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Arsenic trioxide (As_2O_3) induces apoptosis through activation of Bax in hematopoietic cells

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This study explores the roles of Bax and other Bcl-2 family members play in arsenic trioxide (As_2O_3)-induced apoptosis. We showed that As_2O_3 treatment triggered Bax conformational change and subsequent translocation from cytosol to mitochondria to form various multimeric homo-oligomers in IM-9 cells. On the other hand, human leukemic Jurkat cells deficient in Bax showed dramatically reduced apoptosis in response to As_2O_3. Stable overexpression of Bcl-2 in IM-9 cells (IM-9/Bcl-2) inhibited As_2O_3-mediated Bax activation and apoptosis, and this inhibition could be partially averted by cell-permeable Bid-Bcl-2 homology (BH)3 peptide. Meanwhile, Bax conformational change and oligomerization induced by As_2O_3 were not inhibited by the pancaspase inhibitor z-VAD-fmk, although Bid cleavage could be completely abolished. Bax activation by As_2O_3 seemed to require stress-induced intracellular reactive oxygen species (ROS), since the ROS scavengers (*N*-acetyl-L-cysteine and lipoic acid) could completely block the conformational change and translocation of Bax from cytosol to mitochondria. These data suggest that As_2O_3 might exert the cell killing in part by inducing Bax activation through a Bcl-2-suppressible pathway in hematopoietic cells that is caspase independent and intracellular ROS regulated.
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Introduction

Arsenic trioxide (As_2O_3) is an ancient remedy, which has attracted a renewed attention owing to its therapeutic application in the induction of complete remission of newly diagnosed or even relapsed APL (Shen *et al*., 1997; Soignet *et al*., 1998; Zhang *et al*., 2001). Although the reason for this action is unclear, apoptosis may be involved. More recently, it was shown that the apoptosis-promoting effects of As_2O_3 are not limited to APL cells alone (Dai *et al*., 1999; Li and Broome, 1999; Rousselot *et al*., 1999; Anderson *et al*., 2002). This agent apparently affects multiple intracellular signal transduction pathways, which are associated with the regulation of apoptosis. Several mechanisms, including caspase activation, enhanced generation of reactive oxygen species (ROS), mitochondrial permeability transition pore opening, suppression of the apoptosis-inhibitory Ras/MAP kinase cascade and enhanced translocation of PML protein to nuclear bodies, have been suggested to participate in the process of cell death triggered by As_2O_3 (Akao *et al*., 1998; Chen *et al*., 1998b; Doza *et al*., 1998; Quignon *et al*., 1998; Jing *et al*., 1999; Larochette *et al*., 1999; Miller *et al*., 2002; Zheng *et al*., 2004). However, currently the precise mechanism through which As_2O_3 induces apoptosis is still not clear.

The Bcl-2 family proteins appear to function as molecular gatekeepers, determining whether or not the apoptotic program will commence (Yang *et al*., 1997; Vander Heiden and Thompson, 1999). This ever-expanding family of proteins now encompasses over 30 members, which can be subdivided into three separate categories. The first category is comprised of antiapoptotic proteins, such as Bcl-2 and Bcl-xL, which have all four of the evolutionarily conserved Bcl-2 homology (BH) domains 1–4. The remaining two categories make up the proapoptotic members of the Bcl-2 family, which consist of either the multidomain death effectors such as Bax and Bak or the BH3-only proteins such as Bid (Huang and Strasser, 2000; Adams and Cory, 2001). The mitochondria has emerged as a central component of the apoptotic cascade, serving both as a major amplification step of apoptosis as well as the principal site of action for pro- and antiapoptotic members of the Bcl-2 family (Green and Reed, 1998; Desagher and Martinou, 2000). One current model suggests that the BH3-only proteins could induce the activation of Bax and Bak at the level of mitochondria, leading to the loss of mitochondrial integrity. On the outer membrane of the mitochondria, oligomerized Bax and Bak may form a channel or membrane pore, which allows the release of

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apoptogenic factors such as cytochrome *c* (cyt *c*), Smac/Diablo and AIF (Desagher *et al*., 1999; Eskes *et al*., 2000; Murphy *et al*., 2000; Mikhailov *et al*., 2001; Yamaguchi and Wang, 2002). In addition, other mitochondrial protein such as VDAC could potentially interact with Bax or Bak to form a mega-channel (Shimizu *et al*., 2001; Tsujimoto and Shimizu, 2002; Shi *et al*., 2003). We and others have recently shown that the mitochondria seem to be the primary target for As_2O_3-induced apoptosis (Petronilli *et al*., 1994; Costantini *et al*., 1996; Larochette *et al*., 1999). Specifically, we recently demonstrated that the mitochondrial outer membrane protein VDAC is a biological target for As_2O_3, which may be involved in its ability to induce apoptosis (Zheng *et al*., 2004). Given the critical function of Bax in the initiation of apoptosis at the level of mitochondria and the mechanism underlying the activation of Bax in As_2O_3-induced apoptosis was not systematically addressed, we sought to examine the role of Bax and Bcl-2 family proteins in As_2O_3-induced apoptosis.

Results

As_2O_3 induces apoptosis blocked by ectopic Bcl-2 expression in IM-9 cells

As reported previously (Zheng *et al*., 2004), our current data reaffirmed the observation that clinic achievable concentrations of As_2O_3 could kill IM-9 cells by apoptosis in a Bcl-2 inhibitable manner. Phosphotidylserine exposure, which is a defining characteristic of apoptotic cells, was examined in As_2O_3-treated IM-9 and IM-9/Bcl-2 cells. FACS analysis showed that 2 μM As_2O_3 induced a significant increase of the Annexin V-positive population (apoptotic cells) in IM-9 cells, which could be suppressed by stably overexpressed Bcl-2 (Figure 1a). It is known that cyt *c* release and caspase 3 activation are two major biochemical events for the occurrence of apoptosis. We thus examined cyt *c* release (Figure 1b) and the status of caspase 3 (Figure 1c) in As_2O_3-induced cell death. Western blotting revealed that cyt *c* was released and caspase 3 was activated as early as 4 h after As_2O_3 treatment in IM-9 cells before the appearance of Annexin V positivity. All these data suggest that As_2O_3 induces typical apoptosis in IM-9 cells and overexpression of Bcl-2 prevents the cell death induced by As_2O_3.

