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Down-regulation of four arsenic antagonists on apoptosis and telomerase activity induced by arsenic trioxide in three myelocytic leukemia cell lines[1]

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KEY WORDS arsenic trioxide; telomerase; apoptosis; leukemia; down-regulation (physiology); K562 cells; HL-60 cells

ABSTRACT

AIM: To investigate regulative effects of thiol reagents, *N*-acetyl-*l*-cysteine (NAC) and natrii dimercaptosussinas (NDMS), catalase (CAT), and calcium chelator 2-[(2-bis-[carboxymethyl]-amino-5-methyl-phenoxy)-methyl]-6-methoxy-8-bis-[carboxy-methyl]-aminoquinoline (Quin 2) on apoptosis and telomerase activity induced by arsenic trioxide (As_2O_3) in three myelocytic leukemia cell lines. **METHODS**: Flow cytometry was used to examine apoptosis and a PCR ELISA kit was used to detect telomerase activity. **RESULTS**: As_2O_3 induced about 40 % - 60 % of apoptosis in NB4, K562, and HL-60 cells at the concentration of 0.6, 2.7, and 8.1 μmol/L respectively, as well as down-regulated telomerase activities in three cell lines. NAC 4 mmol/L, NDMS 200 μmol/L, CAT 80 kU/L, and Quin 2 20 μmol/L could down-regulate apoptosis variously induced by As_2O_3. NAC and CAT alone could decline telomerase activity in three cell lines and further decline telomerase activities that had been decreased by As_2O_3, whereas Quin 2 antagonized the decline in K562 and HL-60 cells. **CONCLUSION**: Thiol activity loss, free radical alteration, intracellular calcium changes, and decline of telomerase activity might be involved in As_2O_3-induced apoptosis. NAC, NDMS, CAT, and Quin 2 antagonized in some extent the effect of As_2O_3 on the three tested cell lines.

INTRODUCTION

Telomerase was first identified in the ciliated protozoan *Tetrahymena thermophila*[1] and then detected in human germ cells, most malignant tumor cells, and a few somatic cells such as lymphocytes, but not in the majority of somatic cells[2,3]. It plays an important role in the control of cellular proliferation capacity and senescence by utilizing its own RNA component as a template for the synthesis of multiples of telomeric repeats onto the end of replicating chromosomes. Telomerase activity has been proved to be associated with immortallization of cells and cancer, it is expected as a target for cancer treatment. Several chemotherapy drugs have been reported that they could inhibit telomerase activity[4-6].

Arsenic has been shown a specific treatment effect on acute promyelocytic leukemia (APL) in clinic without bone marrow depression or other severe side effects, its complete remission (CR) were as high as 66 % - 84 % including retina acid-resistant APL cases[7,8]. A more detail study reported that arsenic trioxide triggered apoptosis in APL NB4 cell line in a dose-dependent way via down-regulating Bcl-2 and degradating PML-RARα chimerical protein at a high concentration (1 μmol/L), and partial differentiating at a lower concentration (0.1 μmol/L)[9]. Although arsenic agent may react with cellular thiols to exert its toxicity, the pathway from thiol reaction to genetic damage is elusive. Glutathione peroxidase, catalase, and calcium have been proved to play important roles in arsenite-induced micronuclei in CHO-K1 cells[10-12]. How do these factors work in human myelocytic cells during apoptosis induced by arsenic trioxide and how do they affect on telomerase activity in this process? The aim of this paper is to investigate the regulative effects of thiol reagents *N*-acetyl-*l*-cysteine (NAC)

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and natrii dimercaptosussinas (NDMS), catalase (CAT), and calcium chelator 2-[(2-bis-[carboxymethyl]-amino-5-methyl-phenoxy)-methyl]-6-methoxy-8-bis-[carboxymethyl]-aminoquinoline (Quin 2) on apoptosis as well as telomerase activity induced by As_2O_3 in three myelocytic leukemia cell lines, NB4, K562, and HL-60.

