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Research Paper

Acute telomerase components depletion triggers oxidative stress as an early event previous to telomeric shortening

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ABSTRACT

Loss of function of dyskerin (DKC1), NOP10 and TIN2 are responsible for different inheritance patterns of Dyskeratosis congenita (DC; ORPHA1775). They are key components of telomerase (DKC1 and NOP10) and shelterin (TIN2), and play an important role in telomere homeostasis. They participate in several fundamental cellular processes by contributing to Dyskeratosis congenita through mechanisms that are not fully understood. Presence of oxidative stress was postulated to result from telomerase ablation. However, the resulting disturbed redox status can promote telomere attrition by generating a vicious circle, which promotes cellular senescence. This fact prompted us to study if acute loss of DKC1, NOP10 and TINF2 can promote redox disequilibrium as an early event when telomere shortening has not yet taken place. We generated siRNA-mediated (*DKC1, NOP10 and TINF2*) cell lines by RNA interference, which was confirmed by mRNA and protein expression analyses. No telomere shortening occurred in any silenced cell line. Depletion of H/ACA ribonucleoproteins DKC1 and NOP10 diminished telomerase activity via TERC down-regulation, and produced alterations in pseudouridylation and ribosomal biogenesis. An increase in the GSSG/GSH ratio, carbonylated proteins and oxidized peroxiredoxin-6 was observed, in addition to MnSOD and TRX1 overexpression in the siRNA DC cells. Likewise, high PARylation levels and high PARP1 protein expression were detected. In contrast, the silenced *TINF2* cells did not alter any evaluated oxidative stress marker. Altogether these findings lead us to conclude that loss of DKC1 and NOP10 functions induces oxidative stress in a telomere shortening independent manner.

1. Introduction

An increasing number of inherited diseases, referred to as telomeropathies, have been correlated with mutations in the genes that encode the proteins required for telomere structure, replication, repair and length maintenance [1], which indicate the genetic complexity of all these syndromes. Telomeres are special functional complexes placed at the end of linear eukaryotic chromosomes, and consist of noncoding tandem repeat DNA sequences and associated shelterin complexes, which together play a dual role in protecting chromosome ends, and in mediating cell proliferation and senescence [2].

The dynamics of telomeres comes under the control of telomerase and shelterin. Telomerase is a reverse transcriptase (hTERT) capable of utilizing an integrated RNA component (hTERC) as a template to add protective tandem telomeric single-strand DNA repeats at the end of chromosomes [3]. Several telomerase-associated accessory proteins like TCAB1, dyskerin (DKC1), NHP2, NOP10, GAR1, among others, guarantee the biological specificity of the enzymatic complex [4]. Shelterin has a core of six proteins, TRF1, TRF2, POT1, Rap1, TPP1 and TIN2, which work together with telomerase in physiological circumstances. In fact the disruption and aberrant activation of either shelterin and/or the telomerase complex lead to deleterious effects for the cell [5].

Dyskeratosis congenita (DC; ORPHA1775), a rare inherited multisystem disorder of premature aging, was the first impaired telomere maintenance syndrome to be described [6]. To date, mutations in 12 genes have been linked to the DC phenotype, many of which share a link to telomere/telomerase biology, such as those that encode for telomerase complex components (TERT, TERC, DKC1, NHP2 and NOP10), shelterin components (TIN2), the proteins involved in T-loop dissociation (RTEL1), telomerase trafficking (TCAB1) and replication

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(CTC1) [7,8].

The mutated genes involved in DC result in three modes of transmission: X-linked recessive (X-L), autosomal dominant (AD), and autosomal recessive (AR). Mutations in *DKC1* are related with X-L, the commonest in DC. Mutations in *TERC*, *TERT*, or *TINF2* were found in AD-DC, and *TINF2* is the second most commonly mutated gene in DC. Lastly, *TCAB1, NOP10* and *NHP2* have been described in AR-DC [9].

Many DC-related genes perform more than one fundamental cellular function; e.g., DKC1, NOP10, NHP2 are the H/ACA-motif RNA-binding proteins required for the pseudouridylation of rRNAs and snRNAs [10], and alter ribosome and spliceosome functions [11]. Currently, debate continues as to whether the primary cause of DC is a defect in telomerase activity or ribosome biogenesis. In fact there are good arguments for accepting both points of views, and it seems reasonable that both could contribute to the broad range of clinical features found in this syndrome [12].

It is worth bearing in mind that TERT was the first telomeric protein detected in both the mitochondrial matrix and nucleus, and it performs different functions in these two subcellular compartments [13]. Chen and co-workers [14] reinforce the opinion that telomere-related proteins can regulate intermediary metabolism by demonstrating that TIN2 can localize into the mitochondria, where it regulates intermediary metabolism and reactive oxygen species (ROS) production. Altogether, these findings support a link between telomeric proteins and metabolic control by presenting oxidative stress as an additional mechanism by which telomeric proteins can trigger cancer or aging-related phenotypes [14].

In this context, it is noteworthy that DC lymphocytes with different DC mutations (TERT, TIN2, and TERC) have displayed a stressed phenotype characterized by high levels of ROS, DNA damage response (DDR), apoptotic markers and proliferative defects [15]. The opposite has also been postulated; oxidative stress can promote telomere attrition, which suggests a potential feedback loop to sustain elevated ROS and to favor entry into senescence. In fact oxidative stress has been suggested to be a major cause of telomere shortening [15]. So although it has been contended that the role of DDR and elevation of ROS are secondary to telomere shortening in DC [16], the possibility of oxidative stress contributing to the physiopathology of the disease as an early contributor cannot be ruled out.