As_2O_3 induces translocation of Bax protein from cytosol to mitochondria in IM-9 cells

To investigate the role of Bax in regulating As_2O_3-induced apoptosis, we compared the change of expression and subcellular localization of Bax protein in IM-9 and IM-9/Bcl-2 cells before and after As_2O_3 treatment. Immunoblotting analysis revealed that there was an increase (1.50-fold after 12 h and 1.75-fold after 24 h treatment) in the Bax protein in IM-9 cells challenged with 2 μM As_2O_3, and the levels of Bax expression did not change in IM-9/Bcl-2 cells (Figure 2a). The increase

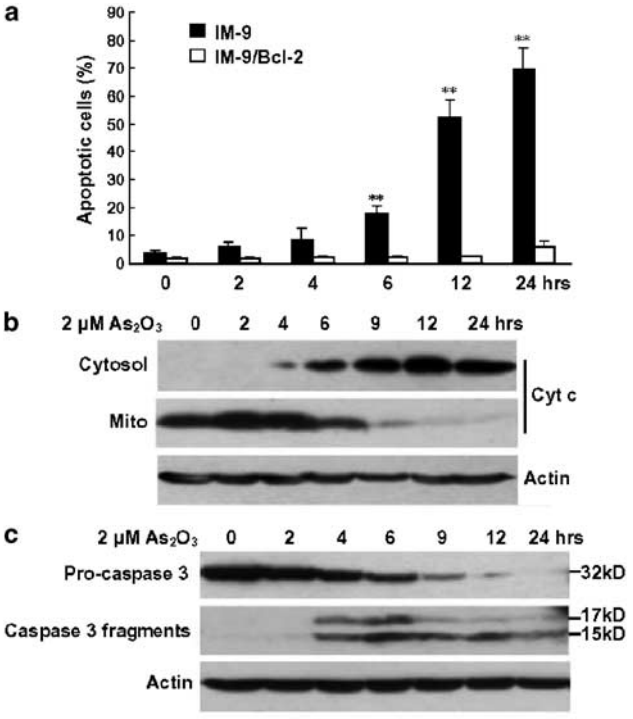

Figure 1 As_2O_3 treatment induces apoptosis of IM-9 cells, which is inhibited by Bcl-2. (**a**) IM-9 or IM-9/Bcl-2 cells were treated with 2 μM As_2O_3 for the indicated times, and the percentage of apoptotic cells were characterized as those that stained with Annexin V using the Annexin V assay kit (see 'Materials and methods'). Data represent the mean values of three independent experiments (**$P<0.01$). (**b**) IM-9 cells were exposed to 2 μM As_2O_3 for the indicated times and subjected to subcellular fractionation. Then, the cytosolic fraction (Cytosol) and mitochondrial fraction (Mito) were analysed by immunoblotting (30 μg of protein per lane) with antibody specific for cyt *c*. β-actin was used as a protein loading control. Data were representative of three separate experiments. (**c**) IM-9 cells were collected at the indicated times after being treated with 2 μM As_2O_3, and were analysed by Western blotting for caspase 3 cleavage. β-actin was used as a protein loading control. Data were representative of three separate experiments

of Bax expression in IM-9 cells at late time points may be due to the As_2O_3-induced oxidative stress, which could be inhibited by overexpressing of Bcl-2 (see Figure 7). On the other hand, no perceptible change in the protein levels of Bcl-2 could be observed in IM-9 or IM-9/Bcl-2 cells after treatment with 2 μM As_2O_3 (Figure 2a). Cell fractionation results showed that Bax protein translocated from cytosol to mitochondria following the As_2O_3 treatment in IM-9 cells. As shown in Figure 2b, the protein levels of Bax decreased in the cytosolic fractions and, concomitantly, increased in the mitochondria-enriched heavy membrane fractions of IM-9 cells starting at 4 h after As_2O_3 treatment. In contrast, no Bax translocation was detected in IM-9/Bcl-2 cells even 24 h after As_2O_3 treatment (Figure 2b). These results suggest that Bax translocation from cytosol to mitochondria happens at the time of cyt *c* release from mitochondria to cytosol and the activation of caspase 3, all of which are preceding the appearance of morphological change of apoptosis.

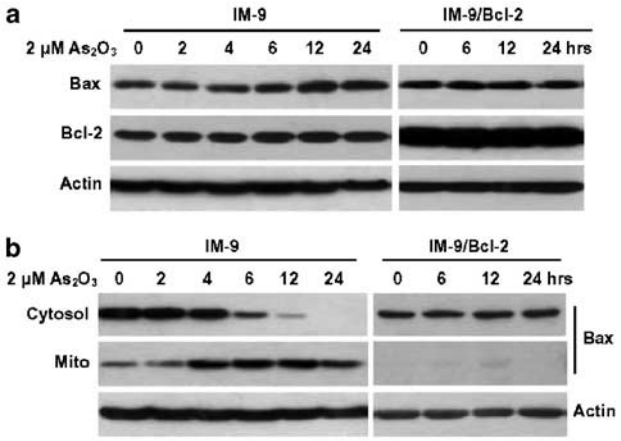

Figure 2 As_2O_3 induces increase of Bax expression and Bax translocation from cytosol to mitochondria in IM-9 cells, which are absent in IM-9/Bcl-2 cells. (**a**) IM-9 or IM-9/Bcl-2 cells were treated with 2 μM As_2O_3 for the indicated times, followed by preparation of cell lysates and SDS–PAGE/immunoblot analysis with the indicated antibodies. β-actin was used as a protein loading control. (**b**) IM-9 or IM-9/Bcl-2 cells were exposed to 2 μM As_2O_3 for the indicated times, and subjected to subcellular fractionation. Then, the cytosolic fraction (Cytosol) and mitochondrial fraction (Mito) were analysed by immunoblotting (30 μg of protein per lane) with antibodies specific for Bax using β-actin as a protein loading control