MATERIALS AND METHODS

Cell culture and chemicals Human leukemia cell lines NB4 (APL), K562 (chronic myelocytic leukemia, CML), and HL-60 (acute myelocytic leukemia, AML) were cultured in fresh RPMI 1640 medium (GIBCO-BRL), supplemented with 10 % heat-inactivated fetal bovine serum, benzylpenicillin 100 kU/L, and streptomycin 100 kU/L, and with or without different concentrations of As_2O_3 solutions, at 37 ℃ in a 5 % CO_2 humidified atmosphere. To avoid possible effects of cell density on cell growth and survival, cells were adjusted not more than 5×10^8/L by addition of fresh culture medium containing the corresponding concentration of As_2O_3. As_2O_3 (Shuikoushan Second Reagent Factory, Hunan, China) was dissolved in physiological saline as stock solution of 0.5 mmol/L and diluted to working concentration before use. NDMS was purchased from Shanghai Pharmacy Company. Propidium iodide (PI), RNase, catalase(CAT, 11 kU/mg), NAC, and Quin 2 were purchased from Sigma.

Assessment of apoptosis by flow cytometry Cells were seeded at a density of 1×10^8/L with As_2O_3 0.1 – 8.1 μmol/L and NAC 4 mmol/L, NDMS 200 μmol/L, CAT 80 kU/L, or Quin 2 20 μmol/L respectively for 72 h in the above incubation conditions. About 1×10^6 cells were harvested and washed with ice cold PBS (NaCl 150, K_2HPO_4 0.61, NaH_2PO_4 0.20 mmol/L) then injected into cold ethanol (−20 ℃, 70 %), which was kept overnight at 4 ℃. Cells were rinsed with PBS and stained with PI 50 mg/L in PBS solution containing 0.1 % Triton-X100, egzatic acid 0.1 mmol/L, and RNase 100 kU/L, incubated in the dark at room temperature for 30 min, then PI was washed with PBS, 10 000 cells were determined by flow cytometer (Facscalibure, Becton-Dickison, San Jose, CA) within 1 h. All data were collected, stored, and analyzed by Cellquest version 1.2.2 software (B-D).

Telomerase detection Telomerase PCR ELISA Kit (Boehringer Mannheim) is an extension of original method described by Kim *et al*[2]. It allows highly specific amplification of telomerase-mediated elongation products combined with non-radioactive detection following an ELISA protocol. Cells of 2×10^6 were harvested and centrifuged twice at 1500 × *g* for 10 min in PBS. The PBS, water, and tips should be pretreated with diethyl pyrocarbonate (DEPC) in order to prevent RNase from destroying telomerase activity. The cell extracts were prepared by resuspending cells in 200 μL of lysis reagent and incubated on ice for 30 min, then centrifuged at 22 000 × *g* for 20 min at 4 ℃. For PCR amplification, 2 μL of extract (equal to 20 000 cells) and 25 μL of reaction mixture were transferred into a suitable tube, then added sterile water to a final volume of 50 μL. An elongation/amplification reaction was performed by the following protocol: mixture was kept at 25 ℃ for 30 min, 94 ℃ – 30 s, 50 ℃ – 30 s, 72 ℃ – 90 s (30 cycles), and at 72 ℃ for 10 min. Amplification product (equal to 2000 cells) 5 μL was mixed with 20 μL of denaturation reagent and incubated at room temperature for 10 min. Hybridization buffer 225 μL was added and mixed thoroughly, then 100 μL of mixture (equal to 800 cells) per well was transferred into the precoated MTP modules and incubated at 37 ℃ on a shaker for 2 h. Anti-DIC-POD working solution 100 μL was added and incubated at room temperature (18 – 22 ℃) for 30 min with shaking. The solution was removed completely and the precipitate was rinsed 5 times with 250 μL of washing buffer per well for a minimum of 30 s. After removing the washing buffer, 100 μL of TMB substrate solution was added and incubated for color development at room temperature for 20 min with a slight shaking. Finally, 100 μL of stop reagent per well was added to stop color development. The absorbance of the samples at 450 nm and 690 nm (a reference wavelength) within 30 min was measured. The result was reported as $\Delta A = A_{450\ nm} - A_{690\ nm}$ (blank). Negative control should be less than $0.25\Delta A$ units. Positive control should be high than $1.5\Delta A$ units. Sample was regarded as telomerase-positive if the difference in absorbance (ΔA) is higher than $0.2\Delta A$ units.

Statistics Means from different groups were compared by *t*-test and expressed as $\bar{x} \pm s$.

