Telomere maintenance in telomerase-positive human ovarian SKOV-3 cells cannot be retarded by complete inhibition of telomerase

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Abstract The two known mechanisms for telomere maintenance in eukaryocytes are telomerase in telomerase-positive cells and alternative lengthening of telomeres (ALT) in telomerase-negative cells. We report here that telomere maintenance in the telomerase-positive human ovarian SKOV-3 cells was not affected by inhibition of telomerase. For comparison, the effect of telomerase inhibitors on telomere maintenance in another telomerase-positive cell line (i.e. human pharynx FaDu cells) and the telomerase-negative human osteosarcoma Saos-2 cells was examined. Telomerase activity was measured using a modified telomeric repeat amplification protocol and telomere length was measured using a solution hybridization-based method and fluorescence in situ hybridization. A reverse transcriptase inhibitor (3’-azido-deoxythymidime or AZT) and an antisense against a component of human telomerase RNA (antisense hTR) were used to inhibit telomerase. FaDu and SKOV-3 cells showed comparable baseline telomerase activity. Telomerase activity in both cells was inhibited about equally by AZT (maximal inhibition of \( \sim 80\% \)) and by expression of antisense hTR (complete inhibition in SKOV-3 cells and maximal inhibition of \( \sim 80\% \) in FaDu cells). However, treatment with telomerase inhibitors resulted in \( \sim 50\% \) telomere shortening in FaDu cells but had no effect on SKOV-3 nor Saos-2 cells. SKOV-3 cells did not show the characteristic features of ALT (i.e. heterogeneous telomere length and promyelocytic leukemia bodies), whereas these ALT features were observed in Saos-2 cells. Collectively, these results suggest the existence of a telomerase-independent mechanism of telomere maintenance in the telomerase-positive SKOV-3 cells. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Telomerase; Telomere; Ovarian cancer; Alternative lengthening of telomeres

1. Introduction

Telomeres are DNA–protein complexes that cap the chromosome ends. Telomeres have multiple functions, including preventing chromosomes from fusion and degradation (reviewed in [1]). In normal somatic cells, the telomere repeats are shortened progressively after each cell division, due to the incomplete replication at the telomeric ends. Cell senescence occurs when the telomeres reach critical length, leading to the hypothesis that telomere length is the biological clock that determines the number of divisions and life span of a cell [2]. The two known mechanisms for telomere maintenance in eukaryocytes are telomerase in telomerase-positive cells and alternative lengthening of telomeres (ALT) in telomerase-negative cells.

Most germ-line and stem cells, immortalized cells, and cancer cells contain telomerase, a ribonucleoprotein DNA polymerase, that adds telomeric repeats onto the 3’-ends of chromosomes. Telomerase activity is observed in \( >85\% \) of cancer cell lines and human tumors [3]. Inhibition of telomerase using an antisense to human telomerase RNA component (antisense hTR) or dominant negative expression of mutant telomerase reverse transcriptase (TERT) caused telomere shortening, cell senescence, apoptosis and chromosome instability in telomerase-positive cells [4,5]. On the other hand, ectopic expression of TERT in telomerase-negative human fibroblasts, retinal epithelial cells and vascular endothelial cells results in elongation of telomere length, extension of life span and allowance of cells to bypass the senescence limitation [6–8]. About 15% of human tumors and cancer cell lines do not express detectable telomerase activity. Telomeres in these cells are maintained by mechanisms that are independent of telomerase, referred to as ALT [9]. For example, studies using telomerase RNA component-deficient mice (mTER\(^{-/-}\)) demonstrated a telomerase-independent mechanism for telomere maintenance and elongation [10]. In yeasts lacking telomerase activity and in mosquito Anopheles gambiae, telomeres are maintained by a homologous recombination-based mechanism [11,12]. Drosophila melanogaster and related Dipteran species maintain their telomere length by transposition of a set of retroposons [13,14]. The characteristic features of ALT are highly heterogeneous telomere length, in a range of \( <5\; \text{kb} \) to \( >20\; \text{kb} \) and the presence of specific promyelocytic leukemia (PML) bodies that are donut-shaped structures in nuclei initially observed in PML cells [9,15,16]. The ALT-associated PML bodies are unique, consisting of telomeric DNA and telomere-binding proteins TRF1 and TRF2, and are universally present in ALT cells but absent in mortal cells and telo-
merase-positive cell lines or tumors [15–17]. ALT in human cells was surmised from their ability to maintain the telomere length in the absence of telomerase and from the presence of telomeres with highly heterogeneous lengths [9]. Recently, an ALT mechanism of telomere-to-telomere recombination was found in GM847 cells derived from human skin fibroblasts [18].

Zaffaroni and colleagues reported that partial inhibition of telomerase by either peptide nucleic acids or a hammerhead ribozyme targeting the hTR did not result in telomere shortening in the telomerase-positive human melanoma JMR8 and M14 cells, and postulated the emergence of activation of the ALT mechanism as a response to telomerase inhibition [19,20]. However, these cells did not show the characteristics of ALT before or after treatments with telomerase inhibitors. It is also not known whether the residual telomerase activity was sufficient for telomere maintenance in these cells.

