Evaluation of Effect of As2O3 on Cell Growth, Cell Cycle and Apoptosis in Human Leukemia Cell Line HL-60

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Abstract

Introduction: Arsenic Trioxide (As2O3) causes antitumor effects by various mechanisms, including degradation of PML-RARA in the acute promyelocytic cells, growth inhibition and induction of apoptosis. However, the precise mechanisms of these processes remain incompletely understood. The purpose of this study was to evaluate the effects of arsenic trioxide on cell growth, cell cycle regulation and apoptosis in PML/RARA negative acute promyelocytic leukemia cell line HL-60.

Methods: The cytotoxic effect of As2O3 was assessed by MTT assay, Apoptosis was detected by flow cytometry analysis using annexin V–FITC/PI and cell cycle distribution was evaluated by propidium iodide staining.

Results: MTT assay showed that cell growth was inhibited in a time- and dose-dependent manner. As2O3 exerts growth inhibitory effect by arresting the cells at G2/M phase. Apoptosis analysis revealed that As2O3 treatment induced apoptosis at the lower concentrations, while necrosis occurred at the higher concentrations.

Conclusion: As2O3 inhibits growth and induces apoptosis of HL-60 cells through induction of cell cycle arrest. Further studies are required to elucidate the molecular mechanisms of As2O3-induced anti-tumor effects.

Key words: As2O3, apoptosis, cell cycle

Introduction

Arsenic has been used for over 2400 years as a part of traditional Chinese medicine in the treatment of a number of human diseases including malaria, syphilis, epilepsy, asthma, ulcers, rheumatism, psoriasis and cancer,(1-3)Acute promyelocytic leukemia (APL) is a well-characterized subtype of acute myelogenous leukemia (AML), which is associated with a distinct cytogenetic finding t(15;17) and the production of a fusion protein, PML/RARa. PML/RARA fusion protein is known to deregulate many of cellular signaling pathways and block terminal differentiation of human promyelocytic leukemia cells. However, the fusion protein is likely to be responsible for the unique sensitivity of APL cells to all-trans-retinoic acid and arsenic trioxide (As2O3) (4).

A series of clinical trials have shown promising results for treating relapsed or refractory acute promyelocyte leukemia.(5-7) In 2000, arsenic trioxide was approved by the U.S. Food and Drug Administration (FDA) for treatment of relapsed/refractory APL patients.(8) The following clinical trials on new cases of APL have confirmed the initial results and have shown the effectiveness of As2O3 for first-line treatment of APL patients.(9-11) The effects of As2O3 are not restricted to PML/RARA positive cell, but it has been shown to have anti-cancer effects against a wide range of hematologic malignancies and solid tumor cell lines such as chronic myeloid leukemia (CML) , MDS, multiple myeloma , lymphoma and solid tumors.(12-14)

Though As2O3 has been proved to be very effective in treating APL, many of its anti-tumor mechanisms have not been elucidated. Our recent study showed that As2O3 leads to increase expression of p38, ERK1 and Bax proteins in APL patients.(15)

In the present study, we used HL-60, a PML/RARA negative human promyelocytic leukemia cell line (16), to further evaluate the anti-tumor activity of As2O3. To obtain insights into its mechanism of action, we explored the effects of As2O3 on cell proliferation, cell cycle distribution and apoptosis.

Methods

Drugs and chemicals: Arsenic trioxide was prepared as 10 mg/10 ml vials, manufactured by the pharmaceutical faculty of Tehran University of Medical Sciences. As2O3 with different concentration was prepared by diluting in RPMI medium. Propidium iodide, MTT, DMSO was obtained from Sigma–Aldrich. Annexin V-FITC Kit was purchased from Miltenyi Biotec. RPMI 1640 and fetal bovine serum (FBS) were purchased from Gibco BRL.

Cell culture: HL-60 cell line was grown in RPMI with 10% fetal bovine Serum, 2 mM of l-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 ◦C in an atmosphere of 5% CO2 and 90% relative humidity. Medium was refreshed every 1–2 days to maintain a cell concentration of 2–5×10^5/ml. All cells used in the following experiments were in logarithmic growth**.**

MTT Assay: Microculture tetrozolium test (MTT) was used to measure Cells were seeded in triplicate into 96-well plates the growth inhibitory rate of the cells treated with AS2O3. The cell line in the exponential growth phase were seeded in triplicate onto 96-well cell growth plates **(**$5X10^4$ cells/well**).** At 24 , 48 h and 72h after the cells were exposed to different concentrations of AS2O3, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (5 mg/ml) was added to each well. After incubation at 37^0C for additional 4 h, The MTT-formazan crystals were dissolved in 150 µl of DMSO**.**

The optical density *(*O.D) was measured at 570 nm. Each condition was tested in triplicate. Three independent experiments were performed**.** The inhibition ratio of cell growth *(*I**)**R was calculated via the following formula: (MTT OD value of control–MTT OD value of the sample)/MTT OD value of control] X100.

