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Telomeric DNA induces p53-dependent reactive oxygen species and protects against oxidative damage

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ABSTRACT

Background: Reactive oxygen species (ROS) are generated by cellular metabolism as well as by exogenous agents. While ROS can promote cellular senescence, they can also act as signaling molecules for processes that do not lead to senescence. Telomere homolog oligonucleotides (T-oligos) induce adaptive DNA damage responses including increased DNA repair capacity and these effects are mediated, at least in part, through p53.

Objective: Studies were undertaken to determine whether such p53-mediated protective responses include enhanced antioxidant defenses.

Methods: Normal human fibroblasts as well as R2F fibroblasts expressing wild type or dominant negative p53 were treated with an 11-base T-oligo, a complementary control oligo or diluents alone and then examined by western blot analysis, immunofluorescence microscopy and various biochemical assays.

Results: We now report that T-oligo increases the level of the antioxidant enzymes superoxide dismutase 1 and 2 and protects cells from oxidative damage; and that telomere-based γH2AX (DNA damage) foci that form in response to T-oligos contain phosphorylated ATM and Chk2, proteins known to activate p53 and to mediate cell cycle arrest in response to oxidative stress. Further, T-oligo increases cellular ROS levels via a p53-dependent pathway, and these increases are abrogated by the NAD(P)H oxidase inhibitor diphenyliodonium chloride.

Conclusion: These results suggest the existence of innate telomere-based protective responses that act to reduce oxidative damage to cells. T-oligo treatment induces the same responses and offers a new model for studying intracellular ROS signaling and the relationships between DNA damage, ROS, oxidative stress, and cellular defense mechanisms.

1. Introduction

Human telomeres, tandem repeats of the sequence TTAGGG and its complement that cap the ends of chromosomes [1], play important roles in DNA damage responses [2–4] and aging [5,6]. Telomeres exist in a loop structure that is stabilized by telomeric repeat binding factor 2 (TRF2) [7]. Disruption of the loop by a dominant negative construct ($TRF2^{DN}$)[2] leads to apoptosis of certain mammalian cells [8] and senescence of others [9], a process mediated at least in part through ATM and p53 activation [8], suggesting that telomere loop disruption initiates a DNA damage signal.

Interestingly, provision of telomere TTAGGG homolog oligonucleotides (T-oligos), known to rapidly accumulate in the nucleus [10–12], also stimulates DNA damage signals and adaptive responses mediated, while control oligonucleotides complementary or unrelated to the TTAGGG repeat sequence do not [10,13–16]. Specifically, we have shown that exposure of fibroblasts to T-oligos leads to dose-dependent DNA damage responses, such as increased DNA damage repair capacity [17,18], S-phase cell cycle arrest, apoptosis [10–12] and senescence [14,15], mediated at least in part through ATM and p53 [13–15,19]. These cellular responses occur without affecting the cells' own telomeres [10,14,19] and are independent of telomerase [15,20]. Most recently, these T-oligo-induced responses were shown to involve formation of DNA damage foci at the telomere via WRN [19], the helicase and exonuclease mutated in the cancer-prone progeroid Werner Syndrome [21,22]. Furthermore, p53 is known to interact with

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WRN both *in vivo* and *in vitro* [23–25] and fibroblasts derived from individuals with Werner Syndrome display reduced p53-mediated apoptosis, restored by introducing wild type WRN into the cells, suggesting that WRN is involved in p53 activation [24].

High levels of ROS are procarcinogenic [26,27] and can damage cellular proteins, lipids and DNA [Reviewed in [28–30]], and a network of antioxidant enzymes has evolved to decrease ROS levels that would otherwise damage cells [Reviewed in [31–33]]. Antioxidant defense mechanisms include enzymes such as glutathione peroxidase (GPX) [Reviewed in [34,35]], glutathione reductase [Reviewed in [36,37]], copper and zinc-dependent superoxide dismutase (SOD)1 [38–40], catalase [Reviewed in [41]], and manganese-dependent SOD2[38–40] that acts preferentially in the mitochondria.

Interestingly, after UV irradiation, a DNA damaging agent that leads to the formation of DNA photoproducts and ROS, the activities of the antioxidant enzymes GPX, SOD1 and particularly SOD2 are induced [42], suggesting an adaptive or protective response of fibroblasts to UV-induced oxidative DNA damage. Continuous exposure to the damaging agent precipitates the fibroblast response of stress-induced premature senescence (SIPS) [43], a response similar or identical to the induction of senescence following serial cell division with critical telomere shortening [44,45], activation of tumor supressors such as p53 [46] or over-expression of Ras [47] or Raf [48] oncogenes. Oxidative stress preferentially targets guanine (G) residues, leading to formation of 8-oxo-G [49], and telomeres are particularly sensitive to oxidative stress because of their high G content. ROS exposure is well documented to cause telomere shortening and SIPS in fibroblasts [50].

