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Human telomerase represses ROS-dependent cellular responses to Tumor Necrosis Factor-α without affecting NF-κB activation

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ABSTRACT

In addition to its well-established role in telomere synthesis, telomerase exerts non-canonical functions that may promote cancer and stem cell survival, notably by lowering reactive oxygen species (ROS) levels and acting as transcriptional cofactor in Wnt-β-catenin signaling pathway. We investigated the impact of telomerase on ROS-dependent and -independent cellular responses to Tumor Necrosis Factor-α (TNF-α), a potent inducer of endogenous ROS production and activator of NF-κB signaling pathway. Strikingly, telomerase overexpression in normal human fibroblasts treated with TNF-α strongly repressed ROS-dependent activation of both ERK1/2 mitogen-activated protein kinases and cell death. Telomerase overexpression also considerably diminished TNF-α-induced transcription of *SOD2* Superoxide Dismutase 2 gene by reducing ROS contribution to *SOD2* gene induction, both in normal fibroblasts and in cancer cells. Conversely, telomerase did not impair TNF-α-induced transcription of various ROS-insensitive NF-κB target genes. These data were in apparent contrast with the striking observation that telomerase overexpression induced strong constitutive nuclear accumulation of NF-κBp65. Accumulated NF-κBp65, however, lacked Ser-536 activating phosphorylation, was not associated with global constitutive NF-κB activation and did not impair subsequent nuclear translocation of phosphorylated NF-κBp65 in response to TNF-α. Our results demonstrate that human telomerase represses ROS-dependent intracellular signaling and gene induction in response to TNF-α.

1. Introduction

Telomerase is a ribonucleoprotein enzyme whose main function resides in telomere repeat synthesis and chromosome end protection. The minimal components of active enzyme include hTERT[2], the human telomerase reverse transcriptase catalytic subunit, and h*TR*, the RNA template component. While most normal human somatic cells display a finite lifespan due to the lack of telomerase activity, ectopic expression of hTERT prevents *in vitro* replicative senescence and extends cellular lifespan [1]. In cancer cells, telomerase is frequently up-regulated and ensures cellular immortality and metastasis [2]. In the last decade, accumulating evidences pointed toward the existence of telomere-independent functions of telomerase that may promote survival of cancer cells. These include protection against apoptosis or oxidative stress, enhanced DNA repair activity and modulation of gene expression profiles [3]. A protective role of telomerase against oxidative stress was clearly documented and associated with reduced intracellular reactive oxygen species (ROS) levels and improved mitochondrial function [4–9]. Additional studies demonstrated that telomerase – whether overexpressed or at endogenous levels – can be exported out of the nucleus to mitochondria, thanks to the presence of a mitochondrial targeting sequence at the N-terminus [6,9–14]. Nuclear export of hTERT is exacerbated under oxidative stress conditions, resulting in increased mitochondrial membrane potential, higher levels of overall respiratory chain activity and increased activity of cytochrome c oxidase, the rate-limiting enzyme in the mitochondrial electron transport chain [6,7,9,10]. Improved mitochondrial function of telomerase-expressing cells is likely to account for the protective role of telomerase against apoptosis that has been previously reported in numerous studies [4,5,7,9,15,16]. Additional extra-telomeric roles of telomerase include modulation of gene expression through an impact on unrelated cellular mechanisms like RNA interference [17] or Wnt/β-catenin signaling pathway [18]. Notably, telomerase was identified as a transcriptional modulator of the Wnt/β-catenin signaling pathway through its

Abbreviations: hTERT, human telomerase reverse transcriptase catalytic subunit; h*TR*, human telomerase RNA component; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; NAC, N-acetyl-L-cysteine; CHX, cycloheximide; ERK1/2, extracellular signal-regulated kinases 1 and 2; SOD2, superoxide dismutase 2; IL-6, interleukin 6; TGF-β, transforming growth factor-β; IKK, IκB kinase; IK-SR, IκB-α "Super-Repressor"; MAPK, mitogen-activated protein kinase; H_2DCF-DA, 2′, 7′-dicholorodihydrofluorescein diacetate; AACOCF$_3$, arachidonyl trifluoromethyl ketone; cPLA2, cytosolic phospholipase A2; 5-LOX, 5-lipoxygenase; SV40, T/t antigens of Simian virus 40; ALT, alternative lengthening of telomeres; U0126, inhibitor of MEK1/2 ERK-activating kinases.

