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Inhibitory Effects of Selenium on Telomerase Activity and hTERT Expression in Cadmium-transformed 16HBE Cells[1]

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Objective To investigate the effects of sodium selenite on telomerase activity and expression of hTERT mRNA in cadmium-transformed 16HBE cells. **Methods** Telomerase activity and expression of genes were measured after cultured cadmium-transformed 16HBE cells were exposed to sodium selenite at different doses (0.625, 1.25, 2.50, 5.00 μmol/L) for 24 hours. **Results** Selenium decreased telomerase activity in cadmium-transformed 16HBE cells. There existed an obvious dose-effect relationship between the selenium concentration and these changes. The expression of hTERT and c-myc mRNA also decreased but the expression of mad1 mRNA increased after exposure to selenium for 24 hours. No difference was found in expression of hTRF1 and hTRF2 mRNA after incubated with sodium selenite for 24 hours, compared with control group. **Conclusion** Selenium inhibits telomerase activity by decreasing hTERT and c-myc mRNA expression and increasing mad1 mRNA expression in cadmium-transformed 16HBE cells and selenium concentration is significantly correlated with these changes.

Key words: Selenium; Cadmium; Telomerase; Human telomerase reverse transcriptase

INTRODUCTION

Cadmium (Cd) is a high toxic metal, with an estimated half-life in humans of 15-20 years[1], and is widely dispersed in the workplace and living environments. Exposure to cadmium occurs through intake of contaminated food or water, or after inhalation of tobacco smoke or polluted air. Because of its characteristics as a lung carcinogen, cadmium has been classified as a category 1 carcinogen (human carcinogen) by IARC[2], which is supported by a study which showed a significant association between risk of lung cancer and environmental exposure to cadmium[3].

Selenium is an essential trace element with several important biological functions and has received considerable attention as a naturally occurring effective anticarcinogenic agent[4]. Selenium supplementation in laboratory animals decreases tumorigenesis in several tumor models including those of skin, liver, colon, and pancreas[5-6]. The preventive effect of selenium compounds on tumors has also been shown in human cancers[7], but the mechanisms of its anti-tumor effect are still not fully understood.

Telomerase is a specialized RNA template-containing reverse transcriptase that allows replication of the telomeric repeats present at the end of all eukaryotic chromosomes. Being involved in cellular immortality, the telomerase activity is present in most types of human tumors and absent in noncancerous cells[8]. The human telomerase complex comprises several components, such as human telomerase RNA component (hTR) which is used as a template in DNA replication; human telomerase reverse transcriptase (hTERT), a

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human telomerase catalytic subunit; and human telomerase-associated protein 1 (hTEP1). In addition, although hTR contains the essential template region specifying the addition of telomerase sequence, studies have shown that the expression of hTR is not correlated with the level of telomerase activity[9]. Concomitant up-regulation or down-regulation of the hTERT mRNA expression and telomerase activity during cell immortalization or differentiation has been observed, suggesting that control of the hTERT expression at the mRNA level mainly contributes to the regulation of telomerase enzymatic activity[10-14]. Thus hTERT is the key component for the control of telomerase activity[15].

In this study, we investigated the inhibitory effect of sodium selenite on the growth inhibition and telomerase activity in cadmium-transformed human bronchial epithelial cells (cadmium-transformed 16HBE cells). Moreover, the effects of sodium selenite on the expression of hTERT, c-myc, and mad1 mRNA were tested.

MATERIALS AND METHODS

General Chemicals

Sodium selenite (Na_2SeO_3) was purchased from Tianjin Chemical Reagent Corporation (Tianjin, China). Bovine serum, agarose, and DMEM cell culture powder were obtained from GIBCO BRL (Paisley, Scotland). Ethidium bromide, trypsin, and dimethylsulfoxide (DMSO) were purchased from Sigma (Deisenhofen, Germany).

Cell Culture and Treatment

Cadmium-transformed 16HBE cells[16] (kindly provided by Professor Yi-Xiong LEI, Guangzhou Medical College) were maintained in DMEM (high glucose) supplemented with 10% bovine serum, penicillin (100 unit/mL), and streptomycin (100 μg/mL). Cells grown at 80%-90% confluence were trypsinized and replated at a 1:3 split ratio for a passage. Immediately prior to experimental treatment, medium was replaced. Exponentially growing cells were divided into a control group treated with PBS and 4 treatment groups treated with 0.625, 1.25, 2.50, 5.00 μmol/L sodium selenite respectively and incubated for 24 hours.