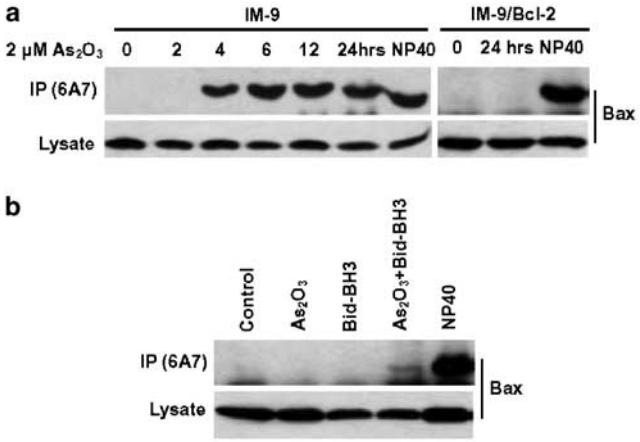

Figure 3 As_2O_3 induces Bax conformational change in IM-9 cells in a Bcl-2 inhibitable manner, and membrane-permeable Bid-BH3 sensitizes Bcl-2-overexpressing cells to As_2O_3-induced Bax conformational change. (**a**) After being treated with 2 μM As_2O_3, IM-9 or IM-9/Bcl-2 cells were lysed in Chaps lysis buffer and subjected to immunoprecipitation with anti-Bax 6A7 antibody for detection of conformationally changed Bax protein. Cell lysate obtained by NP-40 lysis was used as positive control. In addition, the total lysates were applied directly to SDS–PAGE/immunoblot analysis with specific anti-Bax polyclonal antibody. (**b**) IM-9/Bcl-2 cells were treated with 2 μM As_2O_3, 50 μM TAT-Bid-BH3 alone or in combinations for 24 h. The conformationally changed Bax protein was detected by immunoprecipitation with anti-Bax 6A7 antibody

As_2O_3 induces Bax conformational change in IM-9 cells in a Bcl-2 inhibitable manner, and membrane-permeable Bid-BH3 peptide sensitizes Bcl-2-overexpressing IM-9 cells to As_2O_3-mediated Bax conformational changes

It has been previously reported that Bax conformational change could be induced by several types of apoptotic stimuli (Desagher *et al*., 1999; Yamaguchi and Wang, 2002; Dewson *et al*., 2003). These findings prompted us to investigate whether As_2O_3 could induce Bax to undergo a conformational change. As_2O_3-treated IM-9 cells were lysed in 1% Chaps, and immunoprecipitation was carried out with anti-Bax 6A7 monoclonal antibody that specifically recognizes the conformationally changed Bax protein (Hsu and Youle, 1998; Murphy *et al*., 2000; Yamaguchi and Wang, 2001; Yamaguchi *et al*., 2002). The zwitterionic detergent Chaps was chosen due to the fact that it has been shown to retain the Bax protein in its native conformation, whereas nonionic detergents, such as NP-40, can induce a conformational change in the Bax protein and as such can be used as a positive control (Hsu and Youle, 1997). As shown in Figure 3a, conformationally changed Bax was detectable in IM-9 cells starting from 4 h after treatment with 2 μM As_2O_3. As expected, Bcl-2 has the ability to retain Bax conformation in IM-9/Bcl-2 cells after As_2O_3 treatment since there was no detectable conformationally changed Bax protein in IM-9/Bcl-2 cell lysates prepared with 1% Chaps even after 24 h of treatment with 2 μM As_2O_3 (Figure 3a). These results indicate that As_2O_3 can evoke Bax conformational change while it induces the Bax translocation, the cyt *c* release and its activation of caspases for apoptosis.

Since Bcl-2 blocked As_2O_3-induced Bax conformational change, we reasoned that inhibition of Bcl-2 should reverse this effect, and restore the wild-type phenotype, thereby sensitizing Bcl-2-overexpressing cells to apoptosis and restore Bax activation induced by As_2O_3. It has been shown that the membrane-permeable Bid-BH3 peptide kills cells by binding to and antagonizing the antiapoptotic proteins Bcl-2 and Bcl-xL (Wang *et al*., 1996; Xia *et al*., 2002). Therefore, we treated IM-9/Bcl-2 cells with either As_2O_3, the Bcl-2 inhibitor Bid-BH3 peptide, or a combination thereof, and assayed Bax conformational change via immunoprecipitation. Our results indicated that the cell-permeable Bid-BH3 peptide indeed promoted As_2O_3-mediated Bax conformational change (Figure 3b) and apoptosis (not shown) in IM-9/Bcl-2 cells.

Bax oligomerization is a late event induced by As_2O_3 in IM-9 cells

The oligomerization of Bax has previously been reported to only occur in apoptotic cells, possibly playing a role in mediating cyt *c* release (Antonsson *et al*., 2001). It was for this reason that we investigated whether As_2O_3 could trigger Bax oligomerization. Following treatment with As_2O_3, IM-9 cells were exposed to the membrane-permeable crosslinking agent disuccinimidyl suberate (DSS) and subjected to SDS–PAGE/immunoblot for the analysis of Bax oligomerization. As shown in Figure 4a and b, a Bax immunoreactive band of ~42–46 kDa, previously reported as a Bax homodimer (Eskes *et al*., 2000), could be detected

from the As_2O_3-treated IM-9 cell lysate. In addition, a ~90 kDa band or higher, which could be a Bax tetramer or oligomer, were also detectable. Bax multimers could not be detected in untreated IM-9 cells or cells treated with As_2O_3 for 6 h, and in Bcl-2-overexpressing cells (Figure 4a and b). Bax oligomerization could be detected in IM-9 cells starting from 8 h (data not shown) after treatment by 2 μM As_2O_3. These results demonstrate that As_2O_3 induces Bax dimmer- or oligomerization, and it is a late event compared with the appearance of Bax conformational change, translocation and caspase 3 activation. To further confirm the existence of Bax dimer or oligomer, we used DSS to crosslink the As_2O_3-treated cells, and then performed immunoprecipitation with Bax antibody. We found that Bax monomers and oligomers were present in As_2O_3-treated IM-9 cells (Figure 4c), but we were unable to detect the presence of Bcl-2 in these complexes (data not shown). These results indicate that either the Bax-Bcl-2 dimer is a dynamic complex or only a portion of the Bax protein becomes homo-oligomerized in the mitochondria during apoptosis.