RESULTS

Apoptosis induced by different As_2O_3 concentrations The apoptosis induced by As_2O_3 in three cell lines was determined by flow cytometer after incubation for 72 h, it showed a concentration-dependent (0.1 – 0.9

μmol/L) apoptosis in NB4 cells. The apoptotic rate was 17.0 % - 73.8 % compared with that of control (13.1 %). But in K562 cells, an obvious apoptosis (58.2 %) was found at 2.7 μmol/L of As_2O_3, while in HL-60, apoptosis (62.3 %) was found at the concentration as high as 8.1 μmol/L of As_2O_3. It showed that the concentrations of As_2O_3 that could induce apoptosis in three cell lines were very different, even 10 times higher in HL-60 than that in NB4 cells (Tab 1).

Tab 1. Flow cytometric analysis of apoptosis induced by different As_2O_3 concentrations after 72 h incubation in NB4, K562, and HL-60 cells. $n = 3$ wells. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control group.

As_2O_3/ μmol·L^{-1}	Apoptosis/% NB4	K562	HL-60
0	13.1±2.9	10.9±2.1	11.2±2.9
0.1	16.9±2.5	12.7±1.8	10.4±1.3
0.3	26.2±4.1^b	15.2±2.5	10.1±1.5
0.6	44.5±4.5^c	-	-
0.9	73.8±7.9^c	16.3±2.9	12.0±1.4
2.7	-	58.2±5.8^c	18.2±3.9
8.1	-	-	62.3±8.1^c

Down-regulation of NAC, CAT, NDMS, and Quin 2 on apoptosis induced by As_2O_3 Cells were cultured in RPMI 1640 medium described above with NAC 4 mmol/L, NDMS 200 μmol/L, CAT 80 kU/L, Quin 2 20 μmol/L, respectively, added with or without As_2O_3 0.6 μmol/L in NB4 cells; As_2O_3 2.7 μmol/L in K562 cells; As_2O_3 8.1 μmol/L in HL-60 cells. The protective effects of four antagonists on As_2O_3-induced apoptosis were shown in Tab 2. NAC, CAT, NDMS, or Quin 2 alone did not induce apoptosis in three tested cell lines. The apoptosis in NB4 cells decreased from 47.5 % (at As_2O_3 0.6 μmol/L) to 44.2 % by NAC, to 20.4 % by NDMS, to 18.8 % by CAT, to 34.0 % by Quin 2; In K562 cells, apoptosis decreased from 54.2 % (at As_2O_3 2.7 μmol/L) to 44.1 %, 13.4 %, 17.6 %, and 33.9 % respectively; In HL-60 cells, the apoptosis decreased from 55.8 % (at As_2O_3 8.1 μmol/L) to 40.9 %, 21.0 %, 39.0 %, and 37.1 % respectively. NDMS, which was generally used to prevent from toxicity caused by heavy metals in clinical, showed the strongest protective effect among the four arsenic antagonists at the above concentration on As_2O_3-induced apoptosis in three cell lines.

Inhibition of As_2O_3 on telomerase Telomerase activity decreased variously in three cell lines following induction of apoptosis by As_2O_3. The TRAP assay (the final cell number is 800 per well) was used to determine the telomerase activities in three leukemia cell lines. After 72 h incubation with different concentrations of As_2O_3, NB4, K562, and HL-60 cells telomerase activities decreased with the increasing of concentration of As_2O_3. The sensitivity of telomerase to As_2O_3 was various in three leukemia cell lines. At 0.6 μmol/L of As_2O_3, telomerase activity in NB4 cells was inhibited to about a half of the control, whereas that in K562 and HL-60 cells, it happened at about 2.7 μmol/L of As_2O_3 (Tab 3). The direct effect of As_2O_3 on telomerase activity was examined in extract with adding different concentrations of As_2O_3, the results exhibited no changes compared with their courtpartners (data not shown).

Tab 2. The regulative effects of NAC, NDMS, CAT, and Quin 2 on apoptosis induced by As_2O_3 in three myelocytic leukemia cell lines, NB4, K562, and HL-60. $n = 3$ wells. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* As_2O_3 group.

