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Modulation of p53, *c-fos*, RARE, cyclin A, and cyclin D1 expression in human leukemia (HL-60) cells exposed to arsenic trioxide

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Abstract

Arsenic trioxide (As_2O_3) has recently been successfully used to treat all-trans retinoic acid (ATRA) resistant relapsing acute promyelocytic leukemia. However, its molecular mechanisms of action are poorly understood. In the present study, we used the human leukemia (HL-60) cell line as a test model to study the cellular and molecular mechanisms of anti-cancer properties of As_2O_3 . We hypothesized that As_2O_3 -induced expression of stress genes and related proteins may play a role in the cellular and molecular events leading to cell cycle modulation in leukemic cells. To test this hypothesis, we performed Western blot analysis to assess the expression of specific cellular response proteins including p53, *c-fos*, RARE, Cyclin A, and Cyclin D1. Densitometric analysis was performed to determine the relative abundance of these proteins. Western Blot and densitometric analyses demonstrated a strong dose-response relationship with regard to p53 and RARE expression within the dose range of 0-8 $\mu\text{g}/\text{mL}$. Expression of *c-fos* was slightly up-regulated at 2 $\mu\text{g}/\text{mL}$, and down-regulated within the dose-range of 4-8 $\mu\text{g}/\text{mL}$. A statistically significant down-regulation of this protein was detected at the 6 and 8 $\mu\text{g}/\text{mL}$ dose levels. No statistically significant differences ($p > 0.05$) in Cyclin D1 expression was found between As_2O_3 -treated cells and the control. Cyclin A expression in As_2O_3 -treated HL-60 cells was up-regulated at 6 $\mu\text{g}/\text{mL}$, suggesting that it is required for S phase and passage through G_2 phase in cell cycle progression. Taken together, these results indicate that As_2O_3 has the potential to induce cell cycle arrest through activation of the 53-kDa tumor suppressor protein and repression of the *c-fos* transcription factor. Up-regulation of RARE by As_2O_3 indicates that its cytotoxicity may be mediated through interaction/binding with the retinoic acid receptor, and subsequent inhibition of growth and differentiation.

Keywords

As_2O_3 ; HL-60 cells; cyclin A; cyclin D1; RARE; *c-fos*; p53; APL

Introduction

Recent studies have shown that arsenic trioxide (As_2O_3) can induce a clinical remission in patients with acute promyelocytic leukemia (APL). Arsenic-containing compounds have been reported to induce apoptosis in leukemic cells both *in vivo* and *in vitro* [1]. Many studies on APL-derived cell lines and transgenic mice carrying the PML/RAR α fusion proteins indicate that As_2O_3 induces degradation of both PML/RAR α and native PML from the nuclei of the malignant cells [2,3]. This process allows partial differentiation of leukemia population to proceed. *In vitro* studies on APL-derived leukemia cells have also indicated that As_2O_3 causes

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disappearance of both mutant PML/RAR α and wild type PML from the nucleus, eliminating its dominant negative oncogenic effect and leading to terminal maturation of the malignant cells [3].

Recent studies have also indicated that arsenic induces neoplastic cell transformation and apoptosis in tumor cells, by significantly affecting specific signal transduction pathways and by activating the expression of AP-1 and nuclear factor kappa B (NF- κ B) in *JB*₆ cells [4,5]. Other laboratory studies using myeloid leukemia cell lines that do not express PML-RAR α have shown that melarsoprol and As₂O₃ inhibit cell growth, downregulate Bcl-2 protein, and induce apoptosis [6].

Although As₂O₃ *in vitro* influences signal transduction pathways in tumor cells, its specific molecular mechanisms of action remain to be elucidated. In the present study, we hypothesized that As₂O₃-induced expression of stress genes and related proteins plays a role in the molecular events leading to cell cycle modulation in leukemic cells. To test this hypothesis, we performed the western blot and densitometric analyses to assess the expression and relative abundance of specific cellular proteins including p53, c-fos, RARE, cyclin A, and cyclin D1 in human leukemia cells exposed to As₂O₃.