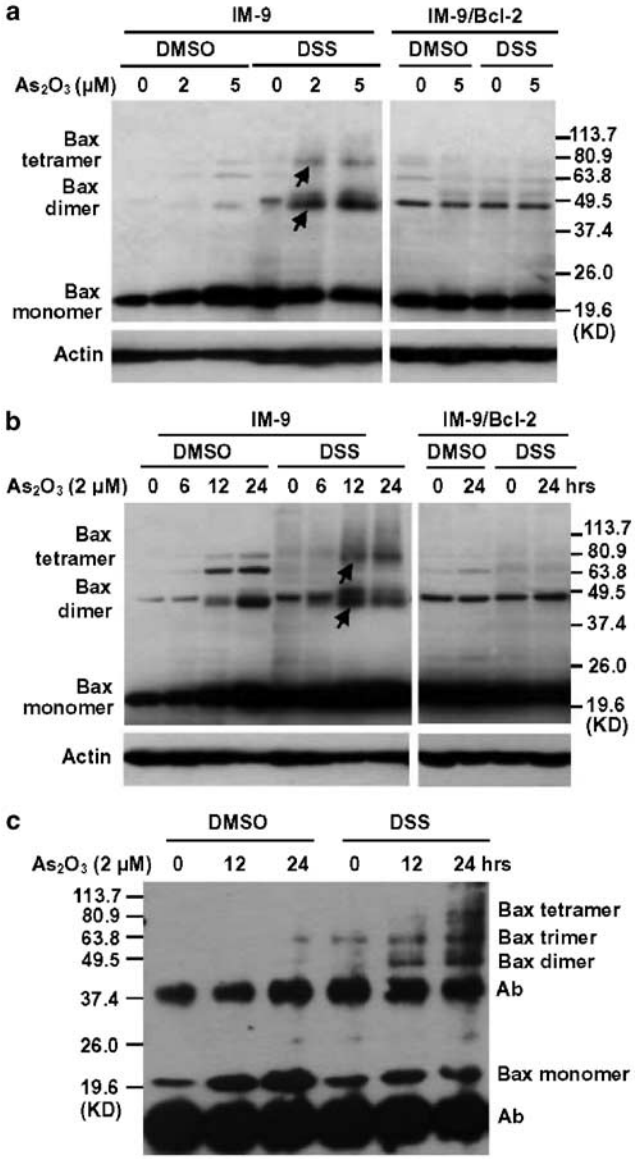

Figure 4 As_2O_3 induces Bax oligomerization in IM-9 cells, which can be blocked by stably overexpressing of Bcl-2. IM-9 or IM-9/Bcl-2 cells (**a**) were treated with 2 or 5 μM As_2O_3 for 12 h, or (**b**) were treated with 2 μM As_2O_3 for the indicated times. The oligomerization of Bax was assessed by crosslinking with DSS followed by immunoblot analysis. DMSO was used as the vehicle control and β-actin was used as a loading control. Arrows indicate Bax dimer or tetramer (**c**) IM-9 cells were treated with 2 μM As_2O_3 for the indicated times, followed by crosslinking with DSS. Bax complexes were immunoprecipitated using Bax monoclonal antibody and Western blotted with Bax rabbit polyclonal antibody. Ab denotes IgG chains

Bax-deficient (−) Jurkat cells are resistant to As_2O_3-induced cell death

To further clarify the role of Bax in the As_2O_3-induced apoptosis, we compared the sensitivity of Bax (−) cells and control cells to As_2O_3. To isolate Bax (−) clones, the human T leukemic Jurkat cells were seeded at an average of 0.5 cells/well in 0.1 ml of RPMI 1640 medium containing 10% FBS. Clones lacking Bax protein were identified by SDS–PAGE/immunoblot analysis with anti-Bax antibodies (Figure 5a). Several Bax (−) Jurkat

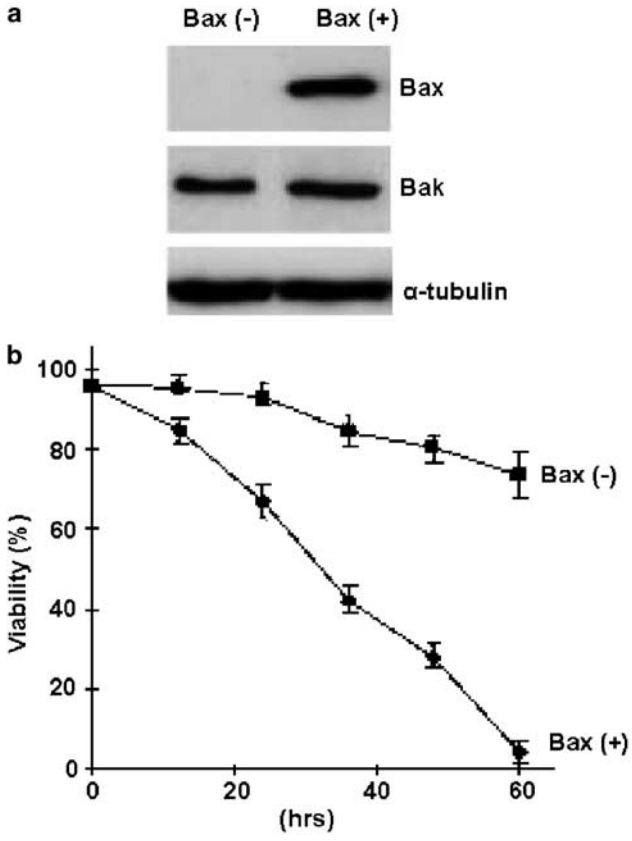

Figure 5 Resistance of Bax (−) Jurkat cells to As_2O_3-induced cell death. (**a**) Bax (+) and Bax (−) Jurkat clones were lysed in 1% NP-40 buffer and applied to SDS–PAGE/immunoblot analysis (50 μg/lane) with specific antibodies for Bax, Bak or α-tubulin. (**b**) Bax (+) and Bax (−) Jurkat cells were treated with 4 μM As_2O_3 for various times and subjected to trypan blue exclusion assay

clones with similar Bak expression were isolated. To determine whether Bax is required for As_2O_3-induced cell death, Bax-positive (+) *versus* Bax (−) Jurkat cells were treated with 4 μM As_2O_3 for various periods, and cell viability was determined by trypan blue exclusion assay. As shown in Figure 5b, the deletion of Bax dramatically reduced cell death of Jurkat cells in response to As_2O_3 treatment. In the experiment shown, for example, nearly 100% of Bax (+) Jurkat cells died at 60 h after 4 μM As_2O_3 treatment, compared to only ~27% of Bax (−) Jurkat cells. Similar results were obtained in multiple experiments and by use of several independent clones. This result further confirms that Bax is important for As_2O_3-induced apoptosis.