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direct interaction with a chromatin remodeling protein involved in Wnt signaling [18]. Interactions between telomerase and other signaling pathways like pRB/E2F, TGF-β (Transforming Growth Factor-β) or EGFR (Epidermal Growth Factor Receptor) [19], were also reported although the impact of telomerase may be indirect in some instances. Notably, increased resistance to the anti-proliferative effect of TGF-β appeared to result from canonical telomerase-dependent reduction of p53 activation in human mammary epithelial cells and was not mediated by a direct impact of telomerase on TGF-β signaling pathway [20]. Tumor Necrosis Factor-α (TNF-α) is a cytokine that plays key roles in inflammation and pathophysiology [21]. In various tissues, TNF-α treatment simultaneously activates apoptotic pathways and NF-κB-dependent signaling pathway, a pathway involved in transcription of a variety of survival genes. The cellular response to TNF-α is determined by a balance between apoptotic- and NF-κB-dependent pathways. Not surprisingly, the NF-κB pathway is frequently aberrantly activated in cancer where it promotes protection against apoptosis and hinders anti-cancer therapies [22]. Addition of TNF-α to cells activates the IκB kinase (IKK) complex which, in turn, phosphorylates both IκB inhibitors of NF-κB and NF-κBp65 itself on Ser-536 residue. These phosphorylation events lead to proteasomal degradation of IκB, nuclear translocation of phosphorylated NF-κBp65 and activation of target genes [23]. TNF-α is also a potent inducer of endogenous ROS production and ROS are important second messengers in cellular responses to TNF-α as they activate mitogen-activated protein kinases (MAPK) and apoptosis [21–24]. TNF-α-induced ROS were further shown to participate in transcriptional induction of *SOD2* superoxide dismutase gene, implying ROS in the modulation of NF-κB-dependent gene transcription [25]. Here, because of the previously reported impact of hTERT on intracellular ROS production, we investigated the impact of telomerase on ROS-dependent and -independent cellular responses to TNF-α, including cell killing, ERK1/2 MAPK phosphorylation and NF-κB target gene induction.

2. Materials and methods

2.1. Cell lines and culture and retroviral plasmids

HFF2 normal human foreskin fibroblasts were purchased from ATCC (Rockville, MD, USA) and maintained in Minimum Essential Medium (MEM) supplemented with 1% non-essential amino acids (GIBCO, Invitrogen, Leek, The Netherlands), 10% fetal bovine serum (FBS) (Hyclone, Thermo Fisher Scientific, Waltham, MA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO, Invitrogen). HFF2hTERT cell line was obtained by retroviral transduction of HFF2 cells at population doubling 9 (PD 9) with pBMN-puro::*hTERT* plasmid (kindly provided by C. Heirman, VUB, Brussels, Belgium). SW39 is a telomerase-positive SV40-immortalized fetal lung fibroblast cell line kindly provided by W. Wright (UT Southwestern Medical Center, Dallas, USA). IMRB (ALT), U2OS (ALT) and U2OS Telo cell lines were described previously [26]. Briefly, U2OS Telo cells were obtained by transfection of U2OS ALT cell line with plasmids encoding hTERT and h*TR* telomerase subunits. pBabe-neo::largeT genomic plasmid was purchased from Addgene (Cambridge, MA, USA). pBabe-puro and pBabe-puro::*IκB-α(S32A-S36A)* (pIK-SR) plasmids were kindly provided by K.B. Marku (University of Bologna, Italy). pSuper-puro::*shp53* plasmid was provided by S. Mattiussi (IBCN, CNR, Roma, Italy).

