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Research Paper

NAC selectively inhibit cancer telomerase activity: A higher redox homeostasis threshold exists in cancer cells

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ABSTRACT

Telomerase activity controls telomere length, and this plays an important role in stem cells, aging and tumors. Antioxidant was shown to protect telomerase activity in normal cells but inhibit that in cancer cells, but the underlying mechanism is elusive. Here we found that 7721 hepatoma cells held a higher redox homeostasis threshold than L02 normal liver cells which caused 7721 cells to have a higher demand for ROS; MnSOD over-expression in 7721 decreased endogenous reactive oxygen species (ROS) and inhibited telomerase activity; Akt phosphorylation inhibitor and NAC both inhibited 7721 telomerase activity. The over-elimination of ROS by NAC resulted in the inhibition of Akt pathway. Our results suggest that ROS is involved in the regulation of cancer telomerase activity through Akt pathway. The different intracellular redox homeostasis and antioxidant system in normal cells and tumor cells may be the cause of the opposite effect on telomerase activity in response to NAC treatment. Our results provide a theoretical base of using antioxidants selectively inhibit cancer telomerase activity. Findings of the present study may provide insights into novel approaches for cancer treatment.

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1. Introduction

Telomeres are nucleoprotein structures, located at the ends of chromosomes and are subject to shortening at each cycle of cell division, which forces human primary cells to stop dividing when a critical minimum telomere length is reached [1,2]. They are synthesized by telomerase consisting of a reverse transcriptase catalytic subunit (TERT) and an RNA template subunit (TERC) [3,4]. Telomerase activity is closely related to aging and tumorigesis. It is generally inhibited in normal cells but highly activated in tumor cells. The differential expression of the telomerase enzyme in normal and cancer cells have led to the evolution of tumor specific anti-telomerase approaches which inhibit the telomerase enzyme activity so as to destabilize and shorten the telomeres, leading to senescence in cancer cells.

In numerous studies on telomerase regulation mechanism, the relationship between oxidative stress and telomerase has successfully attracted worldwide attention. As reactive oxygen species (ROS) are particularly injurious toward the G-rich sequences of telomeres [5], ROS scavengers, also called antioxidants, have been used in protecting telomere and slowing the aging process of cells for many years [2,6,7]. However, in tumor cells, antioxidants exhibit quite the opposite effect. Many drugs which have been reported to possess antioxidant properties could inhibit tumor telomerase activity, promoting telomere shortening and provoking tumor cell apoptosis [8–12]. To the best of our knowledge, there is no research has explained the reason why antioxidants have different effects on telomerase activity of normal and tumor cells, and the present study is trying to explore the potential mechanism underlying this phenomenon.

Antioxidants such as NAC directly affect both ROS levels and the intracellular redox state (REDST). The intracellular oxidative damage/antioxidant defense is maintained at a relatively constant state relying on the combinatory effects of a variety of oxidizing substances, antioxidants and other related enzymatic systems. Redox disorders would cause redox balance to be shifted to the direction of oxidation or reduction, leading to excessive ROS production or elimination. Both excessive and insufficient amount of ROS will bring about bad effect on health, as ROS are no longer viewed just as a toxic by-product of mitochondrial respiration, but a double-edge sword and play an important role in numerous cellular processes. There is a growing body of evidence have indicated that ROS may be an essential element required for regulating a myriad of signaling pathways [13]. In consideration that ROS has both positive and negative effect, it is conceivable that the different intracellular redox state in different types of cell confer themselves different redox balance, and developed different sensitivity to oxidative stress or antioxidant interference, which could

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be the potential reason why telomerase activity of normal cells and cancer cells respond differently to anti-oxidative drugs.

In the present study we took normal liver cell line L02 and liver cancer cell line SMMC-7721 as research object, trying to uncover the reason why antioxidants inhibit cancer cells, and explore the role played by antioxidant system and Akt signaling pathway in regulating cancer cell's telomerase activity.

2. Materials and methods

2.1. Cell culture and hypoxia exposure

L02 and SMMC-7721 cell lines were obtained from Shanghai Institute of Cell Biology. Cells were grown in DMEM (Hyclone) supplemented with 2 mM L-glutamine and 10% FBS in a humidified incubator at 37 °C and 5% CO_2. Hypoxia exposures were done in a tri-gas tissue culture incubator (Binder) which could adjust the oxygen concentration to 5%, 2% and 0.5%.

2.2. MTT assay

1×10^4 cells were seeded in triplicate in a 96-well plate in a final volume of 100 μl and incubated for 4 h. Cells treated with DMSO alone were used as controls. Intervention buffer (100 μl) was then added and cultured the cells for the indicated times. At the end of the treatment, 10 μl MTT (5 mg/ml) was added to each well and incubated for an additional 4 h at 37 °C. DMSO (100 μl)/well were added after dropping the old medium with MTT. The absorbance was measured at 570 nm using a microplatereader (Biotek Synergy 4).

