Arsenic trioxide induces apoptosis via the mitochondrial pathway by upregulating the expression of Bax and Bim in human B cells

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Abstract. Arsenic trioxide (As$_2$O$_3$) has been approved for the treatment of acute promyelocytic leukemia (APML) and it is a promising candidate for the treatment of patients with lymphoproliferative disorders, such as relapsed or refractory multiple myeloma and myelodysplastic syndromes. The effects of As$_2$O$_3$ on B cells, specifically which do not express Bcl-2, have not been studied. In this study, we have demonstrated that As$_2$O$_3$, at clinically achievable therapeutic concentrations, induces apoptosis in Bcl-2 negative human B cell line Ramos. As$_2$O$_3$-induced apoptosis is associated with reduced mitochondrial transmembrane potential ($\Delta\psi$), enhanced generation of intracellular reactive oxygen species (ROS), release of cytochrome $c$ and apoptosis-inducing factor (AIF) from mitochondria into cytoplasm, activation of caspases, and upregulation of Bax and Bim expression. Exogenous glutathione (GSH) reverses the As$_2$O$_3$-induced apoptosis in a dose-dependent manner. Altogether, these data indicate that As$_2$O$_3$ induces apoptosis in B cells, regardless of Bcl-2 expression, via the mitochondrial pathway by enhancing oxidative stress.

Introduction

Arsenic trioxide (As$_2$O$_3$) is a trivalent inorganic compound used as a therapeutic agent particularly in Asia since ancient times. Recently, As$_2$O$_3$ has been shown to be effective in the treatment of patients with acute promyelocytic leukemia (1-3). In vitro studies suggest that arsenic derivatives may actually have a broader antineoplastic activity via inducing apoptosis in malignant cell lines including multiple myeloma, chronic lymphocytic leukemia, non-Hodgkin’s lymphoma, and solid tumors (4-11). Therefore, there is a growing interest in As$_2$O$_3$ as a potential therapeutic drug in malignancies which either do not improve or tend to relapse with conventional treatment regimens. Most of the studies that have examined the apoptotic induced by arsenicals point to the common finding that the mechanism involves activation of caspases and functional regulation of Bcl-2 family member proteins, particularly downregulation of Bcl-2 protein (4-7,10). However, the effect of As$_2$O$_3$ in B cells which lack Bcl-2 expression has not been studied. Hence, the potential use of As$_2$O$_3$ in Bcl-2 negative malignancies remains to be investigated.

Apoptosis (programmed cell death) plays an important role in a variety of physiological events including embryogenesis, metamorphosis, cellular homeostasis, tissue atrophy, tumor regression, selection of lymphocyte repertoires, clearance of activated T cells, regulation of immunological memory and cytotoxicity of target cells (12-14). There are two major pathways of apoptosis: the death receptor pathway (15,16) and the mitochondrial pathway (17-19), both of which involve molecular and biochemical steps that lead to the activation of common effector or executioner cysteine proteases, the caspases, resulting in the cleavage of a number of nuclear and cytoplasmic substrates that culminate in apoptosis. Chemotherapeutic agents along with a variety of stimuli including oxidative stress and radiation induce the mitochondrial pathway of apoptosis. Activation of mitochondria results in dissipation of membrane permeability transport pore (PTP), hence in mitochondrial membrane potential ($\Delta\psi$) depolarization and release of inter-membrane proteins, cytochrome $c$ and apoptosis inducing factor (AIF), from the mitochondria into the cytoplasm (17,18). Cytochrome $c$ binds to an adapter protein, Apaf, which then recruits pro-caspase-9 to form an apoptosome. This complex activates caspase-9 which in turn cleaves and activates effector pro-caspases to active effector caspases, including caspase-3. These effector caspases cleave a number of substrates resulting in morphological and biochemical changes of apoptosis (18-20). The function of PTP pore and release of intermembrane contents of mitochondria are under regulatory control of a number of Bcl-2 family proteins (20,21). Some of these members, including Bcl-2 and Bcl-x$_L$, prevent the release of cytochrome $c$ and AIF and therefore serve as anti-apoptotic proteins, whereas

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Abbreviations: APML, acute promyelocytic leukemia; GSH, reduced glutathione; $\Delta\psi$, mitochondrial membrane potential; PTP, mitochondrial permeability transition pore; ROS, reactive oxygen species; AIF, apoptosis-inducing factor; VDAC, voltage-dependent anion channel

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others, including Bax and Bim, promote the release of cytochrome c and AIF and serve as pro-apoptotic molecules (17-22).