We report here that telomere maintenance in the telomerase-positive human ovarian SKOV-3 cells was not affected by concomitant inhibition of telomerase and telomerase-positive cell line (i.e. human pharynx FaDu cells) was used as the positive control, and the telomerase-negative human osteosarcoma Saos-2 cells which use the ALT mechanism [9] was used as the negative control. A reverse transcriptase and telomerase inhibitor, 3'-azido-deoxithymidine or AZT [21] and antisense to hTR, were used to inhibit telomerase.

2. Materials and methods

2.1. Chemicals and methods

Sulforhodamine B, streptolydin O, p-phenylene diamin and bis-chloronic acid kit for protein determination were purchased from Sigma (St. Louis, MO, USA); polynucleotide kinase, restriction endonuclease HindIII, HindI, HindI and DNA molecular weight markers III and V, hygromycin B, G418, isopropyl-

2.2. Cell culture

Human ovarian SKOV-3, pharynx FaDu and osteosarcoma Saos-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). SKOV-3 cells were cultured in McCoy's medium and FaDu and Saos-2 cells in minimum essential medium. The media were supplemented with 9% heat-inactivated FBS, 0.1% 10 mM non-essential amino acids, 2 mM l-glutamine, 90 μg/ml gentamicin and 90 μg/ml cefotaxime. Cells in exponential growth phase were used for experiments.

SKOV-3, FaDu and Saos-2 cells were continuously treated with AZT for up to 9 weeks. Each week, the medium was exchanged and cells were harvested for analysis of telomerase activity and telomere length.

2.3. Telomere length analysis and detection of PML bodies

Telomere length was measured by two methods. The first method used a solution hybridization-based analysis to detect the mean length of the terminal restriction fragments. This method measured both the amount and length of telomere (i.e. telomere amount and length assay, TALA) [22]. In brief, genomic DNA was isolated and 10 μg of DNA was digested at 37°C overnight with 10 U each of HinfII/CfoI/ HaelIII. The oligonucleotide probe (TTAGGG) was labeled by γ-32P-ATP with polynucleotide T4 kinase. 3 ng of the probe was added to 2.5 μg of DNA solution. After denaturation at 98°C for 5 min, hybridization was performed at 55°C overnight. The samples were electrophoresed on 0.7% agarose gel. After drying under vacuum without heating the gel was exposed to phosphorimage screen and the result was analyzed by ImageQuant software from Molecular Dynamics (Sunnyvale, CA, USA).

The second method for telomere length analysis was fluorescence in situ hybridization (FISH), which detected telomere signal in individual cells, as described previously [22]. FISH was also used to detect PML bodies. The fluorescein-labeled peptide nucleic acid (PNA) probe (CCCTAAT) was synthesized by PerSeptive Biosystems (Framingham, MA, USA). We evaluated the telomere length in interphase and M phase cells. Briefly, cells were treated with 0.1 μg/ml colcemid for 4 h and then harvested. After treatment with hypotonic solution and fixation with acetic acid and methanol, cells were dropped onto slides, air-dried and stored at −20°C. Cells were denatured at 80°C for 2 min and hybridized to the PNA probe at room temperature for 2 h. Slides were then washed at room temperature with 70% formamide and phosphate-buffered saline (PBS). The chromosomes were counterstained with propidium iodide and examined under a fluorescence microscope. The digital images were analyzed by Scion Image software (NIH Image for PC).

2.4. Measurement of telomerase activity

Telomerase activity in intact cells was measured using a modified quantitative telomeric repeat amplification protocol (TRAP) [23]. Briefly, cells were washed twice with PBS solution and reversibly porated by streptolydin O (5 μM) in the presence of TS primer (2 μM), and dNTP (50 μM) in 100 μl of serum-free medium at room temperature for 5 min. The culture medium (900 μl) containing 10% FBS was added to stop the permeabilization process. The TS primer that penetrated the cells was extended in situ by intracellular telomerase by incubation at 30°C for 30 min. The extended TS primer was then extracted by CHAPS-biased lysis buffer, purified using phe- 

2.5. Inhibition of telomerase by antisense hTR

To construct a recombinant expression vector containing antisense or sense hTR, a 185 bp hTR fragment, including the telomere template sequence in hTR, was obtained by RT-PCR with the primers 5'-CACGTGACATTTTTTGGTCTA-3' and 5'-GGTTGCGGA-GGTTGOCCT-3'. The fragment was cloned into pGem-T vector (Promega, Madison, WI, USA). The recombinant plasmid was digested using NofI and the resulting 219 bp DNA fragment was subcloned into the NotI site of the expression vector pOPRSV/ MCS (LaSwitch® II Inducible Mammalian Expression System, Stratagene, La Jolla, CA, USA). The clones that expressed antisense and sense hTR were confirmed by sequencing with T7 or T3 promoter primer (fmol® DNA Sequencing System, Promega). SKOV-3 cells were transfected with the Lac repressor vector, pCMVlac (LaSwitch® II Inducible Mammalian Expression System), using Lipofect- 

Fig. 1. Telomerase activity in SKOV-3, FaDu and Saos-2 cells. Telomerase activity (2.0 μg protein per sample) was detected by the modified TRAP. Six replicates for each cell line. M: pBR322/HaelIII DNA molecular weight marker. NC: negative control using cell lysis buffer instead of lysate.
pOPRSVI/MCS which expressed either antisense or sense hTR. The transfected cells were selected by treatment with hygromycin B and G418. The expression of antisense and sense hTR was induced by IPTG (3 mM). To ascertain the functionality of the antisense hTR and to rule out the effect of transfection and IPTG treatment, we used three controls, i.e. parent cells, cells that expressed sense hTR, and cells transfected with antisense hTR that was not expressed (i.e. without IPTG induction).