MTT Cell Viability Assay

Cadmium-transformed 16HBE cells were plated at a density of 5×10^4 cells/well in a 96-well plate to determine cytotoxic concentrations of sodium selenite. Cells were exposed to selenium at 37℃ under 5% CO_2. After the cells were incubated in the presence of selenium for 24 hours, viable cells were stained with MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, Sigma] (0.5 mg/mL) for 4 h. The media were then removed and produced formazan crystals in the wells were dissolved by addition of 200 mL of DMSO. Absorbance was measured at 490 nm using a microplate reader (Bio-Rad, USA). Cell viability was defined relative to the untreated control cells[17].

Telomeric Repeat Amplification Protocol Assay

The telomerase activity was examined by the telomeric repeat amplification protocol (TRAP), using a TRAPeze® XL telomerase detection kit (Chemicon International). Briefly, cells were harvested by trypsinization, resuspended in CHAPS lysis buffer and incubated on ice for 30 min. Cells (1.0×10^5) were used for each telomerase assay. After centrifugation at 12 000 g for 30 min at 4℃, the supernatants were used as the cell extracts. TRAP assay was performed with 1.0 μg of cell extracts. The assay specificity for active telomerase was determined by inclusion of heat-inactivated samples, in which the extracts were heat-inactivated at 85℃ for 10 min before TRAP assay. The assays were repeated two to four times, and the activity was semi-quantified by fluorescence measurements, as recommended by the manufacturer. Their mean value was taken as the telomerase activity of the samples.

Isolation of RNA and Reverse Transcription-polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was isolated from cells using Trizol (GIBCO, Scotland), according to the manufacturer's recommendations. First strand cDNA synthesis with oligo (dt) primers was performed using M-MLV (reverse transcriptase). The primer sequences and PCR product sizes are shown in Table 1. The PCR products were subjected to agarose gel electrophoresis and visualized by ethidium bromide staining. We confirmed the quality of cell mRNA according to the intensity of β-actin.

Statistical Analysis

Data were expressed as $\bar{x}\pm s$. Statistical analysis was performed with ANOVA (SPSS software). $P<0.05$ was considered statistically significant. All experiment assays were performed in triplicate and repeated 3 times.

RESULTS

Selenium Inhibited the Growth of Cadmium-transformed 16HBE Cells

Cadmium-transformed 16HBE cells were cultured

TABLE 1

RT-PCR Primer Sequence

Target cDNA	Primer Sequence	Product Size (bp)
hTERT	F 5'-TGAACTTGCGGAAGACAGTGG-3'	361
	R 5'-ATGCGTGAAACCTGAACGCCT-3'	
c-myc	F 5'-TAATTCCAGCGAGAGGCAGA-3'	290
	R 5'-GTCCCCAAATGGGCAGAATA-3'	
Mad1	F 5'-GAGATGCCTTAAAACGGAGG-3'	592
	R 5'-AACCAACAGGGAGAACCTTC-3'	
TRF1	F 5'-TTTAGTAGAGGCGGGGTTTC-3'	464
	R 5'-ACTGTGTGATGTTGAGGTTTGG-3'	
TRF2	F 5'-AGTCAATCGCTGGGTGCTCA-3'	636
	R 5'-CCTGGTGCTGGCTGTTTATC-3'	
β-actin	F 5'-GGGTCAGAAGGATTCCTATG-3'	237
	R 5'-GGTCTCAAACATGATCTGGG-3'	

with sodium selenite at different concentrations for 24 h. Selenium showed inhibitory effects on the growth of the cells in a concentration-dependent manner (Fig. 1). A marked decrease was observed after treatment with 1.25, 2.50, and 5.00 μmol/L sodium selenite respectively and the difference was statistically significant, compared with control group (P<0.01).

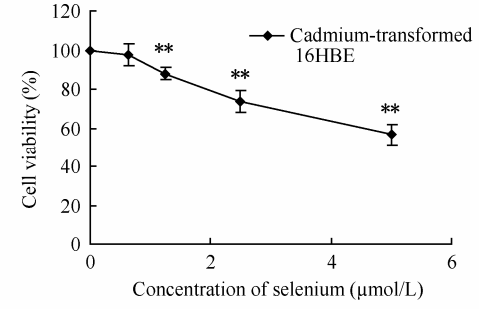

FIG. 1. Effects of selenium on growth of cadmium-transformed 16HBE cells. Cells were incubated with PBS or sodium selenite at different concentrations for 24 h and the effect of selenium on cell growth was detected by MTT assay. The percentage of viability was calculated by defining the absorption of control group as 100%. Bar represents the $\overline{x} \pm s$ from three independent experiments. **P<0.01 *vs* control group.