In this study, we examined the apoptosis-inducing effect of As$_2$O$_3$ in Bcl-2 negative Ramos B cells. Our data showed that, in these cells, As$_2$O$_3$ induced apoptosis via the mitochondrial pathway by enhancing oxidative stress, Bax and Bim expression, and releasing cytochrome c and AIF from mitochondria to cytosol.

Materials and methods

Cell culture. Burkitt's lymphoma B cell line which was negative for Epstein-Barr virus and Bcl-2 expression (23,24) derived from a 3-year-old male Caucasian patient (Ramos, CRL-1596, ATCC, Manassas, VA) was selected. Cells, 1x10$^6$/ml, were grown in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 0.5 mM sodium pyruvate, 0.05 mM non-essential amino acids and 10% fetal bovine serum. Cells were maintained in 5 μM of JC-1, and then cells were acquired and analyzed by FACScan using Cell Quest software (Becton Dickinson, San Jose, CA).

Measurement of mitochondrial membrane potential ($\Delta\Psi_m$). Fluorescent lipophilic cationic dye JC-1 (5,5',6,6'-tetrachloro-1',3',3'-tetraethylbenzimidazol carboxyanine iodide) (Molecular Probes, Eugene, OR) was used to measure the mitochondrial membrane potential. Carbonyl cyanide m-(trifluoromethoxy)phenyl-hydrazone (FCCP) (Sigma), a potent mitochondrial membrane potential uncoupling agent, was used as an internal control. Cells (1x10$^6$/ml) were incubated at 37°C with As$_2$O$_3$, 5 μM alone or in the presence of FCCP 10 μM for 0.5, 2, 4 and 24 h. Five micromolar of As$_2$O$_3$ concentration was selected to investigate the further apoptotic events because that was a therapeutically achievable concentration. For the last 20 min of the incubation period, cultures were maintained in 5 μM of JC-1, and then cells were acquired by FACScan for FL-1 (green) and FL-2 (red) fluorescence. JC-1 exhibits potential-dependent accumulation in mitochondria and changes from green to red fluorescence indicates the accumulation of J-aggregates which correlates with the mitochondrial membrane potential. Green fluorescence represents the monomeric form of JC-1 and increase in green fluorescence represents depolarization.

Measurement of reactive oxygen species. Reactive oxygen species was determined by utilizing an oxidation-dependent fluorescence dye dihydrorhodamine (DHR) (Molecular Probes). Cells (1x10$^6$/ml) were cultured in the absence or presence of As$_2$O$_3$, 5 μM for 0.5, 2, 4 and 24 h. During the last 20 min of incubation, 5 μM of cell permeable DHR was added in culture at 37°C. Then cells were acquired and analyzed by FACScan using cell Quest Software.

Analysis of cytochrome c and AIF release. The release of cytochrome c and AIF from mitochondria to cytosol was analyzed by confocal microscopy. In brief, cells (1x10$^6$/ml) were incubated with As$_2$O$_3$, 5 μM for 24 h and loaded with Mitotracker orange 150 nM (Molecular Probes) by incubating for 15 min at 37°C. Then, cells were fixed with 3% (w/v) freshly prepared paraformaldehyde in phosphate-buffered saline (PBS). After washing three times in PBS (pH 7.4), cells were permeabilized by incubation with 0.25% (w/v) saponin in PBS for 5 min, and washed again three times in blocking buffer (0.05% saponin, 3% BSA in PBS, pH 7.4). Cells were incubated overnight with the primary cytochrome c antibody (Pharmingen, San Diego, CA). Cells were washed six times in blocking buffer, and then incubated with the secondary FITC-conjugated goat anti-mouse IgG antibody (Antibody Incorporated, Davis, CA) for 1 h at room temperature. After washing in PBS, cells were cytopsin onto slides and coverslips were mounted with an antifade reagent (Bio-Rad, Hercules, CA). For AIF release, anti-AIF antibody (ProSci Inc., Poway, CA) and FITC-labeled goat anti-rabbit IgG antibody (Oncogene, Boston, MA) were used as the primary and the secondary antibodies, respectively. Slides were imaged using a laser-based confocal microscope and the percentages of the cells that have a released cytochrome c and AIF were determined.

