— Research Letter —

Exogenous S-adenosyl-L-methionine Could Inhibit c-*myc* Overexpression Induced by As_2O_3 in Normal Human Liver HL-7702 Cells

Yongmei Qi, Ailing Li, Dejun Huang, Yihong Tian, and Yingmei Zhang*

School of Life Sciences, Lanzhou University, 222 South Tianshui Rd, Lanzhou 730000, China

(Received December 1, 2010; Accepted January 25, 2011)

Arsenic trioxide (As_2O_3) is known to promote liver and other cancers due to its ability to decrease the level of S-adenosyl-L-methionine (SAM), thus leading to the abnormal expression of oncogene c-*myc*. The present study investigated the effect of exogenous SAM on As_2O_3-induced c-*myc* expression in the normal human liver cells, HL-7702. c-*myc* expression increased approximately 50% after As_2O_3 treatments (0–20 μM) for 24 hr. However, this elevated c-*myc* expression is significantly inhibited by 0.2 μM SAM. Co-treatment of HL-7702 cells with 1.0 μM As_2O_3 and 0.2 μM SAM for 24 and 30 hr, caused prominent inhibitory effects on c-*myc* overexpression. The results indicate SAM could inhibit c-*myc* overexpression induced by As_2O_3 in HL-7702 cells. These findings may be of significance to the exploration of SAM in preventing arsenic carcinogenesis.

Key words —— arsenic trioxide, S-adenosyl-L-methionine, c-*myc*, reverse transcription-polymerase chain reaction

INTRODUCTION

Arsenic is a well-documented carcinogen, although As_2O_3 has been acknowledged as a valuable therapeutic agent for the treatment of leukemia.[1,2] As_2O_3 is known to induce deletion mutations and chromosomal alterations. Despite its low mutagenic activity, As_2O_3 has high transformation activity.[3] Studies *in vivo* and *in vitro* have suggested that As_2O_3 may act through epigenetic mechanisms. The biomethylation of As_2O_3, as well as other bioactive molecules, uses S-adenosyl-L-methionine (SAM) as a methyl donor during DNA methylation.[4] Decreased level of SAM caused by As_2O_3 could result in DNA hypomethylation which can affect some oncogene expression profiles.[5]

C-*myc* is considered to be a proto-oncogene that is often activated in carcinogenesis and abnormal proliferation.[6] Its overexpression is found to be associated with hepatocellular and other tumors.[7] Furthermore, disrupted c-*myc* expression was also detected in tumors and transformed cells induced by As_2O_3.[8]

Although Ramirez *et al.*[9–11] have done a series of work showing that SAM can reverse micronucleus formation, prevent aneuploidy and counteract mitotic disturbances and cytostatic effects induced by arsenic, there were few studies considering the effect of SAM on arsenic genotoxicity. The present study aims to investigate the effect of exogenous SAM on arsenic carcinogenesis from the molecular perspective; to see how the supplementation of SAM affects the c-*myc* overexpression induced by As_2O_3, thereby providing some valuable reference for the treatment of As_2O_3-induced tumors.

MATERIALS AND METHODS

Cell Cultures and Chemicals —— Normal human liver HL-7702 cells, obtained from Cell Bank of Chinese Academy of Science (Shanghai, China), were grown in RPMI 1640 culture medium containing 15% heat-inactivated newborn bovine serum, 100 IU/ml penicillin and streptomycin at 37°C in a 5% CO_2 incubator. As_2O_3, dissolved in 1 M NaOH and stocked at 100 mM, was from Beijing Chemicals (Beijing, China), and SAM was from Abbott S.P.A. and dissolved in Roswell Park Memorial Institute (RPMI) 1640 medium. Reverse transcription (RT)-PCR related reagents were from Takara Biotechnology Company Limited (Dalian, China). All other chemicals were analytically pure.

Experiment Design —— The experiments were set up as follows: cells were treated with As_2O_3 at different concentrations for 24 hr; cells were co-treated with 1.0 μM As_2O_3 and different concentrations of SAM for 24 hr; and cells were co-treated

*To whom correspondence should be addressed: School of Life Sciences, Lanzhou University, 222 South Tianshui Rd, Lanzhou 730000, China. Tel.: +86-13919123067; Fax: +86-931-8910864; E-mail: ymzhang@lzu.edu.cn

with 1.0 μM As_2O_3 and 0.2 μM SAM for different exposure times. All experiments were performed in triplicate.

RT-PCR Procedures —— HL-7702 cells were homogenized in RNAiso reagent, and total cellular RNA was extracted according to the manufacturer's instructions.

RNA concentration and purity were determined by spectrophotometry at 260 and 280 nm, and its integrity was checked by 0.5% gel electrophoresis.