2.3. GSH assay

Cells were scraped into 50 μl 1 M HPO_3 and freeze/thaw for 2–3 cycles, the suspension was centrifuged with 12,000 rpm for 10 min at 4 °C and then assayed as previously described [14].

2.4. Telomerase activity assay

Extracts of cell lines were prepared as described previously [15] and the lysis buffer contained 10 mM Tris–HCl, pH 8.0; 1 mM $MgCl_2$; 1 mM EDTA; 1% (vol/vol) NP-40; 0.25 mM sodium deoxycholate; 10% (vol/vol) glycerol; 150 mM NaCl; 5 mM β-mercaptoethanol, PMSF. The extracts were quickly frozen in liquid nitrogen and stored at −80 °C. The extracts were centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatant was used for protein quantification and the subsequent testing. Telomerase assay was done according to the protocol previously reported [15] with some modification. 50 μl TRAP reaction mixture containing 2 μg cell protein extracts, 5 μL 10 × TRAP buffer, 1 μL dNTPs, 0.5 μL Phusion-DNA polymerase, 1 μL TS primer (all from Thermo Scientific). After 30 min incubation at 25 °C for TS elongation by telomerase, the reaction was stopped by heating at 90 °C for 3 min. Then 1 μl CX primer was added and subjected to 35 PCR cycles at 94 °C for 10 s, 50 °C for 30 s, and 72 °C for 1 min. Another 10 min at 72 °C was needed for extention. 50 μL of PCR product was loaded and run electrophoresis in Trisborate-EDTA on 8% polyacrylamide nondenaturing gel. The gel was stained in low concentration of ethidium bromide (0.5 μg/mL) for 15 min, and then photographed under UV light.

2.5. ROS detection

For measurement of intracellular ROS, cells were harvested and incubated with 10 μM DCFH-DA (2′-, 7′-dichlorofluorescein diacetate, Sigma) for 30 min at 37 °C. Cell suspension solution was centrifuged with 3000 rpm for 5 min, and washed twice with PBS. The fluorescence intensity was analyzed by FC 500 MCL system (Beckman coulter) immediately at excitation/emission wavelength of 488 nm/525 nm.

2.6. Plasmids and transfection

For overexpression of MnSOD to downregulate ROS levels, or inhibtion of MnSOD to increase ROS level, plasmids containing sense or antisense cDNA of human MnSOD were used. pHβ A-SOD (+) or pHβ A-SOD(−) plasmids (kindly provided by Professor Kunitaka Hirose) were transfected into SMMC-7721 cells and establish human SMMC-7721 hepatoma cell lines with stable expression of MnSOD or with suppressed expression of MnSOD using a standard method as described before [16].

2.7. Immunoblotting

Total cell extracts or nuclear extracts were separated by SDS-PAGE and transferred to PVDF membranes. The following antibodies were used for immunoblot analysis: rabbit phospho-Akt (Thr308), Akt antibody and the PI3K inhibitor LY294002 were from Calbiochem and secondary antibody was purchased from Proteintech. Anti-β-actin antibody was from Cell Signaling Technology.

2.8. Statistical analysis

The results are reported as means ± standard error. Statistical significance was determined using Student's *t*-test and ANOVA, with a value of $p < 0.05$ being considered significant.

3. Results

3.1. L02 and 7721 cells need different redox state to reach their respective maximum cell viability and telomerase activity

Using cell viability as the vertical axis, and using H_2O_2 and NAC concentration as the horizontal axis respectively, a bell-shaped line (as shown in Fig. 1A) could be obtained. Such curve can also be utilized to indicate the relationship between the cellular ROS concentration and cell viability. Interestingly, the top-points of the bell curve of L02 and 7721 appeared at different positions, which indicated their different sensitivity towards the exogenous ROS. L02 cell viability reached a maximum when the concentration of NAC was 5 mM, while the 7721 cell viability reached a maximum when H_2O_2 was 5 μM.

It has been well reported that telomerase activity in normal somatic cells was basically repressed, but are highly activated in embryonic stem cells or tumor cells [15]. Given the close relationship between telomerase activity and cell viability, we suspected that telomerase activity might also represent in the form of "bell-shaped" curve when extracellular redox state changes. As expected, telomerase activity in L02 cells decreased with increasing exogenous H_2O_2 concentration, but increased as NAC concentration was raised. However, 7721 telomerase activity, on the contrary, declined (Fig. 1B). 7721 telomerase activity reached its maximum value when H_2O_2 concentration was around 5 μM, but a further increment in H_2O_2 concentration led to an inevitable decline in its activity.

3.2. Moderate hypoxia-induced oxidative stress increases 7721 cells' telomerase activity

Hypoxia is widely found in solid tumors and is the main cause

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