z-VAD-fmk has no effect on As_2O_3-induced Bax conformational change and oligomerization, while it can completely block the Bid cleavage induced by As_2O_3 in IM-9 cells

Our above results suggest that Bax activation plays an important role in As_2O_3-induced apoptosis. Next, we attempted to investigate the mechanism of Bax activation initiated by As_2O_3. Bid is a BH3-only proapoptotic protein that can be cleaved directly by caspase 8 during apoptosis (Wang *et al*., 1996; Li *et al*., 1998; Luo *et al*., 1998). The resulting truncated Bid plays a role in the induction of Bax conformational change and subsequent translocation to mitochondria (Li *et al*., 1998; Luo *et al*., 1998; Desagher *et al*., 1999; Heibein *et al*., 2000; Yamaguchi *et al*., 2003). Therefore, we examined the role of Bid cleavage in As_2O_3-induced Bax conformational change and oligomerization. As shown in Figure 6a, treatment with 2 μM As_2O_3 produced Bid cleavage in IM-9 cells, which is consistent with a previous report (Perkins *et al*., 2000). Interestingly, treatment of IM-9 cells with the pancaspase inhibitor z-VAD-fmk had no effect on Bax conformational change (Figure 6c), or its oligomerization (Figure 6d) induced by As_2O_3. Nevertheless, z-VAD-fmk efficiently blocked Bid cleavage (Figure 6b) and apoptosis (data not shown) in these cells after treatment with As_2O_3. These results are in agreement with previous reports (Bellosillo *et al*., 2002; Yamaguchi *et al*., 2002), which suggest that Bax conformational change is an early event upstream of caspase activation and Bid cleavage. In addition, our data extended previous findings by showing that Bax oligomerization was also a Bid cleavage-independent event in As_2O_3-mediated apoptosis.

An increase of intracellular ROS is associated with As_2O_3-induced Bax activation in IM-9 cells

It has been reported that arsenic may induce the generation of ROS or modulate intracellular redox to exert its toxicity, although the precise mechanism is not known (Chen *et al*., 1998b; Jing *et al*., 1999; Perkins *et al*., 2000; Woo *et al*., 2002; Maeda *et al*., 2004). In attempt to understand how As_2O_3 causes Bax conformational change, we examined the possibility that ROS is associated with the Bax activation induced by As_2O_3.

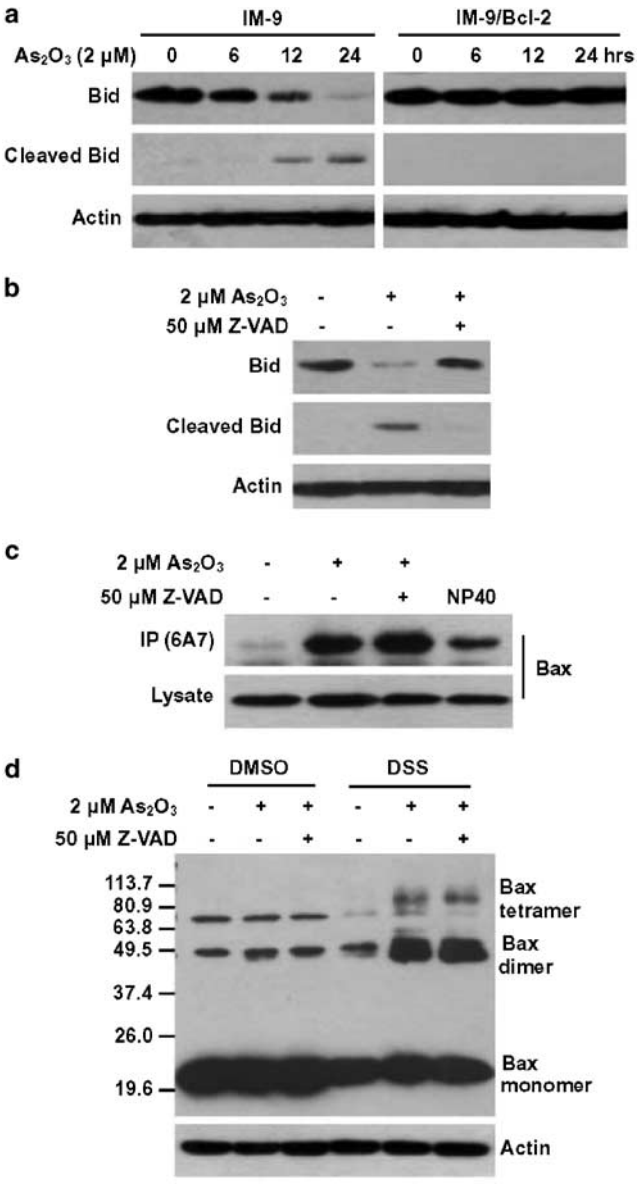

Figure 6 Bid cleavage is not necessary for As_2O_3-induced conformational change and oligomerization of Bax in IM-9 cells. (**a**) After being treated with 2 μM As_2O_3, IM-9 or IM-9/Bcl-2 cells were lysed in 0.5% NP-40 lysis buffer, and the cell lysate was subjected to SDS–PAGE/immunoblot analysis with antibodies specific for Bid and β-actin. In (**b–d**), IM-9 cells were treated with 2 μM As_2O_3 in the presence (+) or absence (−) of 50 μM z-VAD-fmk for 24 h. Bid levels were determined by SDS–PAGE/immunoblot assay (**b**), and Bax conformational change (**c**) and oligomerization (**d**) were assayed as in Figures 3 and 4