For the synthesis of cDNA, 500 ng mRNA from each sample was resuspended in a mixture including 1 μl of Oligo [deoxy thymine (dT)] 15 primer (50 μM) and ribonuclease (RNase)-free water. The reaction mixture was maintained at 70°C for 10 min followed by ice bath for more than 2 min. After several seconds of centrifugation, 50 U Reverse Transcriptase (RTase) M-MLV, 10 U RNase inhibitor, 0.5 μl deoxy-ribonucleoside triphosphate (dNTP, 10 mM), 2 μl of 5 × M-MLV buffer were added to each tube and incubated for 1 hr at 37°C. The reaction mixture was stopped by denaturing the enzyme at 70°C for 15 min. The primer sequences and sizes of amplified products are: c-*myc*, sense-GGTCTTCCCCTACCCTCTCA, antisense-GCTGCGTAGTTGTGCTGATG, amplified PCR fragment, 354 bp; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sense-CCACCCATGGCAAATTCCATGGCA, antisense-TCTAGACGGCAGGTCAGGTCCACC, amplified PCR fragment, 598 bp. 5 μl synthesized cDNA were added to 45 μl of PCR mix containing 5 μl of 10 × PCR buffer, 2 μl dNTP (10 mM each), 1 μl of sense and anti-sense primers (10 μM), 2.5 U DNA polymerase and 35.5 μl dH_2O. Amplification was initiated by 5 min of denaturation at 94°C for 1 cycle followed by 30 cycles at 94°C for 30 sec, 60.8°C for 45 sec, and 72°C for 45 sec. After the last cycle of amplification, the samples were incubated for 10 min at 72°C. Amplification of GAPDH cDNA in the same samples was used as an internal control for all PCR amplification reactions, adjusting for differences in mRNA concentrations.

The PCR products were visualized by UV illumination following electrophoresis through 1.5% agarose at 80 V for 30 min containing 0.5 μg/ml ethidium bromide (Sigma, MO, U.S.A.). Gels were photographed with Gel Imaging System (Bio-Rad, CA, U.S.A.). Areas under the curve, normalized to GAPDH content, were determined using Image J software (National Institutes of Health, MD, U.S.A.) to quantify the signals.

Statistical Analysis —— Data were analyzed using the GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, U.S.A.). Student's *t*-test was used for comparisons between two groups and one-way analysis of variance (ANOVA) was for comparisons among three or more groups. The level of significance was set at $p < 0.05$ in all cases.

RESULTS AND DISCUSSION

As_2O_3 Up-regulated the Expression of c-*myc* in HL-7702 Cells

Arsenic is a potential carcinogen to human beings. It is noteworthy that arsenic exposure can cause gene amplification. This gene amplification may relate to arsenic's carcinogenic effect since amplification of oncogenes is observed in many human tumors.[12] In this study, c-*myc* expression was determined after incubation of HL-7702 cells with 0, 0.5, 1, 5, 10, 20 μM of As_2O_3 for 24 hr. As_2O_3 increased c-*myc* expression by approximately 50% compared with control, with 1 μM As_2O_3 showing the most significant effect (Fig. 1), which is consistent with other studies.[8, 13, 14] Since c-*myc* expression is often activated during cell proliferation and carcinogenesis[6] and also found after arsenic exposure, the up-regulated c-*myc* expression may participate in the development of arsenic-induced cancer.

SAM Could Inhibit c-*myc* Overexpression in HL-7702 Cells

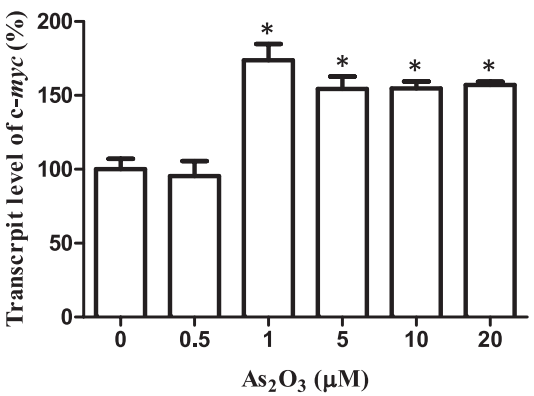

Fig. 1. Effects of As_2O_3 on the Expression of c-*myc* in HL-7702 Cells

HL-7702 cells were treated with 0, 0.5, 1, 5, 10, 20 μM of As_2O_3 for 24 hr. The c-*myc* expression level was determined by PCR. Results are expressed as the percentage of control and represent the mean ± S.E. of three independent experiments. $^*p < 0.05$, compared with control.

