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_2O_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_2O_3 on B cells, specifically which do not express Bcl-2, have not been studied. In this study, we have demonstrated that As_2O_3, at clinically achievable therapeutic concentrations, induces apoptosis in Bcl-2 negative human B cell line Ramos. As_2O_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_2O_3-induced apoptosis in a dose-dependent manner. Altogether, these data indicate that As_2O_3 induces apoptosis in B cells, regardless of Bcl-2 expression, via the mitochondrial pathway by enhancing oxidative stress.

Introduction

Arsenic trioxide (As_2O_3) is a trivalent inorganic compound used as a therapeutic agent particularly in Asia since ancient times. Recently, As_2O_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_2O_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 apoptotis 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_2O_3 in B cells which lack Bcl-2 expression has not been studied. Hence, the potential use of As_2O_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_2O_3 in Bcl-2 negative Ramos B cells. Our data showed that, in these cells, As_2O_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, ATTC, 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 incubated in the absence and presence of As_2O_3 (Sigma, St. Louis, MO) at various concentrations from 2.5 to 10.0 μM.

Determination of apoptosis. Apoptosis was measured by annexin-V staining (Becton Dickinson, San Jose, CA) according to manufacturer's instructions. After incubation with As_2O_3 at concentrations of 2.5, 5 and 10 μM for 24 and 48 h, cells were spun at 1200 g for 5 min and the supernatant was decanted. Cell pellet was resuspended with 100 μl of annexin-V binding buffer and 5 μl of annexin-V dye, and was left in dark at room temperature for 15 min. Following incubation, an additional 400 μl of annexin-V binding buffer was added to each sample. Ten thousand cells were acquired and analyzed by FACScan using Cell Quest software (Becton Dickinson, San Jose, CA).

Measurement of mitochondrial membrane potential ($\Delta\psi$). Fluorescent lipophilic cationic dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol carbocyanine 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_2O_3 5 μM alone or in the presence of FCCP 10 μM for 0.5, 2, 4 and 24 h. Five micromolar of As_2O_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_2O_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_2O_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 cytospun 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_2O_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_2O_3 5 μM for 24 h and harvested by centrifugation. Whole cell extracts were prepared by lysing the cell pellet in 50 μl cold TGNT 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-Polyacrymide 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-x_L, anti-Bax, anti-Bim and anti-VDAC (1:2000 dilution; Transduction Laboratory, San Diego, CA). The

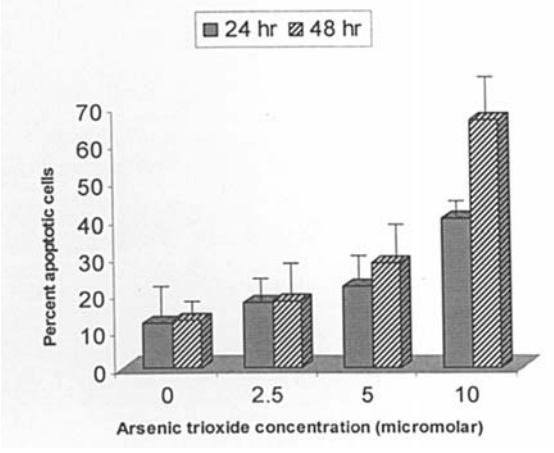

Figure 1. Effect of As_2O_3 on apoptosis in Ramos cells. Cells were treated with As_2O_3 0-10 μM for 24 and 48 h. Apoptosis was detected by annexin-V staining using flow cytometry. Data are shown as the mean ± SD of three separate experiments.