Measurement of caspase activities. Cells (1x10$^6$/ml) were incubated with As$_2$O$_3$, 5 μM at 37°C for 24 h. Caspase-3, -8, and -9 activities were assessed by flow cytometry. Cells were stained with carboxyfluorescein-labeled cell-permeable peptide substrates (Intergen Co., Purchase, NY) that recognize cleaved caspase-9 (FAM-LEHD-fmk), caspase-8 (FAM-IETD-fmk) and caspase-3 (FAM-DEVD-fmk), cells were acquired and analyzed by using FACScan.

Western blotting. Cells, 3x10$^6$, were treated with As$_2$O$_3$, 5 μM for 24 h and harvested by centrifugation. Whole cell extracts were prepared by lysing the cell pellet in 50 μl cold TGN1 buffer with protease and phosphatase inhibitors (100 mM Tris-Cl pH 7.4, 20% glycerol, 100 mM NaCl, 2% Triton X-100, 20 mM EGTA, 100 mM NaF, 4 mM phenylmethylsulfonyl fluoride, 4 mM sodium orthovanadate, and 2 mM p-nitrophenol phosphate) and centrifugation at 4°C for 20 min. Protein concentration of the lysates was determined by Bradford assay (Bio-Rad, Richmond, CA). Aliquots of cell lysates containing 25 μg of total protein were resolved by 10% SDS-Polyacryamide gel electrophoresis and transferred onto PVDF membranes by electroblotting. The membranes were blocked for 2 h at room temperature in TBS-T buffer with 5% non-fat dried milk, and sequentially probed by overnight incubation at 4°C with primary antibodies diluted in TBS-T buffer with 5% non-fat dried milk. These antibodies included anti-Bcl-2, anti-Bcl-xL, anti-Bax, anti-Bim and anti-VDAC (1:2000 dilution; Transduction Laboratory, San Diego, CA).
blots were washed three times for 15 min with TBS-T buffer and then incubated with HRP-conjugated anti-mouse secondary antibody (1:2000 dilution; Cell Signaling Technology, Beverly, MA) for 1 h at room temperature. After washing three times for 20 min in TBS-T buffer, blots were developed with ECL Plus detection system (Amersham Pharmacia Biotech Inc., UK). To normalize protein loading and transfer efficiency, the blots were also probed with anti-β-actin antibody (1:20,000 dilution; Chemicon, Temecula, CA).

Results

As$_2$O$_3$ induces apoptosis in Ramos cells in a concentration- and time-dependent manner. Ramos cells were incubated with various concentrations of As$_2$O$_3$ (2.5-10 μM) for 24 and 48 h. Apoptosis was measured by flow cytometry, using annexin-V labeling (Fig. 1). As$_2$O$_3$, in a concentration- and time-dependent manner, induced apoptosis in Ramos cells at 5 μM concentration, and the percentages of annexin-V (+) cells were 22 and 28% at 24 and 48 h, respectively. The corresponding figures were 40 and 66% at As$_2$O$_3$ 10 μM concentration.

As$_2$O$_3$ causes depolarization of the mitochondrial membrane potential ($\Delta$Ψ). In order to see the effect of As$_2$O$_3$ on the mitochondrial membrane potential, Ramos cells were incubated with As$_2$O$_3$ 5.0 μM for 0.5, 2, 4 and 24 h. This concentration of As$_2$O$_3$ was selected since it was a therapeutically achievable concentration (25). Mitochondrial membrane potential was measured with JC-1 staining using FACS (Fig. 2). As$_2$O$_3$ 5 μM caused maximum depolarization at 0.5 h as shown by a dramatic decrease in FL-2 and a slight increase in FL-1. JC-1 exhibits potential-dependent accumulation in mitochondria and FL-2 indicates the accumulation of J-aggregates which correlates with the mitochondrial membrane potential. FL-1 represents the monomeric form of JC-1 and increase in the proportion of FL1/FL-2 represents depolarization.

As$_2$O$_3$ induces oxidative stress and reduces intracellular GSH.