Groups	Apoptosis/% NB4	K562	HL-60
Control	13.9±2.9	7.9±2.1	9.8±2.1
As_2O_3 (0.6 μmol/L)	47.5±4.9	-	-
(2.7 μmol/L)	-	54.2±7.2	-
(8.1 μmol/L)	-	-	55.8±4.1
NAC (4 mmol/L)	16.3±3.1	9.7±2.5	8.8±2.1
NAC (4 mmol/L) + As_2O_3	44.2±5.6	44.1±4.9	40.9±3.7^b
NDMS (200 μmol/L)	16.4±3.5	6.3±0.5	9.1±0.9
NDMS (200 μmol/L) + As_2O_3	20.4±3.6^c	13.4±2.4^c	21.0±2.8^c
CAT (80 kU/L)	12.0±4.3	11.5±1.1	8.8±1.3
CAT (80 kU/L) + As_2O_3	18.8±3.1^c	17.6±3.2^c	39.0±4.9^b
Quin 2 (20 μmol/L)	8.2±1.9	8.0±2.1	3.6±1.5
Quin 2 (20 μmol/L) + As_2O_3	34.0±4.1^b	33.9±4.3^b	37.1±4.0^b

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Tab 3. Inhibition of different As_2O_3 concentrations on telomerase activities after 72 h incubation in NB4, K562, and HL-60 cell lines. $\Delta A = A_{450\,nm} - A_{690\,nm}$ (blank). n = 3 wells. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control group.

As_2O_3/ $\mu mol \cdot L^{-1}$	Telomerase/ΔA NB4	K562	HL-60
0	1.61 ± 0.10	1.63 ± 0.11	1.63 ± 0.07
0.1	1.24 ± 0.11	1.47 ± 0.07	1.41 ± 0.11
0.3	0.92 ± 0.09^b	1.40 ± 2.5	1.42 ± 0.05
0.6	0.79 ± 0.12^b	–	–
0.9	0.28 ± 0.07^c	0.91 ± 0.08^b	1.01 ± 0.09^b
2.7	–	0.73 ± 0.19^c	0.40 ± 0.15^c
8.1	–	–	0.15 ± 0.10^c

Regulative effects of NAC, CAT, NDMS, and Quin 2 on telomerase activity during As_2O_3-induced apoptosis Telomerase activities were assayed with the same method at the culture condition described above. The results indicated that NAC and CAT alone sharply decreased telomerase activity of three leukemia cells, therefore they declined the telomerase activities further which had been decreased by As_2O_3. On the other hand, NDMS and Quin 2 alone had little effects on telomerase activity. Quin 2 showed a significant protective effect on As_2O_3-declined telomerase activity in K562 and HL-60 cell lines (Tab 4).

DISCUSSION

In 1970s, the drug Ai-lin 1 (mainly composed of As_2O_3) was first applied to treat APL by the First Hospital Affiliated to Harbin Medical University (Harbin, China). It was reported to induce a higher APL clinical CR even if patients were all-trans retinoid acid(ATRA) resistant and showed no significant bone marrow suppression and other severe side effects[7,8]. Chen *et al*[9] demonstrated that arsenic induced down-regulation of Bcl-2 and degradation of chimerical protein, PML-RARα, in APL cell line NB4, but not in another AML cell line HL-60 and mononuclear cell line U937, at the concentration range of 0.5 – 2.0 μmol/L As_2O_3. In the present study, As_2O_3 triggered apoptosis in human chronic leukemia cell line K562, acute myelocytic leukemia cell line HL-60 (non-sensitive to As_2O_3), and acute promyelocytic leukemia cell line NB4 (typi 'cal sensitive to As_2O_3). Lu *et al*[13] reported that As_2O_3 could also induce apoptosis in megakaryocytic leukemia cell lines, such as HEL, Meg-01, UT-7, and Mo7e, via a dose- and time-dependent manner. Some evidence from animal cells indicated that reactive oxygen species and calcium were involved in the arsenic metabolism[11,12,14] and caused apoptosis. So we observed protective effect of two thiol protectors (NAC and NDMS), an antioxidant enzyme (CAT), and a calcium chelator (Quin 2) on apoptosis among three leukemia cell lines after treatment with different concentrations of As_2O_3. The results showed four arsenic antagonists exhibited different protective effects on apoptosis induced by As_2O_3. More recently, a number of chemotherapeutic agents have been reported to down-regulate the telomerase activity in cultured cells[15-17]. In this work, As_2O_3 declined the telomerase activities both in APL cell line (NB4), CML cell line (K562), and AML cell line (HL-60) at

Tab 4. Regulative effects of NAC, NDMS, CAT, Quin 2 on As_2O_3-declined telomerase activities after 72-h incubation in three myelocytic leukemia cell lines, NB4, K562, HL-60. $\Delta A = A_{450\,nm} - A_{690\,nm}$ (blank). n = 3 wells. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control group. $^eP < 0.05$ *vs* As_2O_3 group.