Apoptosis detection: The cells were cultured for 24h in the presence of different concentration of As2O3 in 5 ml culture bottles. Apoptosis was assessed by Annexin V-binding and propidium iodide staining.

Briefly, 10^6 cells were harvested by centrifugation and resuspended in 100ul of binding buffer with 5ul of Annexin V–FITC. After 15-minute incubation in the dark at room temperature, the cells washed by binding buffer and then immediately prior to analysis by flow cytometry 5ul of the PI solution added.

A minimum of 3 independent replications were analyzed. Histograms were analyzed using the Partec FloMax.

Cell cycle distribution Analysis: After being treated with AS2O3 at different concentrations for 24 h cells were collected, washed with PBS and fixed with 70% ethanol at 4^oC overnight. After being washed with PBS again, the cells were treated with 100 µl of 100 µg/ml RNase at room temperature for 30 min and stained with 100 µl of 50 µg/ml propidium iodide at 4^oC for 30 min without light. 10000 cells were measured per histogram and analyzed with the Flomax and Cylchred program. Sub-G1 fraction was calculated and shown as % of total cell population.

Statistics: All data are expressed as the mean±SD. Statistical analysis was performed using SPSS version 18.0 (SPSS Inc.). One-way analysis of variance (ANOVA) was used to analyze the statistical significance between treatments and controls. P values less than 0.05 were considered statistically significant.

Result

Effect of As2O3 on the cell growth of HL-60: MTT test was used to investigate the effect of As2O3 on cell viability in HL-60 cells.

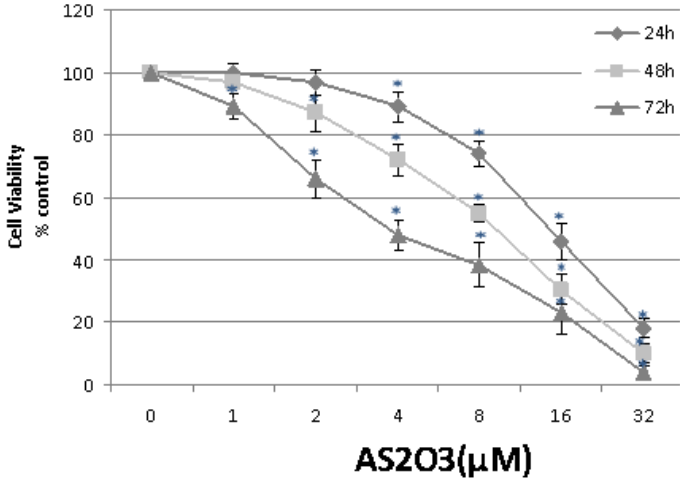

Figure- 1: HL-60 cells were treated with different concentrations of AS2O3 for 24, 48 and 72 h. The cell growth inhibitory rate was determined by MTT assay, as described in Methods. Data are presented as the mean±SD from three independent experiments. Asterisks indicate a significant difference ($p<0.01$).

Cells were seeded into the microplates and treated without or with various concentrations (1μM to 32μM) of As2O3 for different time points as mentioned under materials and methods. As2O3 induced a time- and dose-dependent inhibition of cell growth. After treatment for 24h, 48h and 72h, the IC50 was 16μmol/L, 11.5μmol/L and 5μmol/L, respectively (Figure- 1).

Apoptosis induction by As2O3: To determine whether the growth inhibitory effect of As2O3 is associated with cell death, annexin-V/PI double staining was used on HL-60 cells treated with 5–25 uM of As2O3 for 24 h. As shown in Figure- 2, a concentration-dependent effect of As2O3 in inducing apoptosis in HL-60 cells was observed, as documented by increased annexin- V+ cells. The percentage of early apoptotic cells (Annexin V-positive/PI-negative) was 0.86% for control, 7% for 5 μM As2O3, 12% for 10 μM As2O3, and 18% for 15 μM As2O3, and 27.5% for 20 μM. At higher concentrations (25μM), the number of early apoptotic cells decreased in favor of late apoptosis (Figure- 2). Our results therefore indicated that As2O3, similar to other cytotoxic anticancer drugs, at low doses induces apoptosis and at high doses causes necrosis in HL-60 cells.