Materials and Methods

Chemicals and test media

Arsenic trioxide (As₂O₃), CASRN 1327-53-3, MW 197.84, with an active ingredient of 100% (w/v) arsenic in 10% nitric acid was purchased from Fisher Scientific in (Houston, Texas, U.S.A). Growth medium RPMI 1640 containing 1 mmol/L L-glutamine was purchased from Gibco BRL products (Grand Island, NY). Ninety-six well plates were obtained from Costar (Cambridge, MA). Fetal bovine serum (FBS), antibiotics (penicillin G and streptomycin), and phosphate buffered saline (PBS) were obtained from Sigma Chemical Company (St. Louis, MO).

Tissue culture

The HL-60 promyelocytic leukemia cell line was purchased from the American Type Culture Collection –ATCC (Manassas, VA). This cell line has been derived from peripheral blood cells of a 36-year old Caucasian female with acute promyelocytic leukemia (APL). The HL-60 cells grow as a suspension culture. The predominant cell population consists of neutrophilic promyelocytes [7].

In the laboratory, cells were stored in the liquid nitrogen until use. They were next thawed by gentle agitation of their containers (vials) for 2 min in a water bath at 37°C. After thawing, the content of each vial of cell was transferred to a 25 cm² tissue culture flask, diluted with up to 10 mL of RPMI 1640 containing 1 mmol/L L-glutamine (GIBCO/BRL, Gaithersburg, MD) and supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (w/v) penicillin/streptomycin. The 25 cm² culture flasks (2 × 10⁶ viable cells) were observed under the microscope, followed by incubation in a humidified 5% CO₂ incubator at 37°C. Three times a week, they were diluted under same conditions to maintain a density of 5 × 10⁵/mL, and harvested in the exponential phase of growth. The cell viability was assessed by the trypan blue exclusion test (Life Technologies), and manually counted using a hemocytometer.

Western blot and densitometric analyses

Western blot analysis was conducted to determine specific cellular response proteins including p53, Cyclin A, Cyclin D1, RARE, and *c-fos* at 24 h of arsenic trioxide (As₂O₃) exposure. HL-60 cells were grown in 96 well polystyrene tissue plates. Briefly, 200 μ L cells (5 × 10⁵/mL) were

added to each well of 96 tissue culture plates and treated with 2, 4, 6 and 8 $\mu\text{g}/\text{mL}$ of As_2O_3 for 24 h. These doses were selected based on the results of previous experiments in our laboratory indicating that As_2O_3 is cytotoxic to HL-60 cells, showing a 24 hr LD_{50} of $6.4 \pm 0.7 \mu\text{g}/\text{mL}$ [8]. Control well plates were also made without As_2O_3 . After the incubation period, cells were centrifuged at 800 rpm for 5 min, the supernatant was carefully aspirated, and the cells were washed twice with PBS. The total protein was measured by the Bradford method at 600 nm using a microtiter plate reader [9]. Twenty μL of native sample buffer (0.2 mol/L Tris, pH 6.8, 1% SDS, 30% glycerol, 7.5% mercaptoethanol, 0.1% bromophenol blue) were added to each plate well and the cells were mechanically collected into micro-centrifuge tubes. Cellular protein lysates (15 $\mu\text{g}/\text{mL}$) from human leukemia HL-60 cells containing an equal volume of sample buffer were heated at 100°C for 10 min. Appropriate amounts of total cellular protein were loaded onto 10% SDS polyacrylamide gels and electrophoresed at 100 V constant voltage for 1 hr. Samples were transferred onto a nitrocellulose membrane on ice and the membrane was blocked (Tris buffer saline with 5% nonfat dry milk, 0.1 Tween 20) for 24 hr at 4°C. Detection of membrane-bound proteins was carried out using specific primary antibodies for the proteins of interest (*c-fos* 15:1000, p53 1:1000, RARE 1:500, Cyclin A 1:500, and Cyclin D1 1:750) (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Equal lane loading was assessed using α -tubulin mouse monoclonal primary antibody (Figure 1). Subsequently, the reaction was probed with a 1:750 dilution of alkaline conjugated anti-mouse IgG secondary antibody. NBT/BCIP color substrate was incorporated to develop protein bands. Immunoblot 1-D protein bands were assessed for relative abundances using Total Lab-Image computer software (Nonlinear USA Inc. Durham, NC).