Effects of Sodium Selenite on Telomerase Activity

To determine whether the inhibitory effects of sodium selenite on the growth of cadmium-transformed 16HBE cells are associated with inhibition of telomerase activity, we then compared the effects of selenium on the activity of human telomerase in the cells. We performed TRAP assay using partially purified telomerase from cells cultured in medium with sodium selenite at different concentrations for 24 h. Selenium inhibited human telomerase activity in a dose-dependent manner (Fig. 2). The telomerase activity was decreased in all groups treated with sodium selenite, compared with control group (P<0.01).

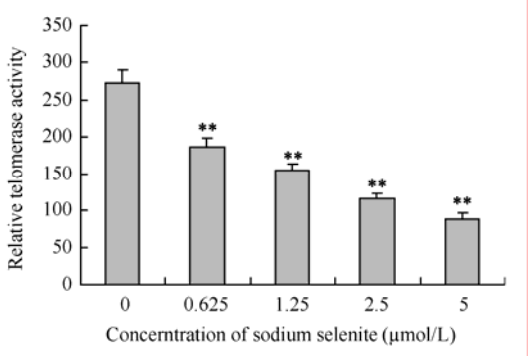

FIG. 2. Effects of selenium on telomerase activity in cadmium-transformed 16HBE cells. Cells were incubated with PBS or sodium selenite at different concentrations for 24 h. Bar represents the $\overline{x} \pm s$ from three independent experiments. **P<0.01 *vs* control group.

Effects of Sodium Selenite on hTERT mRNA Level

To verify the mechanism of the inhibitory effects of selenium on telomerase activity, we investigated whether this agent could modulate the expression of telomerase-component genes in cadmium-transformed 16HBE cells. We focused on the effects of selenium on hTERT mRNA levels. We used RT-PCR to examine the expression of hTERT and found that hTERT mRNA levels were decreased in all groups treated with sodium selenite (Fig. 3) in a concentration-dependent manner and the difference was statistically significant, compared with control group (Table 2).

TABLE 2

Effects of Selenium on Expressions of hTERT, c-myc, mad1, hTRF1, and hTRF2 mRNA in Cadmium-transformed 16HBE Cells ($\bar{x} \pm s$)

Group	hTERT/actin	c-myc/actin	mad1/actin	hTRF1/actin	hTRF2/actin
Control Group	0.709±0.022	0.829±0.030	0.653±0.085	0.470±0.059	0.652±0.078
0.625 μmol/L Se	0.547±0.028**	0.710±0.116	0.763±0.074	0.486±0.055	0.655±0.008
1.25 μmol/L Se	0.466±0.019**	0.601±0.171*	0.820±0.106*	0.487±0.095	0.653±0.030
2.50 μmol/L Se	0.355±0.013**	0.525±0.051**	0.900±0.030**	0.497±0.081	0.680±0.024
5.00 μmol/L Se	0.301±0.017**	0.439±0.130**	0.905±0.056**	0.525±0.063	0.707±0.017

Note. *P<0.05, **P<0.01 *vs* the control group.

Effects of Sodium Selenite on c-myc and mad1 mRNA Level

The expression of c-myc mRNA after incubation with 1.25, 2.50, and 5.00 μmol/L selenium for 24 hours was decreased, in a concentration-dependent manner compared with the control group (Fig. 3, Table 2). Selenium treatment increased the expression of mad1 mRNA in cadmium-transformed 16HBE cells (Fig. 3) and the difference was statistically significant, compared with control group (P<0.05 or P<0.01).

Effects of Selenium Selenite on TRF1 and TRF2 mRNA Level

TRF1 mRNA was observed in selenium groups (Fig. 3), and the expression of TRF1 did not differ from control group (P>0.05). A similar expression of TRF2 mRNA was seen in all groups treated with sodium selenite.