Effects of As2O3 on cell cycle: To better understand the mechanism of growth inhibition of As2O3, HL-60 treated with 5, 10, 15 and 20 μM of drug for 24h, and then were stained with PI and subjected to flow cytometric analysis. Figure 3 illustrates the distributions of the cells in the G0/G1, S and G2/M phases of the cell cycle. The Sub-G1 region (RN1) is indicative of apoptotic cells. In control cells, 47±1.5% of cells are in G0/G1, 39±1% of cells are in S-phase, and 14±0.5% are in G2/M. As2O3 was able to induce the increase of sub-G1 phase and altered cell cycle distribution at 24 hours. The amount of S-phase cells increased to 44±1% and 47±0.9% at 5 and 10 μM, respectively ($P<0.01$), then decreased to 40±0.5% and 39±0.5 % at 15 and 20 μM. The proportion of G2/M phase cells significantly increased following the treatment with As2O3. The number of cells in the G2/M phase increased from 13.5±0.5% in control group to 25.5±1% at 15 μM with a concomitant decrease of cell population in S phase ($P<0.01$). Subsequently the sub-G1 population increased to 23±2%, and the G2/M population decreased to 15±0.5% after Incubation with 20 μM AS2O3. The subG1 phase significantly increased from 2.6±0.3% in control group to 7.5±0.7, 15.6±2 and 23±2% after exposure to 10 μM, 15 μM, 20 μM, respectively ($P<0.01$)(Figure-3B). This result indicates that As2O3-induced apoptosis is cell cycle-dependent.

Discussion

AS3+ belongs to group V of the periodic table and is recognized as an environmental carcinogen. For centuries, arsenic compounds such as As2O3 have been used for the treatment of a variety of diseases including cancer, especially APL (1). Several in vitro studies have shown that As2O3 can be effective in non-APL tumors.(2, 17)

Killing of cancer cells is the main principle in anti-cancer treatment regimens and escape of cell death is a characteristic feature of human malignancies. As2O3, like other chemotherapeutic drugs used in the treatment of malignant disease, tries to overcome this evasion by inducing apoptosis, necrosis or autophagy.(17-22)

Regarding the necrosis-inducing capability of As2O3, Vernhet L et al. have reported that As2O3 at low concentrations (0.1-5 μM) induces necrosis of human CD34-positive hematopoietic stem cells, whereas many studies have shown that it acts mainly by inducing apoptosis.(2, 17, 18) Although it has been shown that the clinically obtainable concentrations of As2O3 inhibit cell growth and induce apoptosis in some neoplasias, such as human cholangiocarcinoma SK-ChA-1 cells, higher concentrations of As2O3 are usually required to induce apoptosis in cancer cells compared to therapeutic concentrations of As2O3 (1–2 μM).(17, 19) In the present study, 24 h IC50 value for As2O3 in HL-60 cell line was determined 16μM, a concentration which is very higher than clinically achievable concentration. This finding is consistent with those of other studies.(20) Dai et al. have evaluated the growth inhibition induced by different concentrations of As2O3 on both NB-4 and HL-60 cell lines. They have found a reverse correlation between drug sensitivity and cellular GSH content.(23)

To determine whether the As2O3 inhibitory effect on cell growth is due to necrosis or apoptosis, we analyzed the treated cells after staining with annexin V and PI. The externalization of PS to the exposed membrane surface is an early event in apoptosis. Lower concentrations (5 μM - 20 μM) of As2O3 mainly induced annexin-V+/PI-, early apoptotic cells, and relatively higher concentrations (25 μM) of As2O3 induced annexin-V+/PI+ late apoptotic and necrotic cells (Figure- 2).

Next, we sought to determine whether As2O3-induced apoptosis of HL-60 cells is associated with changes of cell cycle distribution.