Statistical analysis

To determine the lysate volumes to be loaded on the gels, protein samples collected from controls and As_2O_3 -treated cells were measured using the Bradford method based of the optimal density readings at 600 nm. After electrophoresis, the expression levels of specific cellular proteins were photographed using the Gel Documentation System (Nucleotech Corporation, Inc, San Mateo CA). The experiments were performed three or more times to ensure reproducibility. Densitometric analysis was performed using the Gel Documentation System (Nucleotech Corporation, Inc, San Mateo CA) to determine the relative abundance of protein expression. Comparison of protein levels between control cells and As_2O_3 -treated HL-60 cells was performed using one way analysis of variance (ANOVA) for multiple samples and Student's t-test for paired sample sets. All p-values <0.05 were considered to be significant.

Results

The expression and relative abundance levels of p53 in human leukemia (HL-60) cells exposed to arsenic trioxide (As_2O_3) are represented in Figure 2. According to ANOVA Dunnett's test ($p < 0.05$), western blot and the densitometric analyses demonstrated a significant increase of p53 expression in As_2O_3 -treated HL-60 cells showing a gradual increase in protein expression with increasing doses of As_2O_3 .

The relative level of *c-fos* expression was slightly up-regulated at 2 $\mu\text{g}/\text{mL}$, and down-regulated within the dose-range of 4-8 $\mu\text{g}/\text{mL}$. Statistically significant down-regulation of this protein was detected at the 6 and 8 $\mu\text{g}/\text{mL}$ dose levels. Hence, *c-fos* expression data indicated a somewhat biphasic response that encompasses up-regulation at 2 $\mu\text{g}/\text{mL}$ level of exposure and down-regulation at higher doses of exposure (Fig 3).

The data of RARE expression in As_2O_3 -treated cells is represented in Figure 4. As shown in this figure, the expression of RARE in HL-60 cells exposed to As_2O_3 at 2, 4, and 6 $\mu\text{g}/\text{mL}$ dose levels are not significantly different ($p > 0.05$) compared to the control. However, an up-

regulation of this protein was observed in As₂O₃-treated cells with a statistically significant increase at 8 µg/mL compared to the control.

Western blot analysis showed a slight increase in cyclin D1 expression in As₂O₃-treated HL-60 cells. However, the statistical analysis based on the densitometric analysis did not show any significant differences ($p > 0.05$) between As₂O₃-treated cells and the control (Fig 5).

Data on cyclin A expression and its relative abundance in As₂O₃-treated HL-60 cells are represented in Figure 6. As shown in this figure, there was no statistically significant difference between the control and As₂O₃-treated cells up to 4 µg/mL dose levels. An up-regulation of this protein was detected at 6 µg/mL As₂O₃, suggesting that it is likely required for S phase and passage through G2 phase in cell cycle progression. However, at 8 µg/mL of As₂O₃ treatment, the expression of this protein was similar to that of the control.

Discussion

The present study was designed to investigate the influence of arsenic trioxide (As₂O₃) treatment on the expression of *p53*, *c-fos*, RARE, cyclin A, and cyclin D1 in human leukemia (HL-60) cells. Data from western blot and densitometric analyses show a gradual increase of p53 expression in HL-60 cells with increasing doses of As₂O₃. This increased expression of the 53 KDa tumor suppressor protein detected in the As₂O₃-treated HL-60 cells suggests the potential of As₂O₃ to cause either G₁ cell cycle arrest and/or apoptosis in HL-60 cells. However, despite the ability of As₂O₃ to induce cell cycle arrest, little is known concerning the precise role of this agent in cell cycle progression and the function of p53 in this process. The increase in p53 expression in As₂O₃-treated cells may be indicative of a cellular response to oxidative and DNA damage. A series of recent studies have demonstrated DNA damage [10], oxidative stress [11,12], UV irradiation [13,14], and transcriptional blockade [15] induce translocation of p53 to the mitochondria. Research has suggested that arsenic induces DNA damage, such as chromosome aberration [16], and sister chromatid exchange [17]. Because p53 mutations are common in most human cancers and apoptosis plays a key role in the bioactivity of most chemotherapeutic agents, As₂O₃ seems to be very useful for the treatment of APL and certain human cancers. This notion is supported by the findings that p53 is the most commonly mutated tumor suppressor gene, and the lack of p53 activation or expression is associated with an increased risk of tumor formation [18-20]. Previous studies in our laboratory have demonstrated that As₂O₃ is able to transcriptionally induce the expression of p53, *c-fos*, and HSP70 in human liver carcinoma (HepG₂) cells [21]. The p53 transcription factor has been shown to mediate apoptosis through its direct action at the mitochondria [22,23].