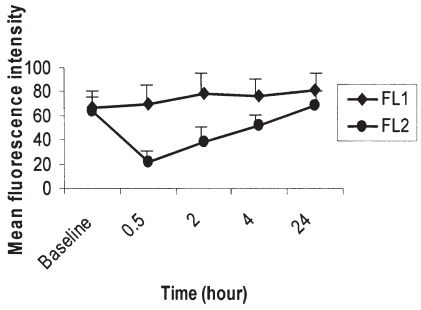

Figure 2. Effect of As_2O_3 on mitochondrial membrane potential ($\Delta\psi$) depolarization in Ramos cell line (JC-1 staining). Cells were treated with As_2O_3 5 μM for 0.5, 2, 4 and 24 h. As_2O_3 caused maximum ($\Delta\psi$ depolarization at 0.5 h as shown by a dramatic decrease in FL-2 and a slight 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. Data are shown as the mean ± SD of three separate experiments.

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-ß-actin antibody (1:20,000 dilution; Chemicon, Temecula, CA).

Results

As_2O_3 induces apoptosis in Ramos cells in a concentration- and time-dependent manner. Ramos cells were incubated with various concentrations of As_2O_3 (2.5-10 μM) for 24 and 48 h. Apoptosis was measured by flow cytometry, using annexin-V labeling (Fig. 1). As_2O_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_2O_3 10 μM concentration.

As_2O_3 causes depolarization of the mitochondrial membrane potential ($\Delta\psi$). In order to see the effect of As_2O_3 on the mitochondrial membrane potential, Ramos cells were incubated with As_2O_3 5.0 μM for 0.5, 2, 4 and 24 h. This concentration of As_2O_3 was selected since it was a therapeutically achievable concentration (25). Mitochondrial membrane potential was measured with JC-1 staining using FACScan. Fig. 2 shows the effect of As_2O_3 5.0 μM on mitochondrial membrane potentials at different time points up to 24 h. Arsenic trioxide induced depolarization of $\Delta\psi$ at all time-points, most notably at 0.5 h of incubation, demonstrated by a shift from red to green green fluorescence, i.e. an increased proportion of FL1 to FL2 fluorescence intensity.

As_2O_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

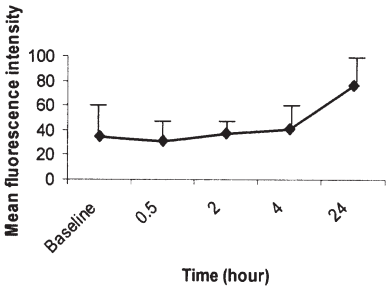

Figure 3. Effect of As_2O_3 on ROS generation in Ramos cells by dihydrorhodamine (DHR) staining. Cells were incubated with As_2O_3 5 μM for 0.5, 2, 4 and 24 h. As_2O_3 enhanced ROS generation as shown by an increase in DHR fluorescence. Data are shown as the mean ± SD of three separate experiments.

the mediators of apoptosis (29,30). Therefore, we examined the effect of As_2O_3 on intracellular levels of ROS. Ramos cells were incubated with As_2O_3 5 μM for 0.5, 2, 4 and 24 h and intracellular ROS was measured with dihydrorhodamine (DHR), an oxidation sensitive dye using FACScan. At 24-h time-point, As_2O_3 enhanced the intracellular levels of ROS significantly (D=>0.2; D values is a computer generated value using K-S software of FACS) (Fig. 3).

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_2O_3-induced apoptosis, Ramos cells were pre-incubated with GSH 2.5 and 5.0 μM for 2 h and then treated with As_2O_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_2O_3-induced apoptosis to baseline level, suggesting that As_2O_3 induces apoptosis in Ramos cells by altering the intracellular redox status.

As_2O_3 induces release of both cytochrome c and apoptosis-inducing factor (AIF) from mitochondria into cytoplasm.