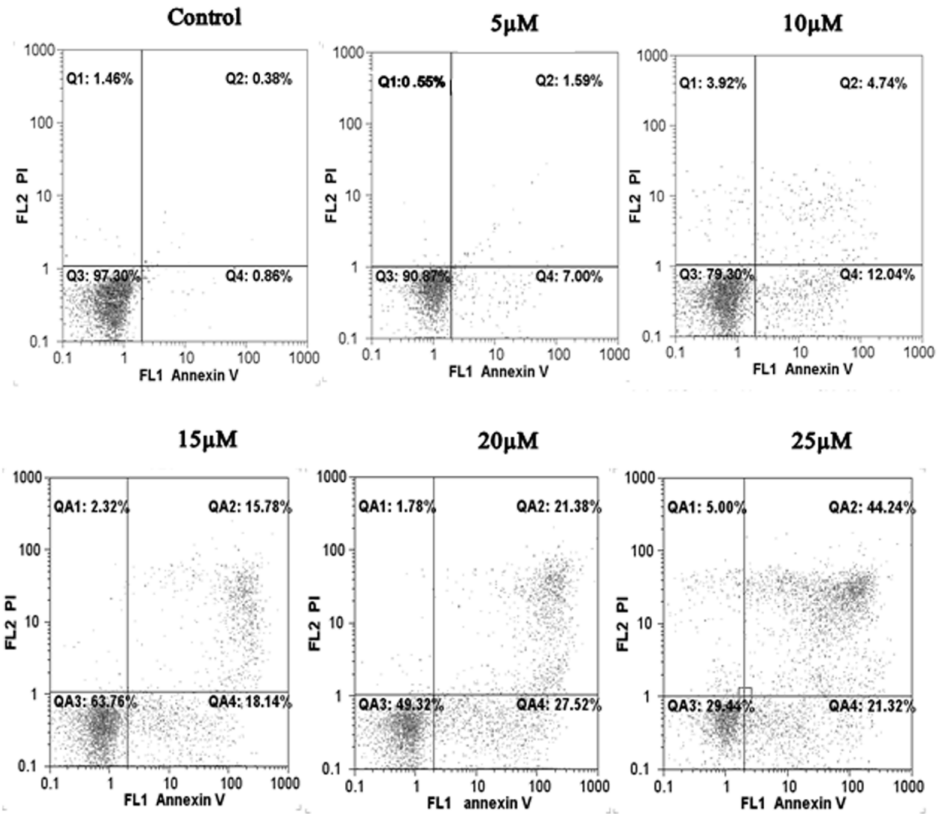

Figure- 2: Flow cytometry analysis of AS2O3-induced apoptosis in HL-60 cells. The cells were treated with the indicated concentrations of AS2O3 for 24 hours, followed by staining with annexin-V and propidium iodide (PI. The lower right quadrant (annexin-V+/PI-) represents early apoptosis, while the upper right quadrant (annexin V+/PI+) represents late apoptosis and necrosis.