The result of *c-fos* expression shows a somewhat biphasic response that encompasses a slight up-regulation at lower doses of exposure, and down-regulation at higher doses of exposure. The inhibition of *c-fos* expression at higher level of As₂O₃ exposure is in agreement with the activation of p53 expression, suggestive of cell cycle arrest at G1 check point of the cell cycle. p53 has been shown to repress regulators of cell proliferation, such as *c-fos* and *c-jun* which are early-response nuclear oncogenes [24]. Many researchers have reported that *c-fos* and p53 share similar function and similar transcriptional regulatory pathways in apoptosis following excitotoxic stimulation [25,26]. Finding from the present study implies that *c-fos* repression may play an important role in HL-60 cells apoptosis induced by As₂O₃. *c-fos* is known to be highly induced in many human cancer cell lines in response to stimulus. This proto-oncogene (*c-fos*) plays a role in cell proliferation, differentiation, and may contribute to tumor promotion. In other cell lines, As₂O₃ has been reported to activate the expression of *c-fos* through numerous signal transduction pathways at low dose of exposure. For example, in a human bladder epithelial cell line, arsenic increased cell proliferation and AP-1 DNA binding [27].

In mammalian cells, cyclins D, E, and A are key cyclins involved in G1 to S phase transition. Cyclin D assembles with cdk4/6 in early G1; cyclin E combines with cdk2 later in G1, and cyclin A associates with cdk2 at the beginning of S phase [28,29]. In the present study, the data of western blot and densitometric analyses show a slight increase of Cyclin D1 expression in As₂O₃-treated HL-60 cells. However, there were no statistically significant differences ($p > 0.05$) in cyclin D1 expression between As₂O₃-treated cells and the control. The lack of a statistically significant difference in cyclin D1 expression in As₂O₃-treated cells compared to the control is in agreement with the activation of the p53 tumor suppressor protein, and the repression of the *c-fos* proto-oncogene, indicative of cell cycle arrest at the G1/S checkpoint. Cyclin D1 is a 36 kDa nuclear protein. The cell cycle in eukaryotic cells is mediated by the formation, activation, and deactivation of complexes containing cyclin-dependent kinases (CDK) and cyclins. Few studies have demonstrated that cyclin D1 over-expression does not correlate with the proliferation rate in rat mammary tumors or in human tumors [30,31].

Data generated from the present study shows that As₂O₃ doesn't significantly affect progression from G₀/G₁ into S phase. This transition is, in part, controlled by the activity of G₁ cyclin-cdk's which include D cyclins associated with either cdk4 or cdk6 and E cyclins associated with cdk2 [32,33], suggesting that the expression of cyclin D1 may be an early event in chemical tumorigenesis, causing an increase in cell proliferation.

Recent publications have accumulated evidence showing an increased expression of cyclin D1 in tumor cell lines [34-36]. Studies have shown that arsenite exposure is able to activate the PI-3K/Akt pathway and induce cyclin D1 expression in mouse epidermal C141 cells [37]. It has been reported that mitogen-activated protein kinase (MAPK) cascades are involved in the modulation of cyclin D1 expression [38]. Other studies have revealed that ectopic expression of cyclin D1 can shorten the G₁ phase, whereas inhibition of cyclin D1 expression blocks G₁-S transition. It has been demonstrated that carcinogenic compounds can induce cyclin D1 expression, which in turn promote tumor cell proliferation [39].

