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Research paper

Alternative splicing of telomerase catalytic subunit hTERT generated by apoptotic endonuclease EndoG induces human CD4$^+$ T cell death

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ABSTRACT

Telomerase activity is regulated by alternative splicing of its catalytic subunit human Telomerase Reverse Transcriptase (hTERT) mRNA. Induction of a non-active spliced hTERT leads to inhibition of telomerase activity. However, very little is known about the mechanism of hTERT mRNA alternative splicing. The aim of this study was to determine the role of the apoptotic endonuclease EndoG in alternative splicing of hTERT and telomerase activity. A strong correlation was identified between EndoG expression levels and hTERT splice variants in human CD4$^+$ and CD8$^+$ T lymphocytes. Overexpression of EndoG in CD4$^+$ T cells down-regulated the expression of the active full-length hTERT variant and up-regulated expression of the non-active spliced variant. A reduction in full-length hTERT transcripts down-regulated telomerase activity. Long-term *in vitro* cultivation of EndoG-overexpressing CD4$^+$ T cells led to dramatically shortened telomeres, conversion of cells into a replicative senescence state, and activation of the BCL2/BAX-associated apoptotic pathway finally leading to cell death. These data indicated the participation of EndoG in alternative mRNA splicing of the telomerase catalytic subunit hTERT, regulation of telomerase activity and determination of cell fate.

1. Introduction

Telomerase is a protein complex that adds the telomere nucleotide repeat TTAGGG at 3′-ends of chromosomes. The most important subunit of human telomerase is human Telomerase RNA (hTR), which contains the telomere template and human Telomerase Reverse Transcriptase (hTERT), which acts as reverse transcriptase to synthesize telomeric DNA using the hTR template (Blackburn 2000). Telomerase activity is not detected in normal somatic human cells (Ewald et al., 2013). Somatic cell telomeres shorten during every DNA replication cycle. Shortening of telomeres to critical sizes converts cells into a state of replicative senescence. Such cells stay alive and metabolically active but are unable to proliferate. The replicative senescence state is associated with increased expression and activation of beta-galactosidase (b-Gal) (Ewald et al., 2013; Lee et al., 2006) followed by activation of apoptosis and cell death (Harley et al., 1990). Telomerase is active in normal germ cells, stem cells, activated lymphocytes and most types of cancer cells (Kaszubowska 2008). Telomerase activity in these cells supports telomere length and increased proliferation potential (Kim et al., 1994).

Telomerase activity is known to be regulated by hTERT expression (Meyerson et al., 1997) and by alternative splicing (AS) of its mRNA (Listerman et al., 2013; Krams et al., 2001). Regulation of hTERT gene expression has been rather well investigated (Daniel et al., 2012), whereas very little is known about telomerase regulation by AS hTERT. To date, 22 splice variants of hTERT have been described; however, only full-length hTERT is catalytically active (Saebøe-Larssen et al., 2006). Two splice variants comprise the major forms of total hTERT mRNA. Deletion of 36 nucleotides from exon 6 (α–variant) leads to the loss of a portion of the reverse transcriptase domain in the hTERT protein and results in the loss of catalytic activity. Deletion of 182 nucleotides from exons 7 and 8 (β–variant) leads to a reading frame shift and results in formation of a premature stop-codon in exon 10 and synthesis of a truncated hTERT protein (Ulaner et al., 1998; Ulaner et al., 2000). This hTERT splice variant acts as a dominant negative protein (Lydeard et al., 2007).