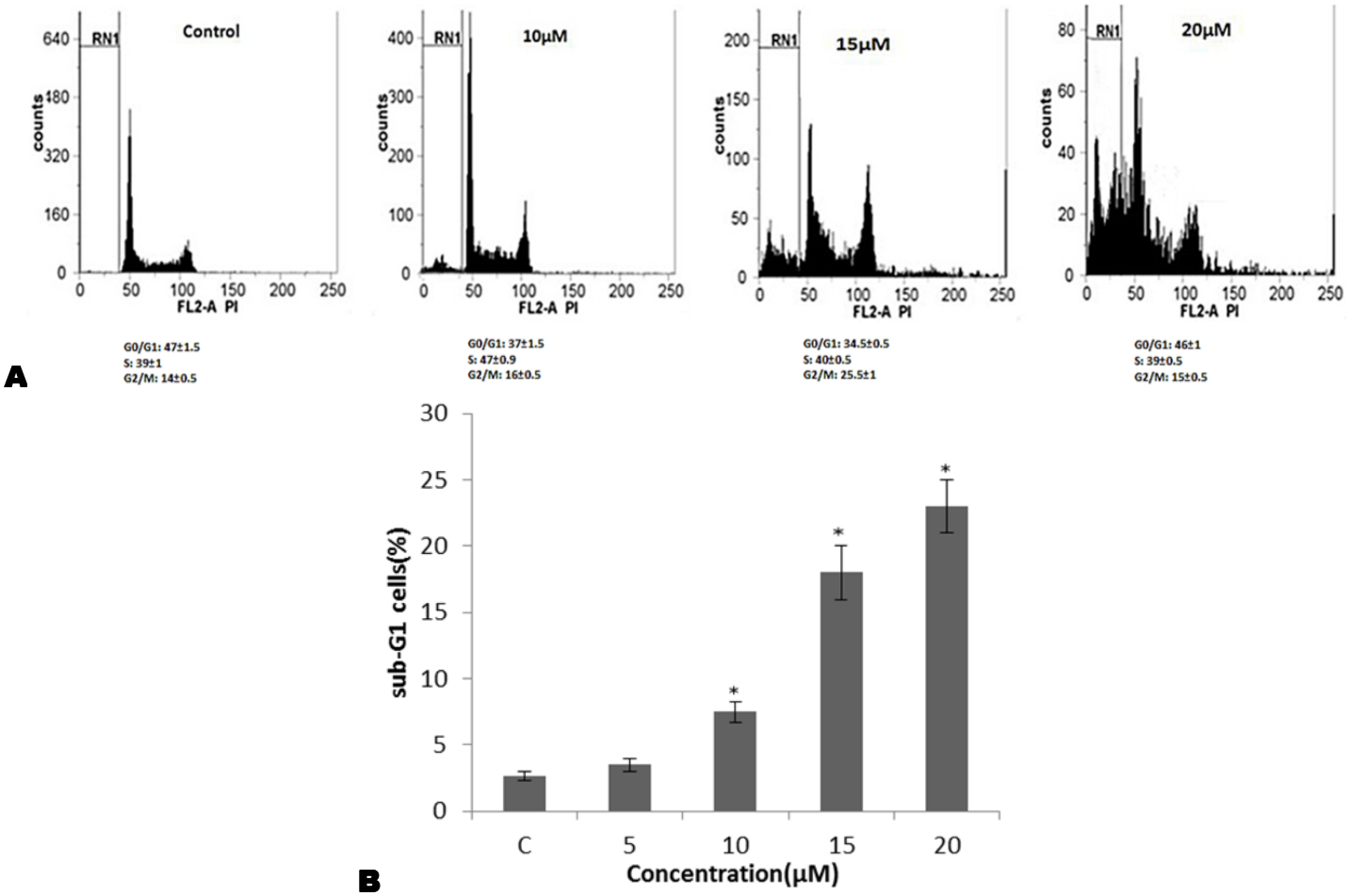

Figure- 3: The concentration-dependent effects of AS2O3 on cell cycle : **A)**Following 24 hours of AS2O3 treatment flow cytometric analysis was used to measure of cellular DNA content. Values are means ± SD of 3 independent experiments. The Sub-G1 region (RN1) represents the percentage of cells undergoing apoptosis. **B)** sub-G1 fraction was calculated and shown as % of total cell population.

Cytotoxic agents could inhibit the cell cycle at different phases and then induce apoptosis. As2O3 has been shown to induce reactive oxygen species and ROS can directly cause DNA damage in HL-60 cells.(24)

The G2-M DNA damage checkpoint is a common mechanism by which cells respond to genotoxic stress and prevent the cell from entering mitosis if the genome is damaged.

However, if DNA damage is severe, cells may undergo apoptosis to eliminate the damaged cell.(25, 26) In the current study, HL-60 cells treated with 15μM As2O3 remarkably accumulated in the G2/M phase of the cell cycle, with a parallel reduction in the percentage of cells in G0/G1 and S phases (Figure- 3). Furthermore, we found a significant S-phase delay before a G2-M arrest after 5-10 μM exposure (Figure- 3). It is known that arsenic trioxide induces G1, S or G2M phase arrest depending on the time, dose and types of tumor cells.(1-3) Previous studies have indicated that treatment with As2O3 resulted in G2/M arrest in NB4 line in a time- and dose-dependent manner.(27) Similar to our study, Huang et al, have showed incubation of HL-60 cells with 25μM As2O3 resulted in a marked cytotoxicity with no change in cell cycle distribution.(28) In contrast the cell-cycle perturbation was observed at 5μM concentrations. Flow cytometric analysis revealed an increase in the cell population in G2M-phase.(28) It seems that As2O3 induces G2/M arrest like anti-tubulin agents.

This is an agreement with the results reported by other research group. Ling YH *et al*. have demonstrated that As2O3 is a potent tubulin polymerizer (paclitaxel-like effect) but show no cross-resistance with paclitaxel in P-glycoprotein, MRP and mutated tubulin mediated drug resistance cell lines. They suggested the use of As2O3 for treating drug resistant patients.(29) Therefore, it is not surprising that a synergistic effect achieved in the combination therapy of As2O3 and paclitaxel.(30)

By and large, these studies support the notion that As2O3 may impart a wide range of biological and anti-tumor effects by affecting a number of targets that influence cell cycle progression and apoptosis depending upon tumor cell type, level and duration of exposure.

The major challenge for clinical use of arsenic in non-APL tumors is lowering the dose while maintaining the effectiveness of the drug.

Identification of the mechanisms that As2O3 exert its act regardless of whether the cells carried the PML/RARα fusion protein may offer new avenues in the treatment of non-APL hematologic malignancies and solid tumors.

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