Data generated from the present study have demonstrated a positive expression of cyclin A in As₂O₃-treated HL-60 cells showing an up-regulation at 6 µg/mL of As₂O₃ exposure. Cyclin A is required in more than one phase of the cell cycle. It is one of the first cyclins to be identified and is believed to function between that of cyclin E and cyclin B. Generally, it is expressed in late S and G₂ phase and degraded during mitosis just prior to metaphase. It associates with two cyclin dependent kinases: cdk2 in the S phase of the cell cycle and Cdc2 in the G₂/M phase. These associations are required for both DNA replication and mitosis [40-42]. Cyclin A is required for S phase and passage through G₂ phase in cell cycle progression. Interestingly, our results indicated that treatment of HL-60 cells with As₂O₃ may lead to cell cycle progression at the G₂ checkpoint as demonstrated by the significant increase of cyclin A expression at 6 µg/mL of As₂O₃ exposure. The activation of cyclin A expression at higher level of As₂O₃ exposure is in agreement with slight increase in cyclin D1 expression; suggestive that exposure of human leukemia HL-60 cells to As₂O₃ may result in an increase in the proportion of cells in mitosis.

Up-regulation of the retinoic acid response element (RARE) in HL-60 cells by As₂O₃ suggests that its toxicity may be mediated through interaction or binding with the retinoic acid receptor, and subsequent inhibition of growth and differentiation. Substantial data showed that As₂O₃ exposure induces remission in patients with APL at least in part through a mechanism that results in the degradation of the aberrant PML-retinoic acid receptor- α fusion protein. In the early 1980s, it was noted that all trans retinoic acid (ATRA) could induce differentiation of myeloid cell lines such as HL-60 [43], and of primary cells from patients with APL [44]. Retinoids, a group of structural and functional analogues of vitamin A, are known to mediate cellular signals critical for embryonic morphogenesis, cell growth, and differentiation. The use

of retinoids to suppress tumor development has been evaluated in several animal models of carcinogenesis, including models of skin, breast, oral cavity, lung, hepatic, gastrointestinal, prostatic, and bladder cancers [45]. Clinically, retinoids are able to reverse premalignant lesions and inhibit the development of primary tumors [46,47].

Conclusions

The present study provides new insights into the biochemical effects of arsenic trioxide (As_2O_3) in HL-60 cells and the potential of As_2O_3 as a chemotherapeutic agent for the treatment of acute promyelocytic leukemia. Using western blot and densitometric analyses, our data provide evidence that As_2O_3 performs key functions of an anti cancer agent against human leukemia; it is antiproliferative and apoptotic at high doses (p53 activation and *c-fos* repression), and induces cellular differentiation at low doses (cyclins D1 and A expression). These results are consistent with previous studies reporting that As_2O_3 (an effective drug for the treatment of APL) exerts dose-dependent dual effects in APL cells by triggering apoptosis and inducing partial differentiation [48-51].

Taken together, we have demonstrated in the present study that As_2O_3 serves as an activator of the p53, RARE, cyclin A and cyclin D1, and a repressor of *c-fos* in HL-60 cells. It is evident that As_2O_3 exposure places HL-60 cells under a degree of toxic stress that evokes response acting at different levels on the cell cycle. This toxic action of As_2O_3 induces transcription of specific genes that affect mitogen response, cell cycle progression, and programmed cell death. Findings from our studies suggest that activation of cyclin D1, cyclin A, and RARE by As_2O_3 in HL-60 cells may be an important part of the multistep process involved in cell cycle progression, whereas the activation of the p53 tumor suppressor protein and repression of the *c-fos* transcription factor at higher level of exposure may be involved in cell cycle arrest and apoptosis. Further *in vitro* studies are underway to refine the molecular mechanisms of action and to determine whether signal transduction pathways vary according to the cell type, form of arsenic, or other factors.

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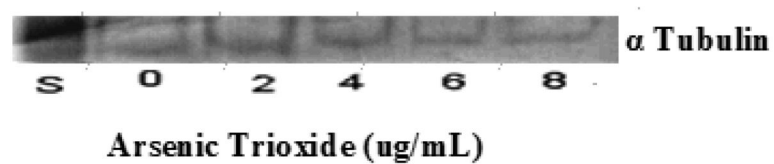


Figure 1. Expression of α -tubulin in human leukemia (HL-60) cells exposed to arsenic trioxide. HL-60 cells were treated with different doses of arsenic trioxide, and Western blot analysis of α -tubulin expression was performed as indicated in the Materials and Methods.