Among the proteins involved in the pathogenesis of DC, DKC1 has been the subject of the vast majority of depletion studies [17,18]. However, the effects of the targeted depletion of other H/ACA proteins, like NOP10, have been poorly characterized. Likewise, the roles of TIN2 mutations in telomere maintenance, DDR and antioxidant defense are still a challenge that requires further research.

We aim to silence essential genes (*DKC1, NOP10* and *TINF2*) in Hela cells, as was previously done in other studies [19–21], for the functionality of telomerase and shelterin complexes, which are involved in several inheritance patterns of DC, in order to evaluate their role in oxidative stress, antioxidant responses and DDR without interfering with telomere length.

2. Experimental procedures

2.1. Cell culture

HeLa cells (American Type Culture Collection, Manassas, VA. USA) were grown in DMEM (Gibco Life Technologies, Karlsruhe, Germany), supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 U mL^{-1} penicillin/100 μg mL^{-1} streptomycin) (Sigma-Aldrich, St. Louis, MO. USA) in a 5% CO_2 incubator at 37 °C.

2.2. siRNA transfections

HeLa cells were seeded at a density of 2×10^5 per well in six-well plates that contained 2 mL of DMEM medium supplemented with 10% FCS with no antibiotic. After 24 h, cells were subjected to two sequential transfections, separated by a 24-h interval. Transfections were carried out using validated siRNA (siTARGET) for *DKC1 (siDKC1), NOP10 (siNOP10) and TINF2 (siTINF2)* mRNA (ID: s4111, ID: 215701, and ID: s25355, Ambion, CO, USA) at 100 nM with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Non targeting siRNA (Cat: AM4635, Silencer Negative Control siRNA #1, Ambion), under the same transfection conditions, was considered to be siCONTROL, and a HeLa wild-type was used as the CONTROL. Cells were harvested 48 h after the initial transfection for the next experiments. RNAi efficacy was assessed by quantitative polymerase chain reaction (qPCR) and Western blotting (WB).

2.3. Quantitative RT–PCR

For the reverse transcription reactions (RT), 200 ng of the purified total RNA were reverse-transcribed using random hexamers with the High-Capacity cDNA Archive kit (Applied Biosystems, P/N: 4322171, Foster City, USA) according to the manufacturer's protocol. The RT conditions comprised an initial incubation step at 25 °C for 10 min to allow random hexamers annealing, followed by cDNA synthesis at 37 °C for 120 min, and a final inactivation step for 5 min at 95 °C.

The mRNA levels were determined by a quantitative real-time PCR analysis in an ABI Prism 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The gene-specific primer pairs and probes for *DKC1* (Hs00154737_m1), *NOP10* (Hs00430282_m1), *TINF2* (Hs01554309_g1), *TERC* (Hs03454202_s1), *TERT* (Hs00972650_m1), Superoxide Dismutase 2 (*SOD2*, Hs00167309_m1), Thioredoxin 1 (*TRX1*, Hs00917067_m1), Thioredoxin 2 (*TRX2*, Hs00912509_g1), Poly (ADP-ribose) Polymerase 1 (*PARP1*, Hs00242302_m1), oxoguanine glycosylase (*oGG1*, Hs00213454_m1), Xeroderma Pigmentosum Complementation Group A (*XPA*, Hs00166045_m1), Werner (*WRN*, Hs01087915_m1), *RAD51* (Hs00947967_m1), *RAD53* (Hs00200485_m1), *18S* (Hs0300 3631_g1), and *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase, Hs02758991_g1) were used together with 1x TaqMan® Universal PCR Master Mix (Applied Biosystems, P/N 4304437, Foster City, CA, USA) and 1 μL of the reverse-transcribed sample RNA in 10 μL reaction volumes. The PCR conditions were 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. GAPDH was chosen as the endogenous reference for the normalization of the qRT-PCR-based expression analysis. Each sample was analyzed in triplicate, and the relative quantification of mRNAs was calculated by the $2^{-\Delta\Delta CT}$ method [22].

2.4. Immunoblotting Western blot

Protein extracts were obtained from the HeLa cells lysed in ice using lysis buffer (20 mM Hepes, pH 7.4, 1% Triton X-100, 100 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride (PMSF), and the protein protease inhibitor cocktail (Roche Diagnostics, Barcelona, Spain)). The resultant suspension was centrifuged at 13,000*g* for 10 min at 4 °C and supernatants were stored at − 80 °C until use. Protein content was determined by the Bradford method [23].

Proteins were prepared in LDS sample buffer (Tris 40 mM, EDTA, bromophenol blue 0.01%, sucrose 40%, SDS 4%, β-mercaptoethanol 10%) and heated to 95 °C for 5 min. Proteins were resolved by sodium dodecyl sulfate–polyacrylamide (12%) gel electrophoresis (SDS–PAGE) at 100 V for 2 h. They were then transferred to nitrocellulose membranes (Whatman GmbH, Dassel, Germany). Following transference, the membrane was blocked in 5% skimmed milk in 0.1% Tween-20 (Sigma-Aldrich) and phosphate-buffered saline (PBS). Membrane slices were incubated with specific monoclonal antibodies: Dyskerin (DKC1, 1:1000, Santa Cruz Biotechnology, Texas, USA), NOP10 (1:1000, Santa Cruz Biotechnology, Texas, USA), TIN2 (1:1000, Santa Cruz Biotechnology, Texas, USA), a sulfonic acid form of PRDX6 (PRDX6-SO_3H, 1:1000, Abcam, Cambridge, UK), manganese superoxide