2.6. Analysis of AZT concentrations
Changes in AZT concentrations (starting concentration of 100 μM) in culture medium over time, in the absence or presence of tumor cells, were analyzed using an enzyme-linked immunosorbent assay (Neogen, Lexington, KY, USA). Briefly, 50 μl of each of medium (preadiluted 625-fold with the extraction buffer), AZT antibody, and horseradish peroxidase-conjugated AZT were added to the wells precoated with protein A and incubated at room temperature for 1 h. After washing three times, 150 μl of substrate solution containing tetramethylbenzidine and hydrogen peroxide was added to each well and allowed to stand for 30 min. The absorbance at 650 nm was measured using a microplate reader. The concentration of AZT was calculated according to the standard curve constructed by adding known amounts of AZT to culture medium. The standard curve was linear between 2 and 200 ng/ml AZT.

3. Results

3.1. AZT concentrations in culture medium
Analysis of AZT in the culture medium showed that AZT was stable under the experimental conditions. The AZT concentrations, after incubation in the absence or presence of FaDu, Saos-2 and SKOV-3 cells for 3 and 7 days, ranged from 96±9% to 105±16% of the starting concentrations.

3.2. Baseline telomerase activity
FaDu and SKOV-3 cells showed comparable baseline telomerase activity (Fig. 1); the activity in SKOV-3 cells was 86.5±10.5% of the activity in FaDu cells (P=0.12). In agreement with a previous report [9], Saos-2 cells did not show the characteristic laddering TRAP products. The background signals obtained from Saos-2 cells were 9.5±5.8% of the signals in FaDu cells.
3.3. Inhibition of telomerase by AZT

AZT is converted intracellularly to AZTTP, which inhibits telomerase. Hence, we examined the effect of AZT only in intact cells. Fig. 2 shows the concentration-dependent telomerase inhibition by AZT in SKOV-3 and FaDu cells. In both cell types, the maximal inhibition was \( \approx 80\% \) and was achieved at 100 \( \mu M \) AZT, the highest concentration used in the experiment. Similar IC\( _{50} \) values were found for SKOV-3 and FaDu cells (2.2 ± 1.5 \( \mu M \) and 2.1 ± 1.3 \( \mu M \), respectively).

3.4. Inhibition of telomerase by antisense hTR

Fig. 3 shows the effect of antisense hTR on the telomerase activity in SKOV-3 and FaDu cells. In both cells, inhibition of telomerase was observed in cells expressing antisense hTR (i.e. cells transfected with antisense hTR and induced by IPTG) treatment, whereas no changes were observed in cells expressing sense hTR or cells transfected with antisense hTR but without IPTG induction of antisense expression.

Inhibition of telomerase activity in SKOV-3 and FaDu cells by antisense hTR progressed with time and reached maximal levels at 15 weeks. The maximal inhibition in SKOV-3 cells was greater than in FaDu cells (93.3\% vs. 74.1\%). The residual telomerase activity in SKOV-3 after expressing the antisense hTR for 15 weeks was less than the background signal found in Saos-2 cells, indicating complete telomerase inhibition in SKOV-3 cells.

3.5. Effect of telomerase inhibition on telomere length

Fig. 4 shows the effect of telomerase inhibition on telomere length. Treatment with AZT for up to 9 weeks or with antisense hTR for up to 15 weeks had no effect on the telomere length in SKOV-3 cells nor Saos-2 cells. In contrast, the same treatments resulted in significant telomere erosion in FaDu cells (i.e. 27.3\% by antisense hTR and 18.4\% by 10 \( \mu M \) AZT in 7 weeks).

3.6. Characteristic features of ALT

Fig. 5 compares the morphology of Saos-2 and SKOV-3 cells. Note the presence of donut-shaped PML bodies in the interphase Saos-2 cells and the presence of heterogeneous strength of telomere signals in the M phase Saos-2 cells. The results indicate the variable telomere length. In contrast, both M phase and interphase SKOV-3 cells showed relatively homogeneous telomeric signals with no PML bodies in the nucleus.
In addition to Ku, RAD50, XRS2 and MRE11 are also involved in NHEJ in yeast, and mutations in these proteins lead to telomere shortening, even in the presence of functional telomerase [26]. It is not known whether NHEJ plays a role in the telomere maintenance in SKOV-3 cells.

In summary, results of the present study suggest the existence of a telomerase-independent mechanism for telomere maintenance in the telomerase-positive SKOV-3 cells.

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