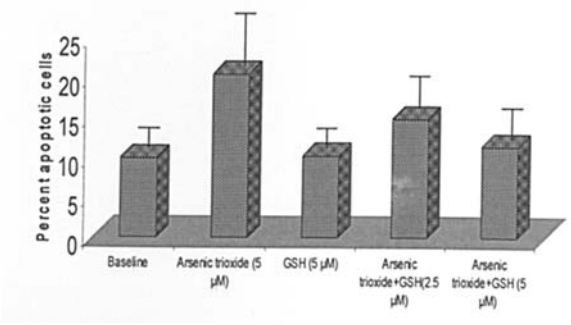

Figure 4. Effect of exogenous glutathione (GSH) on As_2O_3-induced apoptosis in Ramos cells. Cells were cultured for 2 h with GSH 2.5 μM and GSH 5 μM, and subsequently were exposed to As_2O_3 5 μM for 24 h. The percentage of apoptotic cells was determined by annexin-V labeling. Data are shown as the mean ± SD of three separate experiments. Exogenous GSH reversed As_2O_3-induced apoptosis.

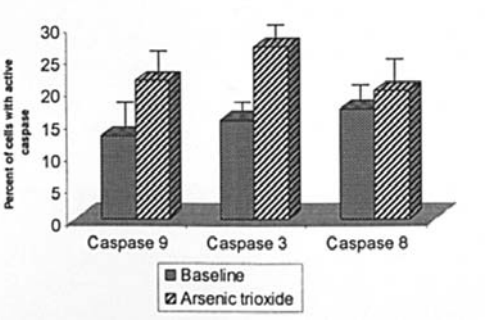

Figure 6. Effect of As_2O_3 on caspase activation in Ramos cells. Cells were treated with As_2O_3 5 μM for 24 h and activation of caspases was examined by staining with carboxyfluorescein-labeled peptide substrates of cleaved caspase-9, -3 and -8 using flow cytometry. As_2O_3 caused a marked activation of caspase-9 and -3, to a lesser extent caspase-8. Data are shown as the mean ± SD of three separate experiments.

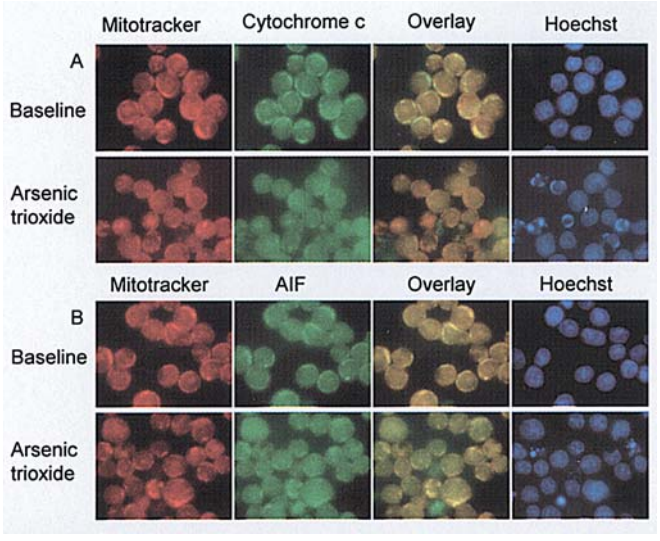

Figure 5. Effect of As_2O_3 on cytochrome *c* (A) and AIF (B) release from mitochondria into the cytoplasm in Ramos cells. Cellular localization of mitochondria is depicted with red fluorescence whereas green fluorescence indicates that of cytochrome *c* or AIF. Co-localization of red and green fluorescence, yellow color in overlaid images, shows the mitochondrial localization of cytochrome *c* or AIF at the baseline (top panels). Distinct cellular green staining points out the cytoplasmic localization, i.e. release of cytochrome *c* or AIF from mitochondria to the cytoplasm in response to As_2O_3 5 μM treatment (bottom panels). Hoechst 33258 staining shows the apoptotic nuclei at the baseline and after As_2O_3 treatment.