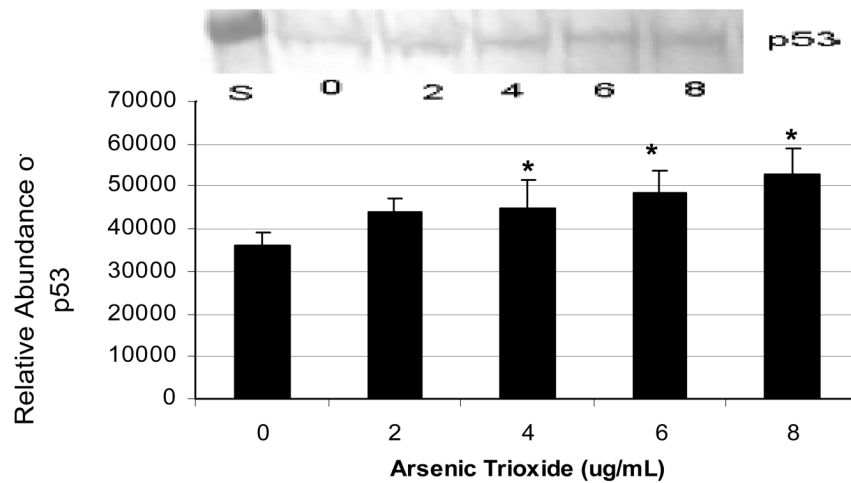


Figure 2. Expression and relative abundance of p53 in human leukemia (HL-60) cells exposed to arsenic trioxide. HL-60 cells were treated with different doses of arsenic trioxide, and Western blot and densitometric analyses of p53 expression were performed as indicated in the Materials and Methods. α -tubulin expression was used to assess equal lane loading. Inset shows a representative Western Blot analysis. Bars represent p53 relative abundance. Each point represents the mean value and the standard deviation of three experiments. * Significantly different from control (0 $\mu\text{g/mL}$), $p < 0.05$.