2.2. Cell treatments

Recombinant human TNF-α was purchased from R&D Systems (Minneapolis, MN, USA). Cycloheximide, N-acetylcysteine and rotenone were from Sigma-Aldrich (St Louis, MO, USA). Arachidonyl trifluoromethyl ketone ($AACOCF_3$) was purchased from Merck Biosciences (Damstadt, Germany) and U0126 was from Cell Signaling Technology (Beverly, MA, USA). When indicated, cells were pre-treated for 1 h before TNF-α addition.

Quantitative RT-PCR–Total RNA was extracted using TriPure Isolation Reagent (Roche Applied Science, Mannheim, Germany). Reverse transcription was performed with random hexamers and M-MLV reverse transcriptase (Invitrogen, Leek, The Netherlands) as described before [26]. Gene expression levels were measured quantitatively using either *Taq*Man or SYBR Green technology. Primers, probes, qPCR Core kit and SYBR Green were purchased from Eurogentec (Seraing, Belgium). Sequences of primers and probes are described in Supplementary Table 1. Annealing and elongation were performed at 60 °C for 1 min for all genes except for *ACTB* (62 °C, 2 min).

2.3. Total cell lysates, nucleo-cytoplasmic separations and Western blots

Total cell lysates were prepared in the presence of Complete mini protease inhibitor cocktail (Roche Applied Science), PhosphoSTOP phosphatase inhibitor (Roche Applied Science) and 5 mM DTT (Sigma-Aldrich). Nuclear and cytosolic fractions were prepared with ProteoJET kit (Fermentas GMBH, St Leon-Rot, Germany) according to the manufacturer's protocol. Proteins were subjected to SDS-PAGE and electroblotted onto PVDF membranes (Millipore, Billerica, MA, USA). The following primary antibodies were used (1/1000): anti-p65, anti-β-tubulin, anti-IkB-α (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-P-p65Ser536, anti-P-ERK1/2 (Cell Signaling Technology), anti-p80Ku (Upstate Bio-Scientific, Lake Placid, NY) and anti-β-actin (1/25000) (Sigma-Aldrich). Peroxidase-conjugated anti-mouse (1/2000) (Santa Cruz Biotechnology) or anti-rabbit (1/1000) (Cell Signaling Technology) IgGs were used. Protein bands were visualized using the Westpico chemiluminescence reagent (Pierce, Rockford, IL, USA). Western blots quantifications were performed with Image Station 440 CF (Kodak Digital Science).

2.4. Measurement of intracellular ROS concentration

For ROS detection, cells were grown for 24 h in low FBS (5%). After trypsinization, cells were washed with PBS (Sigma-Aldrich) and incubated for 30 min with 10 μM 2′, 7′-dicholorodihydrofluorescein diacetate (H_2DCF-DA, Molecular Probes, Invitrogen) at 37 °C. Cells were washed again with PBS and ROS levels were measured by fluorescence-activated cell sorter (FACS) analysis (Becton Dickinson, Mountain View, CA).

2.5. Measurement of cell viability

Cell viability measurements were performed with the WST-1 Cell Proliferation Reagent (Roche Applied Science) according to the manufacturer's protocol. Briefly, 10 μl of WST-1 reagent and 100 μl of medium containing 5% FBS were added to cells grown in multi-well plates before incubation at 37 °C for 4 h. Formazan dye produced by metabolically active cells was quantified with an ELISA plate reader at 450 nm with a reference wavelength of 640 nm.

2.6. Statistical analyses

All qPCR measurements were done in triplicate on 5–10 independent RNA extractions. ROS (H_2DCF-DA) and cell viability (WST-1) measurements have been repeated three times. Values in graphs are given as mean ± standard deviation. Student's *t*-test was applied to compare data sets (paired with unequal variances) using Microsoft Excel software. *P*-values are indicated on the graphs.