To this end, we used DCFH-DA to measure the intracellular ROS in IM-9 cells by flow cytometry and indeed observed that there was an increase of intracellular ROS levels starting at 4 h following As_2O_3 treatment (Figure 7a). However, this induction of ROS was significantly inhibited by *N*-acetyl-L-cysteine (NAC) and lipoic acid (LA), two commonly used antioxidants (Figure 7a). Similarly, overexpression of Bcl-2 also blocked the generation of ROS in IM-9 cells challenged with As_2O_3 (Figure 7a), consistent with its inhibitory

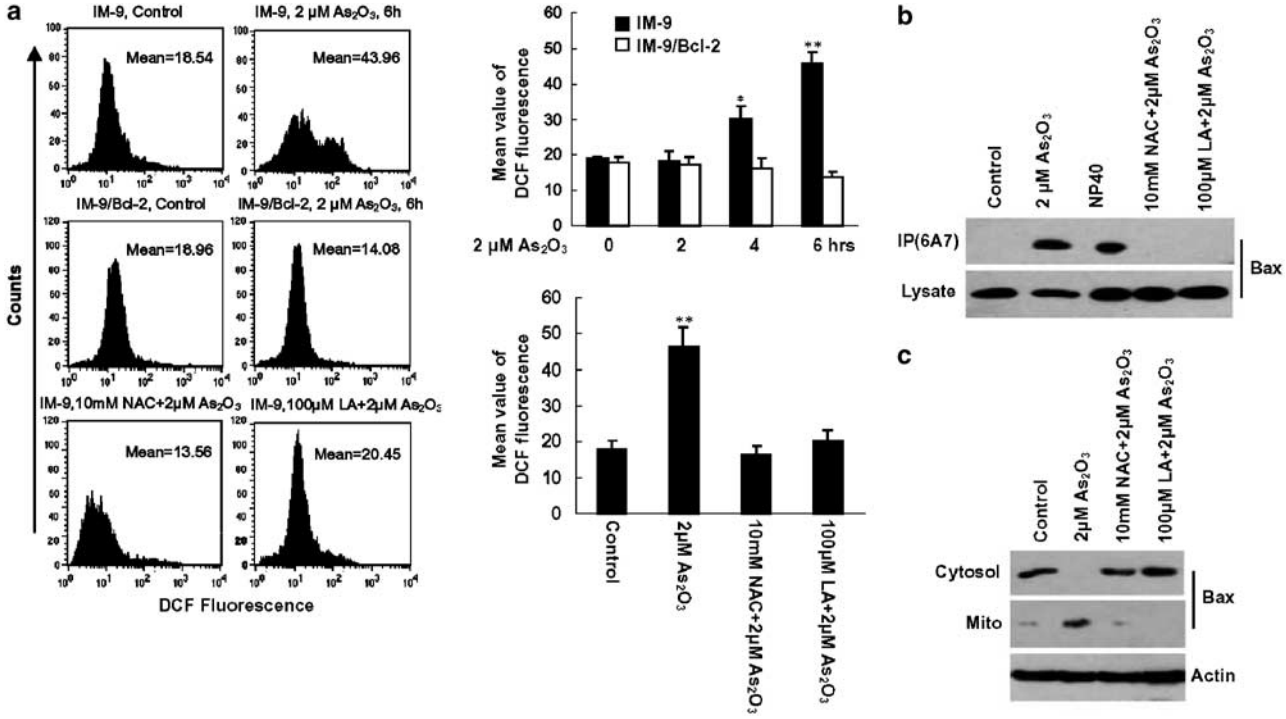

Figure 7 As_2O_3-induced increase of intracellular ROS and Bax activation are completely blocked by overexpression of Bcl-2 and ROS scavengers in IM-9 cells. (**a**) IM-9 or IM-9/Bcl-2 cells were stained with CM-H_2DCF-DA for 1 h, and then were incubated in the absence or presence of 2 μM As_2O_3 at 37°C for the indicated time. After washing with PBS, the cells were immediately analysed using flow cytometry. If required, the cells were preincubated with antioxidants (NAC and LA) for 30 min before being treated with As_2O_3. The left panel is representative of at least three separate experiments, and the right panels are the statistic results ($^*P<0.05$, $^{**}P<0.01$). (**b** and **c**) IM-9 cells were pretreated with 10 mM NAC or 100 μM LA for 30 min, and then were incubated with 2 μM As_2O_3 for another 6 h. Bax conformational change (**b**) and Bax translocation (**c**) were assayed as in Figures 3 and 2b

effect on Bax activation and apoptosis. Importantly, we found that the administration of ROS scavengers completely abolished As_2O_3-induced Bax conformational change (Figure 7b) and its redistribution from cytosol to mitochondria (Figure 7c) as well as apoptosis (data not shown) in IM-9 cells. Taken together, these results clearly suggest that As_2O_3 induces ROS production that contributes to the activation of Bax and apoptosis.

Discussion

In this paper, we addressed the question as to how Bax played a role in As_2O_3-induced apoptosis. We analysed the sequential events of Bax activation and apoptosis, and found that Bax conformational change and its translocation to mitochondria appeared to be important for the initiation of apoptosis, while Bax oligomerization happened at later time point and may be associated with the depletion of cyt *c* from mitochondria. In an attempt to understand how Bax conformational change was activated, we found that intracellular ROS, but not Bid cleavage, was associated with Bax conformational change induced by As_2O_3. Inhibition of ROS by scavengers and Bcl-2 overexpression prevented Bax conformational change and subsequent Bax translocation.

It has been suggested that Bax oligomerization can facilitate the formation of a megachannel in the outer mitochondrial membrane, which can mediate cyt *c* release (Desagher *et al*., 1999; Eskes *et al*., 2000; Murphy *et al*., 2000; Mikhailov *et al*., 2001; Yamaguchi and Wang, 2002). The validity of this hypothesis is yet to be proven since this phenomenon was examined *in vitro* and the molecular details of how a megachannel is formed and how cyt *c* is subsequently released remain uncertain. Our results are not in agreement with the studies, which suggest that Bax dimerization is a prerequisite for mitochondrial translocation (Gross *et al*., 1998). Figures 2 and 4 showed that there were no detectable Bax dimmers or oligomers in the cytosol prior to the translocation event, suggesting that dimerization may not be required for Bax translocation. Our data implicate that Bax oligomerization may be related to destruction of mitochondrial physiology at a late stage of apoptosis, since we detected the loss of mitochondrial membrane potential at later time points (Zheng *et al*., 2004).