Arsenic compounds have been shown to induce generation of ROS in tumor cell lines (26-28) and oxidative stress is one of the mediators of apoptosis (29,30). Therefore, we examined the effect of As$_2$O$_3$, on intracellular levels of ROS. Ramos cells were incubated with As$_2$O$_3$, 5 μM for 0.5, 2, 4 and 24 h and intracellular ROS was measured with dihydrorhodamine (DHR), an oxidation sensitive dye using FACS. At 24-h time-point, As$_2$O$_3$ enhanced the intracellular levels of ROS significantly (25). Glutathione (GSH), an anti-oxidant, protects cells from undergoing stress-induced apoptosis (31,32) and reduced intracellular levels of GSH are associated with increased susceptibility to apoptosis (33). Therefore, to determine a role of decreased intracellular level of GSH in As$_2$O$_3$-induced apoptosis, Ramos cells were pre-incubated with As$_2$O$_3$, 5.0 μM for 2.5 and 5.0 μM for 2 h and then treated with As$_2$O$_3$, 5.0 μM for 24 h, and apoptosis was measured by annexin-V labeling (Fig. 4). Exogenous GSH 5.0 μM dragged the As$_2$O$_3$-induced apoptosis to baseline level, suggesting that As$_2$O$_3$ induces apoptosis in Ramos cells by altering the intracellular redox status.

As$_2$O$_3$ induces release of both cytochrome c and apoptosis-inducing factor (AIF) from mitochondria into cytoplasm.
Because depolarization of the mitochondrial membrane potential and generation of reactive oxygen species are associated with the release of pro-apoptotic intermembrane mitochondrial proteins (17-19), we examined the effect of \( \text{As}_2\text{O}_3 \) on cytochrome \( c \) and AIF release from the mitochondria. Ramos cells were incubated with \( \text{As}_2\text{O}_3 \) 5 \( \mu \text{M} \) for 24 h and release of cytochrome \( c \) and AIF was analyzed by confocal imaging. Fig. 5 illustrates sub-cellular localization of cytochrome \( c \) (green fluorescence) and AIF (green fluorescence) in relation to mitochondria (red fluorescence). Co-localization of green and red fluorescence (bright yellow) indicates mitochondrial localization. Distinct green staining shows the released protein from mitochondria to cytosol. In the presence of \( \text{As}_2\text{O}_3 \), cytochrome \( c \) and AIF release was observed in 85 and 45% of the cells, respectively.

\( \text{As}_2\text{O}_3 \) causes activation of caspases. Cytochrome \( c \) release leads to activation of the caspase cascade, beginning with caspase-9, followed by finally the executioner caspase-3 activation (17-20). Therefore, we examined the effect of \( \text{As}_2\text{O}_3 \) on the activation of caspase-9, caspase-3, and also on death receptor related caspase-8 in Ramos cells by intracellular staining. Ramos cells were incubated with \( \text{As}_2\text{O}_3 \) 5 \( \mu \text{M} \) for 24 h, and activation of caspase-9, -3, and -8 was measured by flow cytometry. \( \text{As}_2\text{O}_3 \) caused a marked activation of caspase-9 and -3, to a lesser extent caspase-8. Data are shown as the mean ± SD of three separate experiments.

\( \text{As}_2\text{O}_3 \) upregulates Bax and Bim expression. Bcl-2 family proteins regulate the release of cytochrome \( c \) and AIF from mitochondria, and subsequently activation of caspases and apoptosis; Bcl-2 and Bcl-\( x_l \), Bax, Bim and VDAC was examined by Western blotting using specific antibodies. \( \beta \)-actin was used as an internal control.
the PTP is VDAC (voltage-dependent anion channel), which is present in the outer membrane of the mitochondria. It is proposed that Bcl-2 family proteins regulate apoptosis by binding to VDAC (34) and PTP opening is controlled by redox status and Bcl-2 family proteins (20-22). Therefore, we examined the effect of As2O3 on the expression of Bcl-2 family proteins and VDAC. Ramos cells were treated with As2O3 5.0 μM for 24 h and the expression of Bcl-xL, Bax, Bim and VDAC was examined by Western blotting using specific antibodies and β-actin as an internal control. As2O3 upregulated the expression of both Bax and Bim (Fig. 7). However, there was no significant effect on Bcl-xL and VDAC expression.

Discussion

In this study, we have shown that As2O3 induces apoptosis in human B cell line Ramos, in which there is no Bcl-2 expression, via the mitochondrial pathway although previous studies conducted with Bcl-2 (+) cells attributed As2O3-induced apoptosis to down-regulation of anti-apoptotic Bcl-2 (4-7,10,35). We have demonstrated that downregulation of Bcl-2 is not required to induce mitochondrial release of pro-apoptotic intermembrane proteins in As2O3-induced apoptosis and upregulation of pro-apoptotic Bcl-2 family proteins, Bax and Bim, may be playing a major role in As2O3-induced apoptosis.

