sample. The corrected values for DH123 fluorescence are expressed as means ± standard error of the mean (SEM) calculated from three assays.

2.3. Telomeric restriction fragment length (TRF) assay

Genomic DNA was prepared using a standard phenol/chloroform/isoamyl alcohol procedure with Phase Lock Gel heavy tubes (Eppendorf, Hamburg, Germany) and precipitated with 0.3 M sodium acetate and two volumes of 100% ethanol. The integrity of the DNA was examined on ethidium bromide-stained agarose gels. After confirming that there was no degradation, the DNA was digested with 10 U each of *Hinf*1 and *Rsa*1 restriction enzymes (New England Biolabs, Ipswich, MA). The TRF assay was performed using the Telo-TAGGG Telomere Length Assay kit (Roche) as previously described [43]. X-ray images were scanned on a flat-bed scanner and analyzed using MacBas V2.5 or MultiGauge V3.X (Fuji, Tokyo, Japan). Mean TRF lengths were determined according to the following formula: $[(S_i/L_i)]/[S_i]$, where S_i is TRF signal at a given location after background subtraction and L_i is the corresponding length at position *i*. Repeated measure of the mean telomere length of the BMhTERT(2A10) cell line at a single time point in three individual TRF assays demonstrated reproducibility within 300 bp. Telomere length of all samples was measured on at least two gels.