We recently found that VDAC could be a biological target of As_2O_3 and that VDAC could mediate As_2O_3-induced apoptosis, at least in reconstituted proteoliposome systems and model system in yeasts (Zheng *et al*.,

2004). While still a matter of debate, VDAC has been implicated in mediating cyt *c* release and related apoptogenic molecules. An interesting model proposes that VDAC could interact with Bax to form a hybrid channel to mediate cyt *c* release (Shimizu *et al*., 2001; Tsujimoto and Shimizu, 2002). Alternatively, VDAC2 could interact with Bak to prevent it from oligomerization, thereby blocking the apoptotic events (Cheng *et al*., 2003). It is also possible that, upon targeting of Bax to mitochondria, it could replace Bak to bind to VDAC2, thereby releasing Bak, allowing it to undergo oligomerization and/or interact with VDAC1. However, our results differ from this model due to the observation that at least in Bax (−) cells, Bak may not be the key regulator for As_2O_3-induced apoptosis. Further work is now underway to more closely examine the dynamic interaction of Bax/Bak and VDAC1/VDAC2, which could govern the release of cyt *c*.

It is being debated with regard to the mechanisms of Bax activation (translocation, conformational change and oligomerization). It has been reported that cleaved Bid could induce Bax conformational change and oligomerization (Desagher *et al*., 1999; Roucou *et al*., 2002; Priault *et al*., 2003). Indeed, treating IM-9 with As_2O_3 resulted in Bid cleavage, which was completely blocked by z-VAD-fmk. However, this pancaspase inhibitor did not inhibit the Bax conformational change and oligomerization induced by As_2O_3. These results suggest that As_2O_3-mediated Bax conformational change and oligomerization occur upstream of caspase activation and Bid cleavage in IM-9 cells. Several groups of investigators have observed ROS generation during exposure to As_2O_3 (Chen *et al*., 1998b; Jing *et al*., 1999; Perkins *et al*., 2000; Woo *et al*., 2002; Maeda *et al*., 2004), and it was suggested that ROS might be associated with the Bax activation in apoptosis induced by some stimuli (Buccellato *et al*., 2004). Our results first linked the possibility that intracellular ROS could induce Bax conformational change and its subsequent translocation to mitochondria in As_2O_3-induced apoptosis. Our data showed that the levels of ROS significantly increased starting at 4 h following the treatment, at the time that Bax conformational change and translocation were also obviously detectable. The increase of intracellular ROS can be completely blocked by the ROS scavengers. Importantly, the ROS scavengers also completely inhibited the Bax conformational change and its translocation. In addition, ascorbic acid and buthionine sulfoxide (two oxidizing agents) could enhance As_2O_3-induced Bax activation and apoptosis, while these agents alone are not able to induce Bax activation (data not shown). Consistent with our previous work and others (Kirkland *et al*., 2002; Chen *et al*., 2003), our results favor the model that the intracellular ROS or perturbation of intracellular redox are upstream factor for Bax activation and cyt *c* release. This may help to explain that pro-oxidant could improve the efficacy of As_2O_3 in clinic (Maeda *et al*., 2004). Further work is underway to investigate the source of ROS and how it is related to the activation of Bax.

Another related question is how Bcl-2 prevents Bax from activation? For long, it has been puzzling that Bcl-2, which is mainly localized at the intracellular membranes (mitochondria and ER), prevents Bax from translocating from cytosol to mitochondria and ER, undergoing conformational changes and forming oligomers. We did not detect the presence of Bcl-2 in the Bax complexes as assayed by immunoprecipitation and crosslinking experiments (data not shown). It is also of interest to note that Bcl-2 could prevent intracellular free radical generation and glutathione depletion, which are key factors involved in protecting intracellular redox environments against diverse death stimuli (Hockenbery *et al*., 1993; Voehringer and Meyn, 2000; Chen *et al*., 2003). These factors also play a role in Bax conformational change induced by As_2O_3. Similarly, Bcl-2 may prevent As_2O_3-mediated Bax conformational change by maintaining the intracellular redox potential, due to the possibility that the sensitivity of the cells is inversely proportional to their GSH content (Dai *et al*., 1999). Our results indicate that Bcl-2 prevents Bax activation by preventing mitochondrial generation of ROS, which leads to Bax conformational change and its translocation. Clearly, additional studies are required to fully delineate the biochemical mechanisms by which Bcl-2 regulates Bax activation and apoptosis in response to As_2O_3 at the levels of both mitochondria and ER.

Materials and methods

Materials

As_2O_3, NAC, LA, anti-actin (A-5441) and anti-Bax 6A7 monoclonal antibody were purchased from Sigma Chemical Co. (St Louis, MO, USA). The Annexin V apoptosis detection kit was obtained from PharMingen (San Jose, CA, USA). DSS was obtained from Pierce (Rockford, IL, USA). 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) was purchased from Molecular Probes (Eugene, OR, USA). Bcl-x mouse monoclonal antibody (AHO0222) was from Biosource International (Camarillo, CA, USA). Anti-caspase 3 (sc-7148) polyclonal, anti-Bcl-2 (sc-509) monoclonal and anti-Bax (N-20, sc-493) polyclonal antibodies were obtained from Santa Cruz Biotechnology Inc. (CA, USA). Anti-cytochrome *c* antibody (556433) was from BD Biosciences PharMingen (San Jose, CA, USA). Anti-Bid polyclonal antibody was from Cell Signaling technology (MA, USA). TAT-Bid-BH3 peptide was synthesized by Abgent (San Diego, CA, USA). Caspase inhibitor z-VAD-fmk was purchased from Alexis (San Diego, CA, USA).