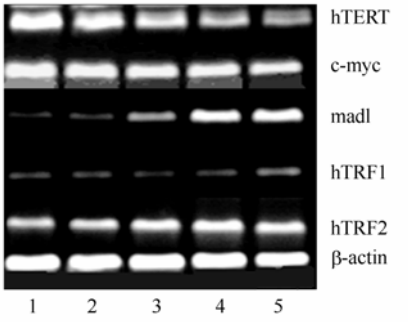

FIG. 3. Expression of hTERT, c-myc, mad1, hTRF1, hTRF2, and β-actin mRNA in cadmium-transformed 16HBE cells after exposure to sodium selenite. RNA samples were isolated and analyzed by RT-PCR. Lane 1: control group and lanes 2-5: cells incubated with 0.625, 1.25, 2.5, and 5.0 μmol/L sodium senenite respectively.

DISCUSSIONS

Telomerase activity is present in most types of human tumors and absent in noncancerous cells[8,17], suggesting that modification of telomerase activity may be a potential therapy for human cancers. In this study, after cadmium-transformed 16HBE cells were exposed to sodium selenite at a micromolar concentration, the telomerase activity was decreased significantly. The nutritional significance and regulatory effect of Se on abnormal metabolism in liver cancer can be useful in designing a new approach to the prevention of cancer or its treatment[18]. Selenium compounds also show preventive effect on other human tumors[6-7]. Selenium may exert its anti-tumor by inhibiting telomerase activity.

Moreover, many studies have suggested that telomerase catalytic subunit gene, hTERT, mainly regulates the expression of human telomerase enzymatic activity. The present results clearly demonstrate that decreased telomerase activity of cadmium-transformed 16HBE cells caused by sodium selenite is accompanied with an inhibition of the hTERT mRNA expression, suggesting that control of the hTERT expression at the mRNA level mainly contributes to the regulation of telomerase enzymatic activity[10-14]. Therefore, investigation of the molecular mechanisms underlying the hTERT gene expression is essential for understanding the molecular basis of telomerase activation and carcinogenesis, and the development of telomerase inhibitors.

c-Myc activates telomerase by inducing expression of hTERT and the hTERT minimal promoter contains two c-Myc binding sites (E-boxes) that mediate hTERT transcriptional activation[20-21]. c-Myc binding leads to activation of the hTERT gene in cells undergoing early neoplastic transformation. This model therefore provides a novel system for analyzing the mechanisms of hTERT induction during early stages of tumorigenesis[22]. The fact that c-Myc binds *in vitro* to the hTERT promoter suggests that the up-regulated c-Myc expression, commonly observed in a wide range of human tumors, is most

likely responsible for the hTERT activation occurring in these cells[23]. Therefore, targeting c-Myc regulatory pathways may inhibit telomerase activity and cell proliferation in human malignancies[22]. Mad1 has been shown to bind to the hTERT promoter at the same sites as c-Myc, thus negatively regulating its transcription. Mad1-mediated repression of the hTERT promoter could be counter-acted by ectopic expression of c-Myc[24]. c-Myc or Mad 1 associates with the ubiquitously produced Max protein to form a heterodimer capable of activating or expressing genes[25]. Rama *et al*.[26] suggested that cells, such as undifferentiated BeWo cells with a high replicative potential (and telomerase activity), are characterized by high levels of c-Myc, while Mad 1 expression is generally minimal in these cells. In this study, the expression of c-myc gene was inhibited and the expression of mad1 gene was increased after treatment with sodium selenite for 24 hours, indicating that decreased expression of hTERT may be due to c-myc reduction and mad1 augmentation, while down-regulation of hTERT expression is largely due to c-Myc reduction[13].

Telomere-specific DNA-binding proteins, such as TRF1 and TRF2, may play a role in modifying telomerase activity and maintaining telomere function[27]. The expression of TRF1 and TRF2 has been examined in a limited number of tumors and cell culture experiments. Over-expression of wild-type and dominant-negative TRF1 can progressively shorten telomere and elongate in human cells[28]. TRF2 is a nuclear protein that coats the length of all human telomeres at all stages of the cell cycle. Similar to the phenotype observed with TRF1, over-expression of TRF2 also results in the progressive telomere shortening. However, there have been few studies of the effect on TRF1 and TRF2 genes after incubated with selenium for 24 hours. To study TRF1 and TRF2 genes in cadmium-transformed 16HBE cells exposed to selenium, we detected the expression of TRF1 and TRF2 mRNA, the results showed that the expression of TRF1 and TRF2 genes was not effected by selenium.

In conclusion, selenium inhibits the growth of cadmium-transformed 16HBE cell line, and telomerase activity, which is associated with, at least in part, down-regulation of hTERT. Therefore, this compound can be used as a leading molecule in further experiments on the molecular mechanism of inhibition of telomerase.

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