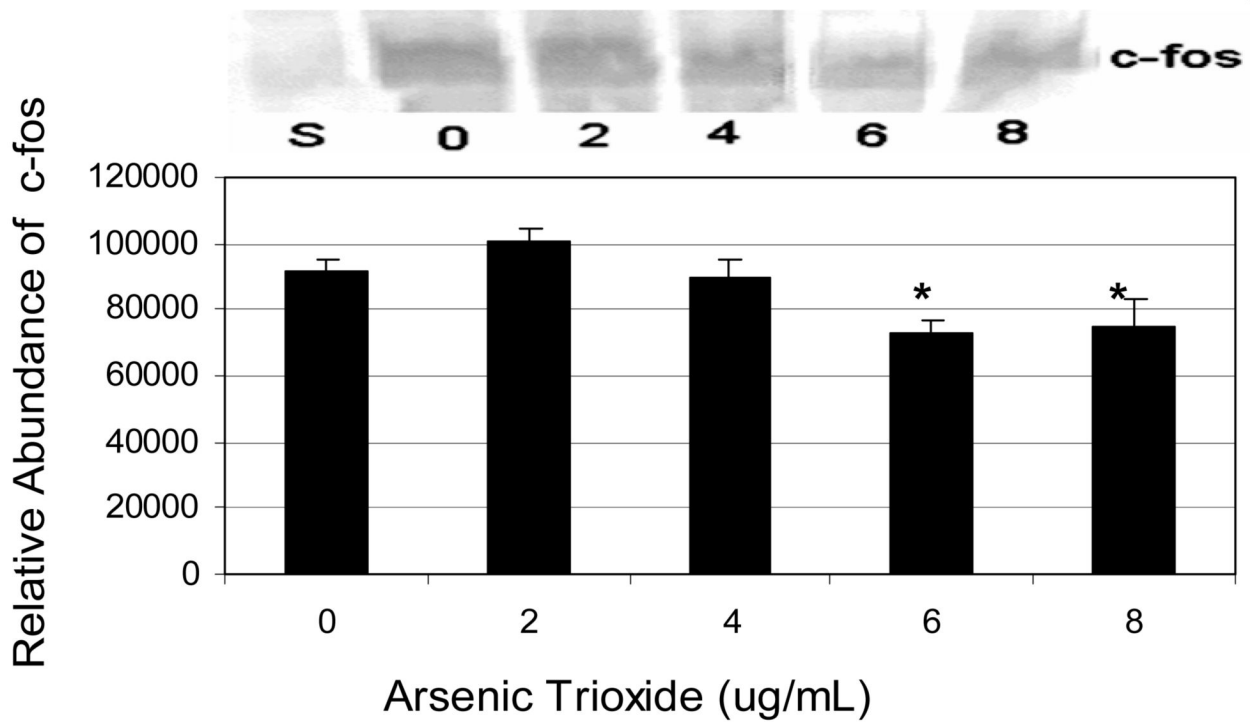


Figure 3.

Expression and relative abundance of *c-fos* in human leukemia (HL-60) cells exposed to arsenic trioxide. HL-60 cells were treated with different doses of arsenic trioxide, and Western blot and densitometric analyses of *c-fos* expression were performed as indicated in the Materials and Methods. α -tubulin expression was used to assess equal lane loading. Inset shows a representative Western Blot analysis. Bars represent *c-fos* relative abundance. Each point represents the mean value and the standard deviation of three experiments. * Significantly different from control (0 μ g/mL), $p < 0.05$.

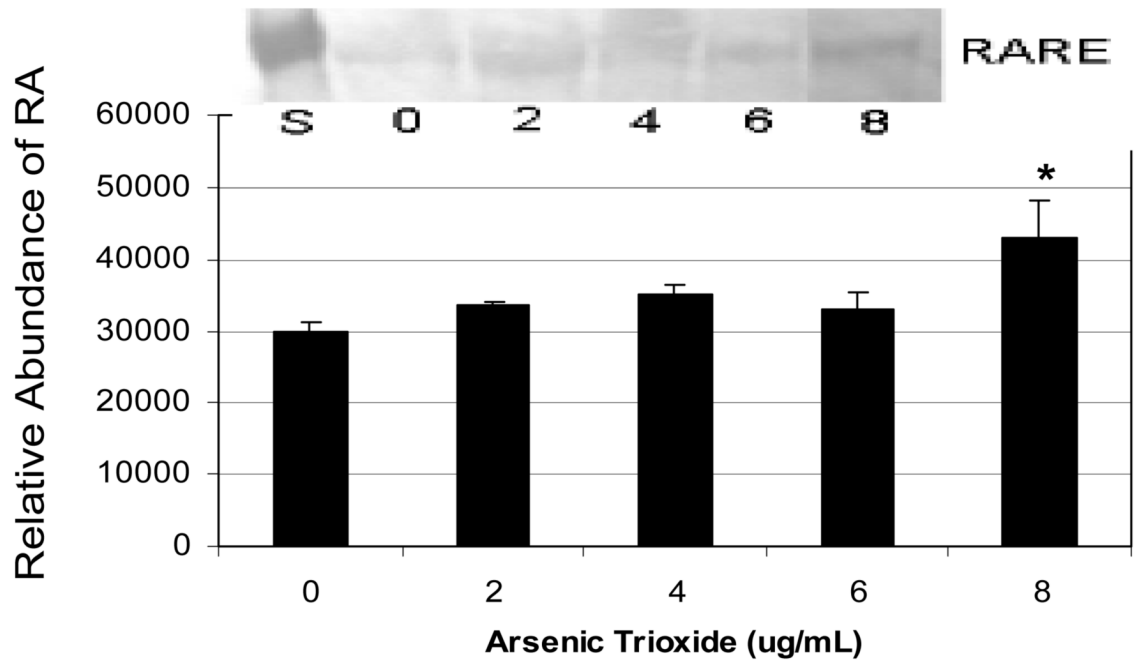


Figure 4.