Cell culture and transfection

IM-9 and Jurkat cells were maintained in RPMI 1640 medium supplemented with 10% FBS (Hyclone) and 1% penicillin–streptomycin at 37°C and 5% CO_2. To establish Bcl-2-stable transfectants, IM-9 cells were transfected with pSFFV-neo/Flag-Bcl-2 plasmid DNA, and positive clones overexpressing Bcl-2 were selected with 1 mg/ml G418 as described previously (Chen *et al*., 2000). The Bax (+) and Bax (−) Jurkat clones were isolated from wild-type Jurkat cells by limit dilution. Briefly, Jurkat cells were seeded at an average of 0.5 cells/well in 0.1 ml of complete medium in 96-well plates, and the Bax

(−) clones were identified by SDS–PAGE/immunoblot analysis with anti-Bax antibody. Exponentially growing cells were subjected to the various treatments as indicated.

Apoptosis assessment by Annexin V staining

Apoptosis was measured using the Annexin V detection kit according to the manufacturer's instructions. Flow cytometric analysis was performed to monitor the green fluorescence of the FITC-conjugated Annexin V (530 ± 30 nm) and the red fluorescence of DNA-bound propidium iodide (PI, 630 ± 22 nm) (Chen *et al*., 1998a). All data were analysed with a Cell Quest software (BD).

Cell fractionation

As_2O_3-treated cells were fractionated by differential centrifugation as described previously (Chen *et al*., 1997; Chen *et al*., 2000). Briefly, cells were harvested and resuspended in three volumes of hypotonic buffer (210 mM sucrose, 70 mM mannitol, 10 mM HEPES (pH 7.4), 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 μg/ml trypsin inhibitor, and 10 μg/ml leupeptin, 5 μg/ml aprotinin and 10 μg/ml pepstatin. After gentle homogenization with a Dounce homogenizer, cell lysates were centrifuged at 1000 *g* for 5 min to remove unbroken cells and nuclei. The postnuclear supernatant was centrifuged at 10 000 *g* to pellet the mitochondria-enriched heavy membrane fraction. The supernatant was further centrifuged at 100 000 *g* to obtain the cytosolic fraction. For alkali extraction, the mitochondrial pellets were resuspended in freshly prepared 0.1 M Na_2CO_3 (pH 11.5) and incubated for 20 min on ice. The membranes were then pelleted by centrifugation.

SDS–PAGE and immunoblotting

SDS–PAGE and immunoblotting were performed as described elsewhere (Chen *et al*., 2003). Briefly, the cells or the membrane fractions were resuspended in NP-40 containing lysis buffer (10 mM HEPES (pH 7.4), 2 mM EGTA, 0.5% NP-40, 1 mM NaF, 1 mM $NaVO_4$, 1 mM PMSF, 1 mM DTT, 50 μg/ml trypsin inhibitor, 10 μg/ml aprotinin and leupeptin) and placed on ice for 30 min. The lysates were centrifuged at 12 000 *g* for 12 min at 4°C, and the protein concentration was determined. Equivalent samples (30 μg protein) were subjected to SDS–PAGE on 12% gel. The proteins were then transferred onto nitrocellulose membranes, and probed with the indicated antibodies followed by the appropriate secondary antibodies conjugated to horseradish peroxidase (KPL, Gaithersburg, MD, USA). Immunoreactive bands were visualized using enhanced chemiluminescence (Pierce). The molecular sizes of the developed proteins were determined by comparison with prestained protein markers (Invitrogen, Carlsbad, CA, USA).

Detection of Bax conformational change

This assay was performed as described previously (Yamaguchi *et al*., 2002). Cells or isolated mitochondria were lysed with Chaps lysis buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 1% Chaps) containing protease inhibitors as described. The cell lysates were normalized for protein content and 500 μg of total protein was incubated with 2 μg of anti-Bax 6A7 monoclonal antibody in 500 μl of Chaps lysis buffer at 4°C for 3 h or overnight. Then, 25 μl of protein G-agarose were added into the reactions and incubated at 4°C for an additional 2 h. Following three washings in Chaps lysis buffer, beads were boiled in loading buffer, and the conformationally changed Bax protein in the immunoprecipitates was subjected to SDS–PAGE (15% gel) and immunoblot analysis with anti-Bax polyclonal antibody as described above.

Crosslinking of Bax protein

Following treatment with As_2O_3, cells were washed with conjugating buffer containing 150 mM NaCl, 20 mM HEPES (pH 7.2), 1.5 mM $MgCl_2$ and 10 mM glucose. DSS in DMSO was added from a 10-fold stock solution to a final concentration of 2 mM (Gross *et al*., 1998; Makin *et al*., 2001). After incubating at room temperature for 30 min, the crosslinker was quenched by the addition of 1 M Tris-HCl (pH 7.5) to a final concentration of 20 mM at room temperature for 15 min. Samples were then solubilized in 0.5% NP-40 lysis buffer and centrifuged at 12 000 *g* for 10 min. Bax was detected by Western blotting with anti-Bax polyclonal antibody, or Bax was immunoprecipitated from the supernatant using Bax monoclonal antibody and then immunoblotted using Bax polyclonal antibody.

ROS production determination

ROS production was detected using CM-H_2DCFDA, an uncharged, cell-permeable fluorescent probe. When being incubated with the cells, this dye can readily diffuse into cells and is hydrolysed by intracellular esterases to yield H_2DCF, which is trapped within the cells. Then, it is oxidized from the nonfluorescent form to a highly fluorescent compound by the hydrogen peroxide or other low-molecular-weight peroxides produced in the cells. Thus, the fluorescence intensity is proportional to the amount of peroxide produced by the cells. Exponentially growing cells (1×10^5 cells/ml) were labeled with 1 μM CM-H_2DCFDA for 1 h before treatment, and then were washed with PBS. The green fluorescence intensity in the cells was examined by FACS (Becton Dickinson, San Jose, CA, USA) with excitation at 488 nm. PI (10 μg/ml) was added 1 min before flow cytometry. To validate the data, 0.1 mM H_2O_2 was added 1 h before the staining (not shown). The data were analysed with a Cell Quest software (BD) from the cell population from which apoptotic cells were gated out against PI positivity (Jing *et al*., 1999).

Statistical analysis

Statistical analysis was performed by using a standard Student's *t*-test analysis, with *P*-values <0.05 considered significant.

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