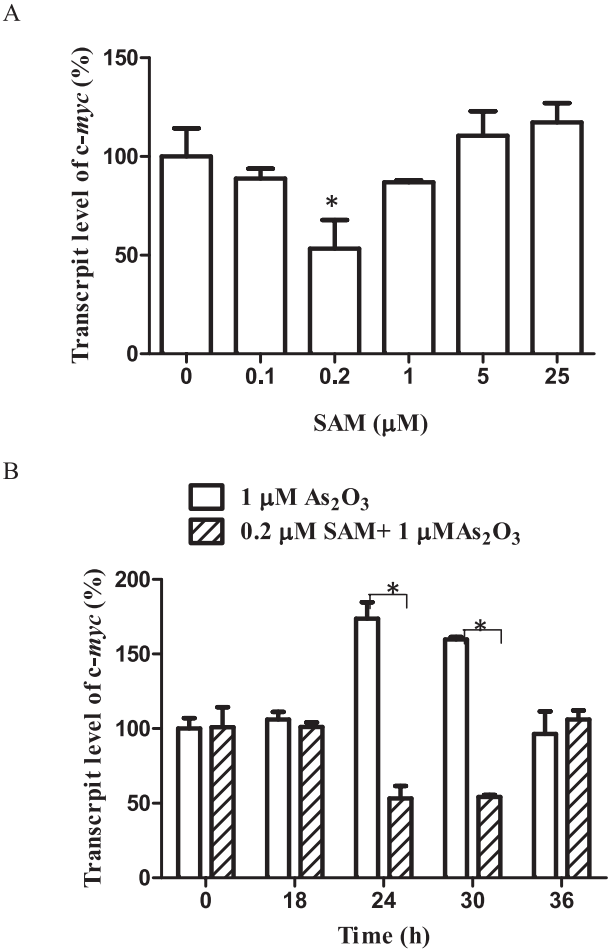

Fig. 2. The Expression Levels of c-*myc* in HL-7702 Cells

A, Effects of SAM at different concentrations on the expression of c-*myc* in HL-7702 cells under 1.0 μM As_2O_3 exposure. $^*p < 0.05$, compared with control. B, Effects of 0.2 μM SAM on the c-*myc* expression in 1 μM As_2O_3-treated HL-7702 cells at different exposure times. $^*p < 0.05$, the groups of SAM and As_2O_3 co-treatment were compared with that of As_2O_3 treatment alone.

SAM is the methyl donor for the majority of methyltranferases, which modify DNA, RNA, histones and other proteins to regulate replication/transcription/translation fidelity.[15] The alteration of SAM levels in cells could lead to abnormal expression of some genes either through DNA hypomethylation or hypermethylation.[16] In this study, the c-*myc* overexpression induced by As_2O_3 was greatly inhibited after the addition of SAM (Fig. 2). To determine the most effective concentration of SAM, HL-7702 cells were treated with As_2O_3 (1 μM) and SAM (0.1, 0.2, 1, 5 and 25 μM) for 24 hr. The c-*myc* expression was confirmed by RT-PCR. As indicated in Fig. 2A, co-treatment with 0.2 μM SAM most dramatically reduced the expression of c-*myc* when compared with 1 μM As_2O_3 treatment alone ($p < 0.05$). However, the results show the inhibitory effect did not increase with the increasing concentrations of SAM, rather the c-*myc* expression increased gradually when the dose of SAM supplemented was greater than 0.2 μM (Fig. 2A). We believe the reason for this is because when the SAM concentration is high, the response of RNA, histone and protein methylation is also activated, all which could lead to the accumulation of S-adenosylhomocysteine (SAH), downstream metabolite of SAM. High levels of this important metabolite, SAH, may then inhibit the activity of DNA methyltransferases which are necessary for demethylation/methylation.[16]

To further investigate time-dependent effect of SAM on the c-*myc* overexpression induced by As_2O_3, HL-7702 cells were treated with 1 μM As_2O_3 and 0.2 μM SAM at different exposure time (18, 24, 30, 36 hr). As shown in Fig. 2B, As_2O_3 significantly enhanced c-*myc* expression at 24 and 30 hr ($p < 0.05$). Notably, co-treatment of As_2O_3 and SAM reduced c-*myc* expression at a significant level when compared with As_2O_3 treatment alone at 24 and 30 hr. Our study showed similar results that inhibitory effect of SAM was prominent at 24 and 30 hr (Fig. 2B).

It has been demonstrated that arsenic can consume cellular SAM during the process of its methylation, giving rise to genomic hypomethylation.[5, 17] In general, hypomethylation promotes gene expression while hypermethylation inhibits gene expression. Thus, we hypothesize the increased c-*myc* expression is a result of its hypomethylation induced by As_2O_3. The supplemented SAM compensated for the loss of cellular SAM induced by As_2O_3, thereby preventing c-*myc* overexpression.

In summary, our research demonstrates that proper supplementation of SAM can prevent the overexpression of c-*myc* induced by As_2O_3. Similarly to how alcohol-induced SAM depletion in hepatic injuries and carcinomas,[18] SAM depletion caused by arsenic also plays a critical role in liver cancer development. However, further studies are needed to confirm the methylation status of c-*myc* and examine the expression of c-*myc* protein.

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