Abbreviations: AS, alternative splicing; b-Gal, beta-Galactosidase; EndoG, Endonuclease G; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase hTR human telomerase RNA; hTERT, human telomerase reverse transcriptase; MFI, mean fluorescence intensity; PI, propidium iodide; RT-PCR, reverse transcription polymerase chain reaction; TRAP, telomeric repeat amplification protocol

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It has been previously shown that telomerase activity depends on the activity of apoptotic endonucleases (Oulton and Harrington 2004; Lydeard et al., 2007) that digest DNA at the latest stages of apoptosis (Nagata et al., 2003). Endonuclease G (EndoG) is a site-specific endonuclease that specifically digests poly-(dG) sequences on double-stranded DNA. Another unique feature of EndoG is its RNase activity (Ruiz-Carrillo and Renaud 1987). EndoG is known to induce cellular senescence and decrease replicative capacity (Diener et al., 2010). Moreover, induced EndoG synthesis significantly decreases telomerase activity (Nagata et al., 2003). EndoG, due to its RNAse activity, produces short oligonucleotides that act as splice-switching oligonucleotides and induce alternative splicing of hTERT mRNA (Zhdanov et al., 2017a, 2017b) and provoke cancer cell apoptosis (Zhdanov et al., 2016). The aim of this work was to determine the association between EndoG and AS of hTERT in human T lymphocytes.

2. Materials and methods

2.1. Cell purification, cultivation and transfection

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the Ethical Committee at the Institute of Biomedical Chemistry; written informed consent was obtained from all participants. Blood from healthy 18–25-year-old donors (n = 12) was collected in Vacuette K3EDTA tubes (Greiner Bio-One, Kremsmünster, Austria). Fresh peripheral blood mononuclear cells (PBMCs) were isolated using Lympholite-H (Cedarlane, Burlington, Ontario, Canada) density gradient centrifugation. $CD4^+$ T cells were purified from PBMCs using the CD4+ Human Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. $CD8^+$ cells were purified using the CD8+ Human Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD4^+$ T cells were seeded at 5×10^5 cells/ml and cultured in 25 cm^2 flasks in RPMI 1640 cell medium (Thermo Fisher Scientific Inc., Waltham, MA) supplemented with 10% FBS (Foetal Serum Bovine, Thermo Fisher Scientific Inc., Waltham, MA), penicillin (50 U/mL; Sigma-Aldrich, St. Louis, MO), and streptomycin (50 mg/ml; Sigma-Aldrich, St. Louis, MO) with 5 μg/ml anti-CD28 (eBioscience Inc., San Diego, CA, USA), 5 μg/ml anti-CD3 MAbs (MedBioSpectr, Moscow, Russia), and 100 U/mL rHu IL-2 (R & D Systems, Minneapolis, MN). Cells were cultivated in 5% CO_2/95% air in a humidified atmosphere at 37 °C and re-stimulated every three days with complete medium supplemented with IL-2 and antibodies. Transfection of $CD4^+$ cells with a pEndoG-GFP plasmid or control plasmid pGFP (both from InvivoGene, San Diego, CA) was performed using Lipofectamine 2000 (Invitrogen, Grand Island, NY) according to manufacturer's protocol.

2.2. RNA isolation and real-time RT-PCR

Total RNA from cells was extracted using the RNeasy Mini kit from Qiagen (Valencia, CA) according to the manufacturer's protocol. Reverse transcription real-time RT-PCR was performed as described by Basnakian and colleagues (Basnakian et al., 2006). Five micrograms of total RNA was reverse-transcribed using the RevertAid RT Kit (Invitrogen, Grand Island, NY) in a 25-μl reaction mixture followed by real-time RT-PCR using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The reaction mix was prepared using the Platinum SYBR Green qPCR Supermix-UDG (Invitrogen, Grand Island, NY) according to the manufacturer's recommendations. The list of primers (Syntol, Moscow, Russia) is presented in Table 1. Two temperature cycles for annealing/extension were used. The fluorescence was measured at the end of annealing step, and melting curve analysis was performed at the end of the reaction (after the 45th cycle) between 60 °C and 95 °C to assess the quality of the final PCR products. The standard curves indicating reaction effectiveness were generated using 4 serially diluted samples (1:40, 1:80, 1:160 and 1:320) of each protein or 18S cDNA. Gene expression levels were normalized relative to the expression of reference gene 18S. The PCR products were separated on 1% agarose gels stained with ethidium bromide and photographed under UV light in a ChemiDoc™ XRS imaging system (Bio-Rad, Hercules, CA). Calculation of the relative RNA concentration was performed using CFX96 Touch software.