Cellular ROS can be produced by enzymatic and non-enzymatic mechanisms [51]. ROS are generated in the mitochondria through the electron transport chain and in other electron transferring cellular systems, a non-enzymatic mechanism. In contrast, ROS are also generated by the plasma membrane-associated NAD(P)H oxidase (NOX), an enzyme complex with multiple components [52–54] and thought to have a regulatory role, stimulated by growth factors and cytokines [30,55]. Although the cellular responses mediated by NOX-generated ROS are not completely understood, it is speculated that ROS generation may enhance cell survival through upregulation of antioxidant defense mechanisms [56]. Thus, it has been suggested that ROS such as superoxide and hydrogen peroxide (H_2O_2) are utilized by cells as signaling molecules for processes that do not necessarily lead to cellular senescence or result in detectable oxidative damage [56,57]. Rather, they propagate signaling through tyrosine phosphorylation of effector proteins, mitogen activated protein (MAP) kinase activation, DNA synthesis, and chemotaxis [58,59]. It is speculated that lower ROS levels lead to adaptive cellular responses, while higher levels result in senescence [33,57,60,61]. It has also been reported that over-expression of p53 leads to cellular ROS elevation, as well as to transcription of redox-associated genes [62].

We now report that T-oligos, known to induce a variety of DNA-protective and anti-cancer responses, also induce the level of antioxidant enzymes, specifically SOD1 and SOD2. Furthermore, T-oligos protect fibroblasts against oxidative challenge by H_2O_2. Finally, T-oligos upregulate ROS levels, consistent with T-oligo induced ROS signaling, a process mediated by p53 and NAD(P)H oxidase activation.

2. Materials and methods

2.1. Materials

Hydrogen peroxide (30% (w/w), with 0.5 ppm stannate and 1 ppm phosphorus as preservatives) was obtained from Sigma (USP grade, St. Louis, MO). The stock solution was stored at 4 °C and all dilutions were made in DMEM immediately before use. 2′,7′-Dichlorodihydrofluorescein diacetate (DCF) from Molecular Probes, Inc. (Eugene, OR) was dissolved in DMSO to a stock concentration of 1 mg/ml and stored under nitrogen at −20 °C. Propidium iodide (PI) was obtained from Sigma. Diphenyliodonium chloride (DPI) powder was obtained from A.G. Scientific, Inc. (San Diego, CA), dissolved in DMSO to a stock concentration of 5 mg/ml and frozen at −20 °C until use. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was dissolved in dimethylformamide and added to a citric acid/NA phosphate buffer at pH 6.0 immediately before use for a final concentration of 1 mg/ml [63].

Purified 5′-phosphorylated oligonucleotides with phosphodiester linkages (Midland Certified Reagents, Inc., Midland, TX) were obtained in lyophilized form, resuspended in sterile dH_2O to generate a 2 mM stock solution and frozen in aliquots at −20 °C as previously described [17]. The stock solution was further diluted to 40 μM in cell culture medium immediately before use.

2.2. Fibroblast cell culture

Normal newborn human dermal fibroblasts were cultured from foreskin specimens and maintained in DMEM supplemented with 10% calf serum (CS) as previously described [64]. In experiments using the DCF assay, cells from three different donors were combined.

R2F fibroblasts were a kind gift from Dr. J. Rheinwald (Harvard Medical School, Brigham and Women's Hospital) The retroviral vector pL(p53DD)SN, that expresses a dominant negative fragment of p53 ($p53^{DN}$) [65,66] was used to transfect newborn foreskin fibroblasts as described [67]. $p53^{DN}$ cells and isogenic wild type p53 R2F cells were maintained in a 1:1 mixture of DMEM and Ham's F12 medium and supplemented with 15% FBS.

2.3. Experimental design

All telomere homolog phosphodiester-linked oligonucleotides (T-oligos) 2-20 bases in length tested to date have been demonstrated to induce genome-protective responses in human cells, with molar efficacy determined by the inter-related parameters of length, percent telomere homology, guanine (G) content and lack of cytosine (C) residues [11]. Experiments reported here were performed with the 11-base 100% telomere homolog GTTAGGGTTAG (40 μM) or, in the case of the immunofluorescence microscopy studies, with a 16-base 100% homolog at half the concentration (20 μM), previously shown to be equipotent in standard T-oligo assays [19].

2.4. ROS induction and resistance to oxidative stress

To determine the effect of T-oligo on ROS induction, cells were stimulated once with GTTAGGGTTAG (T-oligo), the complementary control sequence CTAACCCTAAC (Cont-oligo) and/or diluent alone and were harvested at different intervals after treatment. Oligonucleotide concentrations were based on previous experiments establishing that these concentrations elicit DNA damage-like responses [17,19,68,69]. To determine T-oligo protective effect, 3×10^5 fibroblasts were plated in two 100 mm dishes. Seventy-two hours after plating cells were stimulated with T-oligo (40 μM) or diluent for 72 h. Cells were then harvested and replated at 1×10^5 cells/dish in 35 mm dishes. Twenty-four hours later, cells were treated with 25 μM fresh H_2O_2 or diluent for 1 h and then were provided fresh DMEM supplemented with 10% CS. Cell numbers were determined up to 48 h after medium change.