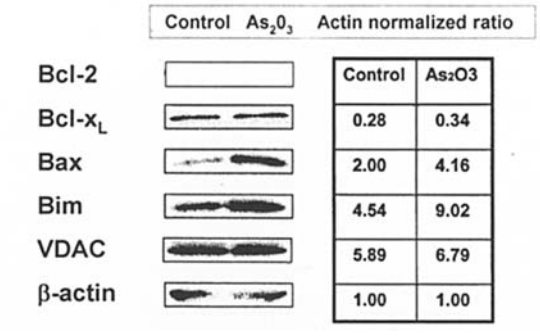

Figure 7. Effect of As_2O_3 on expression of pro- and anti-apoptotic molecules. Cells were treated with As_2O_3 5 μM for 24 h and the expression of Bcl-2, Bcl-x_L, Bax, Bim and VDAC was examined by Western blotting using specific antibodies. ß-actin was used as an internal control.

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 As_2O_3 on cytochrome *c* and AIF release from the mitochondria. Ramos cells were incubated with As_2O_3 5 μM 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 As_2O_3, cytochrome *c* and AIF release was observed in 85 and 45% of the cells, respectively.

As_2O_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 As_2O_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 As_2O_3 5 μM for 24 h, and activation of caspase-9, -3, and -8 was measured by carboxyfluorescein-labeled peptide substrates of cleaved caspase-9, -3 and -8 by flow cytometry. As_2O_3 markedly activated caspase-9 and -3, and minimally caspase-8 in Ramos cells (Fig. 6).

As_2O_3 upregulates Bax and Bim expression. Bcl-2 family proteins regulate the release of *cyto*chrome *c* and AIF from mitochondria, and subsequently activation of caspases and apoptosis; Bcl-2 and Bcl-x_L block, whereas Bax and Bim promote the release of cytochrome *c* and AIF (20-22). Cytochrome *c* may be released via the opening of mitochondrial permeability transition pore (PTP). One of the components of

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 As_2O_3 on the expression of Bcl-2 family proteins and VDAC. Ramos cells were treated with As_2O_3 5.0 μM for 24 h and the expression of Bcl-x_L, Bax, Bim and VDAC was examined by Western blotting using specific antibodies and ß-actin as an internal control. As_2O_3 upregulated the expression of both Bax and Bim (Fig. 7). However, there was no significant effect on Bcl-x_L and VDAC expression.

Discussion

In this study, we have shown that As_2O_3 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 As_2O_3-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 As_2O_3-induced apoptosis and upregulation of pro-apoptotic Bcl-2 family proteins, Bax and Bim, may be playing a major role in As_2O_3-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 ($\Delta\psi$). Depolarization of $\Delta\psi$, 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 As_2O_3 on $\Delta\psi$ and have observed that, As_2O_3 at 5 μM concentration, which is clinically achievable, decreases $\Delta\psi$, suggesting a depolarization of mitochondrial membrane after half an hour incubation. In this study, we have also observed that As_2O_3 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 As_2O_3-induced apoptosis. As_2O_3 has also been shown to reduce GSH levels in tumor cells (39) and *in vitro* depletion of GSH renders tumor cells more susceptible to As_2O_3-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 As_2O_3 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 As_2O_3 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 As_2O_3 also causes AIF release from the mitochondria, it is possible that, in addition to cytochrome *c*-mediated caspase-dependent apoptosis, As_2O_3 might also induce apoptosis in a caspase-independent manner. Interestingly, we have observed that As_2O_3 causes some caspase-8 activation in addition to caspase-9 and -3, a finding that suggests that As_2O_3 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 As_2O_3 increases the expression of pro-apoptotic Bax and Bim whereas there is no significant change in Bcl-x_L.and VDAC expression.

In conclusion, As_2O_3 induces apoptosis in Ramos cells mainly by the release of cytochrome *c* and AIF from the mitochondria as a result of upregulating pro-apopotic Bcl-2 family proteins, Bax and Bim, and of enhancing ROS generation. Therefore, in addition to a number of other malignancies, As_2O_3 appears to be a potential chemotherapeutic agent in B-cell proliferations that do not have Bcl-2 expression.

Acknowledgements

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