Expression and relative abundance of RARE in human leukemia (HL-60) cells exposed to arsenic trioxide. HL-60 cells were treated with different doses of arsenic trioxide, and Western blot and densitometric analyses of RARE expression were performed as indicated in the Materials and Methods. α -tubulin expression was used to assess equal lane loading. Inset shows a representative Western Blot analysis. Bars represent RARE relative abundance. Each point represents the mean value and the standard deviation of three experiments. * Significantly different from control (0 μ g/mL), $p < 0.05$.

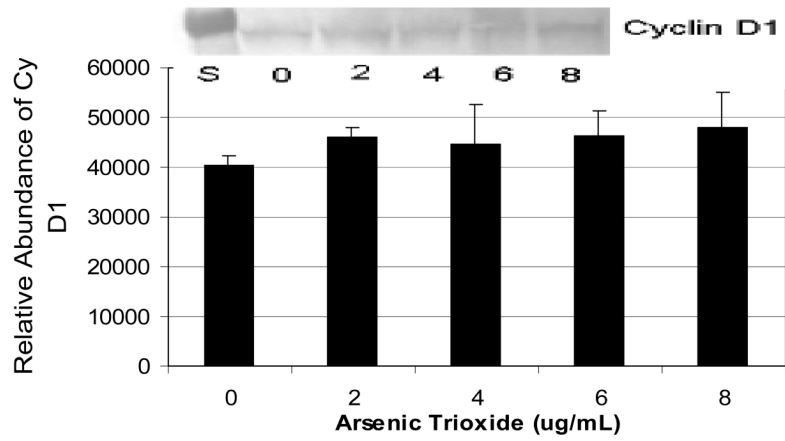


Figure 5.

Expression and relative abundance of Cyclin D1 in human leukemia (HL-60) cells exposed to arsenic trioxide. HL-60 cells were treated with different doses of arsenic trioxide, and Western blot and densitometric analyses of Cyclin D1 were performed as indicated in the Materials and Methods. α -tubulin expression was used to assess equal lane loading. Inset shows a representative Western Blot analysis. Bars represent Cyclin D1 relative abundance. Each point represents the mean value and the standard deviation of three experiments.

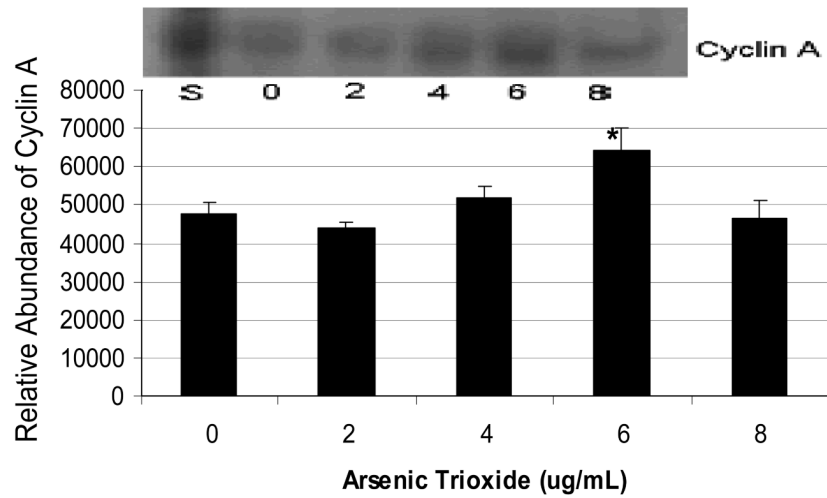


Figure 6.

Expression and relative abundance of Cyclin A in human leukemia (HL-60) cells exposed to arsenic trioxide. HL-60 cells were treated with different doses of arsenic trioxide, and Western blot and densitometric analyses of Cyclin A were performed as indicated in the Materials and Methods. α -tubulin expression was used to assess equal lane loading. Inset shows a representative Western Blot analysis. Bars represent Cyclin A relative abundance. Each point represents the mean value and the standard deviation of three experiments. * Significantly different from control (0 $\mu\text{g/mL}$), $p < 0.05$.