Groups	Telomerase/ΔA NB4	K562	HL-60
Control	1.60 ± 0.10	1.63 ± 0.11	1.63 ± 0.11
As_2O_3 (0.6 μmol/L)	0.81 ± 0.12	–	–
(2.7 μmol/L)	–	0.73 ± 0.19	–
(8.1 μmol/L)	–	–	0.15 ± 0.07
NAC (4 mmol/L)	0.92 ± 0.12^b	1.12 ± 0.12^b	0.53 ± 0.10^b
NAC (4 mmol/L) + As_2O_3	0.48 ± 0.05	0.12 ± 0.04^e	0.14 ± 0.04
NDMS (200 μmol/L)	1.47 ± 0.14	1.41 ± 0.13	1.30 ± 0.09
NDMS (200 μmol/L) + As_2O_3	0.85 ± 0.06	0.79 ± 0.06	0.24 ± 0.10
CAT (80 kU/L)	0.10 ± 0.07^c	0.11 ± 0.05^c	0.14 ± 0.05^c
CAT (80 kU/L) + As_2O_3	0.12 ± 0.09^e	0.21 ± 0.07^e	0.02 ± 0.03
Quin 2 (20 μmol/L)	1.61 ± 0.11	1.53 ± 0.15	1.28 ± 0.17
Quin 2 (20 μmol/L) + As_2O_3	1.02 ± 0.05	1.21 ± 0.11^e	0.40 ± 0.08^e

different efficacy concentrations. Based on this work and other studies[4-6], it appeared that decline of telomerase activity in tumor cells was a general event during chemotherapy. Four arsenic antagonists' regulative effects on telomerase indicated NAC and CAT alone decreased three leukemia cell telomerase activity and had no protective effect on it, whereas Quin 2 showed a quite well protective effect on As_2O_3-declined telomerase.

Taken together, our results showed that As_2O_3 could induce apoptosis and decline telomerase activity in three myelocytic leukemia cell lines (NB4, K562, and HL-60). Apoptosis and telomerase induced by As_2O_3 were regulated or partially regulated by thiol protectors (NAC, NDMS), antioxidant enzyme (CAT), and calcium chelator (Quin 2). It suggested apoptosis induced by arsenic trioxide might be involved in thiol activity loss, free radical alteration, intracellular calcium changes, as well as decline of telomerase activity.

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四种砷拮抗剂对三氧化二砷诱导的三种粒系白血病细胞凋亡和端粒酶活性的减量调节作用[1]

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关键词 三氧化二砷；端粒酶；细胞凋亡；白血病；减量调节(生理学)；K562 细胞；HL-60 细胞

目的：研究巯基供体 *N*-乙酰半胱氨酸(NAC)和二巯丁二钠(NDMS)、抗氧化剂过氧化氢酶(CAT)和 Ca^{2+} 清除剂(Quin 2)对三氧化二砷诱导的三种粒系白血病细胞凋亡和端粒酶活性改变的调控作用。**方法**：用流式细胞仪和 PCR ELISA 法分别检测 NAC、NDMS、CAT 或 Quin 2 与三氧化二砷共同作用于三种粒系白血病细胞后其凋亡和端粒酶活性的变

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化. **结果**：三氧化二砷 0.6、2.7 和 8.1 μmol/L 可分别诱导急性早幼粒细胞白血病细胞株 NB4，慢性粒细胞白血病细胞株 K562，急性粒细胞白血病细胞株HL-60细胞发生 40 % – 60 % 的凋亡，同时下调三种细胞的端粒酶活性. NAC 4 mmol/L，NDMS 200 μmol/L，CAT 80 kU/L，Quin 2 20 μmol/L 不同程度抑制这种凋亡作用. NAC 和 CAT 既可独立降低三种细胞的端粒酶活性，也可促进三氧化二砷对端粒酶的下调作用，而 Quin 2 可抑制 K562 和 HL-60 细胞中的这种下调作用. **结论**：三氧化二砷诱导的三种细胞的凋亡过程涉及了疏基失活、自由基的改变、细胞内 Ca^{2+} 浓度改变及端粒酶活性下降. NAC、NDMS、CAT 及 Quin 2 可不同程度拮抗三氧化二砷对三种细胞的作用.

（责任编辑　吴民淑）

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