Analysis of apoptosis-involved gene expression was conducted with the RT^2 Profiler Human Apoptosis PCR Array (SA Biosciences, Qiagen, Valencia, CA) according to the manufacturer's recommendations using an ABI 7900 real-time PCR machine (Applied Biosystems, Foster City, CA). The data were analysed using the SABiosciences PCR Array Data Analysis Software (http://www.sabiosciences.com/pcr/arrayanalysis.php).

2.3. Western blotting

Cells were lysed in 1 ml of TBE buffer (89 mM Tris, 89 mM H_3BO_3, 2 mM EDTA, pH 8.0) by ultrasonic disruption (50 W, 2 min) using a Sonic Dismembrator (Thermo Fisher Scientific Inc., Waltham, MA). Cell lysates were centrifuged for 10 min at 12000xg to remove cell debris. Sample protein contents were measured using the Bradford protein assay (Pierce Biotechnology, Rockford, IL). Bovine serum albumin was used for serial dilutions to generate a calibration curve. Total protein extracts (50 μg of total protein) were dissolved in 50 mM Tris-HCl, pH 6.8, 1% sodium dodecyl sulfate, 2 mM EDTA, 1% 2-mercaptoethanol, and 7.5% glycerol and denatured by heating at 100 °C for 10 min. Proteins were separated by gradient PAAG (Laemmli 1970) (100 V; 2 h) using NuPAGE® Novex® 4–12% Bis-Tris Protein gels (Life Technologies, Carlsbad, CA). Proteins were transferred onto nitrocellulose membranes in Novex transfer buffer (Invitrogen, Grand Island, NY) at 40 V for 3 h. The membranes were stained with Ponseau S (Sigma-Aldrich, St. Louis, MO) (Hofnagel et al., 2004). After soaking in the Blotting-Grade Blocker blocking solution (Bio-Rad, Hercules, CA), the membranes were incubated with monoclonal antibodies against glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) or hTERT (Abcam, Cambridge, MA) diluted 1:1000 or polyclonal anti-EndoG antibodies (Millipore, Billerica, MA) diluted 1:500. Membranes were washed in Tris-buffered saline, pH 7.6, with 0.1% Tween-20 (Invitrogen, Grand Island, NY) and incubated with secondary antibodies conjugated to horseradish peroxidase (Cell Signaling, Danvers, MA). Membranes were visualized using the Super Signal chemiluminescent kit (Pierce Biotechnology, Rockford, IL) and documented in a ChemiDoc™ XRS imaging system (Bio-Rad, Hercules, CA). Relative protein amounts were determined with densitometry using a GelAnalyzer 2010a (www.gelanalyzer.com).

2.4. Telomerase activity assay

Telomerase activity was determined using the Telomeric Repeat Amplification Protocol (TRAP) (Kim et al., 1994; Kovalenko et al., 2012). Cells were lysed in 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, 5 mM 2-Mercaptoethanol, 0.5% CHAPS and 10% glycerol (all from Sigma-Aldrich, St. Louis, MO) and centrifuged for 30 min at 12000xg. Supernatants were stored at −80 °C. Elongation of the oligonucleotide substrate was completed with the Telomerase Substrate primer (TS-primer) (5′- AATCCGTCGAGCAGAGTT −3′) in a 30-μl reaction mixture containing the following components: 67 mM Tris-HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 1.5 mM $MgCl_2$, 1 mM EGTA (all from Sigma-Aldrich, St. Louis, MO), 0.25 mM each dNTPs (Syntol, Moscow, Russia) and 2 μl of cell lysate (2000 cell equivalent). Elongation was performed for 30 min at 37 °C and 10 min at 96 °C to inactivate the telomerase. Copy Extended primer (CX-primer, 0.1 μl) (5′- CCCTTACCCTTACCCTTACCCTAA −3′) and 2.5 Units of Taq-polymerase were added to the elongation mixture followed