Mitochondria are organelles with two well-defined compartments: the matrix, surrounded by the inner membrane and the intermembrane space, surrounded by the outer membrane. The inner membrane contains various molecules, including ATP synthase, electron transport chain, and adenine nucleotide translocator. Under physiological conditions these molecules allow the respiratory chain to create an electrochemical gradient or membrane potential (ΔΨ). Depolarization of ΔΨ, generation of ROS, release of cytochrome c and AIF are major elements of mitochondrial pathway of apoptosis (17-19,36,37). Therefore, we examined the effect of As2O3 on ΔΨ and have observed that As2O3 at 5 μM concentration, which is clinically achievable, decreases ΔΨ, suggesting a depolarization of mitochondrial membrane after half an hour incubation. In this study, we have also observed that As2O3 increases intracellular levels of ROS in Ramos cells. Arsenites were previously reported to induce oxidative stress and to activate stress gene expression in cultured lung epithelial cells, HeLa cells, and cancer cells (26-28,38). The ROS also regulate apoptosis by modulating PTP (39). Cells contain several anti-oxidant systems to limit the damage caused by increased ROS (30,31). Glutathione is an anti-oxidant that protects cells from oxidative stress-induced apoptosis and glutathione monoethyl ester decreases apoptosis in fibroblasts (31-33). Furthermore, GHS depletion enforces mitochondrial PTP to induce apoptosis (40) and extrusion of GSH causes cytochrome c release (41). In this study, we have seen that exogenous glutathione protects Ramos cells from As2O3-induced apoptosis. As2O3 has also been shown to reduce GSH levels in tumor cells (39) and in vitro depletion of GSH renders tumor cells more susceptible to As2O3-induced apoptosis (32,33).

The permeability transition pore (PTP) is a channel spanning the inner and outer mitochondrial membrane. The intermembrane space contains a number of molecules including cytochrome c, and apoptosis-inducing factor (AIF). The permeabilization of the outer membrane, therefore, results in the release of these molecules. Our data show that As2O3 induces release of both cytochrome c and AIF from the mitochondria. The release of cytochrome c triggers the assembly of Apaf-1 (Apoptotic protease-activating factor) and pro-caspase-9 to form an apoptosome (12,17-19). Pro-caspase-9 is then cleaved to active caspases-9, which activates effector caspases including caspase-3 resulting in cleavage of its substrates and apoptosis. In this study, we have demonstrated that As2O3 activates caspase-9 and -3. AIF, once released from mitochondria, is transported into nucleus, where it stimulates (ATP-independent and caspase-independent) large DNA fragmentation and condensation of chromatin (37). Since As2O3 also causes AIF release from the mitochondria, it is possible that, in addition to cytochrome c-mediated caspase-dependent apoptosis, As2O3 might also induce apoptosis in a caspase-independent manner. Interestingly, we have observed that As2O3 causes some caspase-8 activation in addition to caspase-9 and -3, a finding that suggests that As2O3 may also induce apoptosis in B cells, to some degree, via death receptor signaling pathway (15,16).

Bcl-2 family proteins regulate the release of cytochrome c and AIF (42,43). The cytoplasmic Bax is translocated to the mitochondrial membrane, where it undergoes conformational changes. The docking of Bax to the outer membrane and its subsequent in situ clustering/multimerization causes gating of PTP and release of cytochrome c (22). The VDAC, which is an outer mitochondrial membrane channel protein, is also regulated by Bcl-2 family proteins and plays a role in apoptosis (44). In this study, we have shown that As2O3 increases the expression of pro-apoptotic Bax and Bim whereas there is no significant change in Bcl-xL and VDAC expression.

In conclusion, As2O3 induces apoptosis in Ramos cells mainly by the release of cytochrome c and AIF from the mitochondria as a result of upregulating pro-apoptotic Bcl-2 family proteins, Bax and Bim, and of enhancing ROS generation. Therefore, in addition to a number of other malignancies, As2O3 appears to be a potential chemotherapeutic agent in B-cell proliferations that do not have Bcl-2 expression.

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References


