Caspase-Independent Cell Death by Arsenic Trioxide in Human Cervical Cancer Cells: Reactive Oxygen Species-Mediated Poly(ADP-ribose) Polymerase-1 Activation Signals Apoptosis-Inducing Factor Release from Mitochondria

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ABSTRACT

Although mechanisms of arsenic trioxide (As2O3)–induced cell death have been studied extensively in hematologic cancers, those in solid cancers have yet to be clearly defined. In this study, we showed that the translocation of apoptosis-inducing factor (AIF) from mitochondria to the nucleus is required for As2O3–induced cell death in human cervical cancer cells. We also showed that reactive oxygen species (ROS)–mediated poly(ADP-ribose) polymerase-1 (PARP-1) activation is necessary for AIF release from mitochondria. The treatment of human cervical cancer cells with As2O3 induces dissipation of mitochondrial membrane potential (Δψm), translocation of AIF from mitochondria to the nucleus, and subsequent cell death. Small interfering RNA targeting of AIF effectively protects cervical cancer cells against As2O3–induced cell death. As2O3 also blocks As2O3–induced PARP-1 activation, Δψm loss, nuclear translocation of AIF from mitochondria, and the consequent cell death. Furthermore, pretreatment of 1,5-dihydroxyisouquinoline or 3,4-dihydro-5-(4-(1-piperidinyl)butoxy)-1(2H)-isoquinolinone, PARP-1 inhibitors, effectively attenuates the loss of Δψm, AIF release, and cell death. These data support a notion that ROS-mediated PARP-1 activation signals AIF release from mitochondria, resulting in activation of a caspase-independent pathway of cell death in solid tumor cells by As2O3 treatment.

INTRODUCTION

Arsenic agents have long been used as anticancer agents in traditional Chinese medicine (1). They recently have been used successfully for management of acute promyelocytic leukemia (APL), and their efficacy has been confirmed even in patients resistant to conventional chemotherapy (2, 3). Importantly, it has become evident that mechanisms of arsenic trioxide (As2O3)–induced cell death in hematologic cancers have yet to be clearly defined. In this study, we showed that the translocation of apoptosis-inducing factor (AIF) from mitochondria to the nucleus is required for As2O3–induced cell death in human cervical cancer cells. We also showed that reactive oxygen species (ROS)–mediated poly(ADP-ribose) polymerase-1 (PARP-1) activation is necessary for AIF release from mitochondria. The treatment of human cervical cancer cells with As2O3 induces dissipation of mitochondrial membrane potential (Δψm), translocation of AIF from mitochondria to the nucleus, and subsequent cell death. Small interfering RNA targeting of AIF effectively protects cervical cancer cells against As2O3–induced cell death. As2O3 also induces an increase of intracellular ROS level and a marked activation of PARP-1. N-acetyl-l-cystein, a thiol-containing antioxidant, completely blocks As2O3–induced PARP-1 activation, Δψm loss, nuclear translocation of AIF from mitochondria, and the consequent cell death. Furthermore, pretreatment of 1,5-dihydroxyisouquinoline or 3,4-dihydro-5-(4-(1-piperidinyl)butoxy)-1(2H)-isoquinolinone, PARP-1 inhibitors, effectively attenuates the loss of Δψm, AIF release, and cell death. These data support a notion that ROS-mediated PARP-1 activation signals AIF release from mitochondria, resulting in activation of a caspase-independent pathway of cell death in solid tumor cells by As2O3 treatment.

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mitochondria to the nucleus is required for PARP-1–mediated cell death.

We previously have shown that As2O3 induced apoptotic cell death through a ROS-dependent pathway in human cervical cancer cells (31). Treatment of HeLa cells with As2O3 increased intracellular ROS level and induced loss of the ΔΨm and caspase-3 activation. As2O3-induced cell death was completely blocked by the antioxidant; however, caspase-3 inhibitor failed to reduce cell death induced by As2O3, suggesting a ROS-dependent but caspase-independent cell death pathway is used. Precise mechanism of the ROS-mediated caspase-independent apoptotic cell death triggered by As2O3 treatment still remains unclear, especially in solid cancer cells.

In this study, we investigate molecular mechanism of the ROS-mediated apoptotic cell death triggered by As2O3 in human cervical cancer cells. We show that nuclear translocation of AIF from the mitochondria is required for As2O3-induced cell death in human cervical cancer cells and that ROS-mediated PARP-1 activation is necessary for AIF release from mitochondria. These data suggest that ROS-mediated PARP-1 activation induces AIF release from the mitochondria, resulting in activation of the caspase-independent pathway of cell death in solid tumor cells in response to As2O3 treatment.

MATERIALS AND METHODS

Materials. As2O3 was purchased from Sigma (St. Louis, MO). Antibodies specific for polyclonal anti-AIF, α-tubulin, heat shock protein 60, and Ref-1 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). β-Actin was from Sigma. Anticleared caspase-3 antibody and PARP were from Cell Signaling Technology (Beverly, MA). Polyclonal anti-cytochrome c antibody was from Pharmingen (San Diego, CA). Polyclonal anti-PAR antibody was from Calbiochem (San Diego, CA). The specific PARP inhibitors 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinolinone (DPQ) and 1,5-dihydroxyisoquinoline (DQ) were from Calbiochem and Sigma, respectively. The caspase 3 inhibitor z-DEVD-fmk and the broad-spectrum caspase inhibitor z-Val-d-Val-Ala-Asp (OMe)-fluoromethylketone (ZVAD-fmk) were from Calbiochem. Other chemicals were obtained from Calbiochem.

Cell Culture and Transfection. Human cervical carcinoma cells (HeLa, CaSki, and SiHa) were obtained from the American Type Culture Collection (Manassas, VA). HeLa and CaSki cells were grown in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum, and SiHa cells were grown in modified Eagle’s medium supplemented with 10% fetal bovine serum and nonessential amino acids. All of the media were supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin, and all of the cells were incubated at 37°C in 5% CO2. Cells were transfected with the full-length cDNA of Bcl-2 cloned into the pcDNA 3.1 plasmid (Invitrogen, Carlsbad, CA) or with the control vector (pcDNA 3.1 Zeo) using Lipofectamine PLUS reagent (Invitrogen) according to the manufacturer’s recommendations. Cells were analyzed 24 hours after transfection.

Small Interfering RNA Transfection. RNA interference of AIF was performed using 21-bp (including a 2-deoxynucleotide overhang) small interfering RNA (siRNA) duplexes purchased from Ambion (Austin, TX). The coding strand for AIF siRNA was GGAA/AUUGGGAAGAUCGdTdT. A nonrelated control siRNA that targeted the green fluorescent protein DNA sequence CCAC/TACGTGAGCACCCGAG was used as a control. For transfection, HeLa cells were seeded in six-well plates and transfected at 30% confluency with siRNA duplexes (200 nmol/L) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. Assays were performed 48 hours after transfection. The protein level of AIF was detected in the cell lysate by Western blot analysis.

Quantification of Cell Death. For the cell death assessment, the cells were plated in six-well plates with cell density of 1 × 105 cells per well and treated with As2O3 the next day. At indicated time points, cells were trypsinized, washed in PBS, and then incubated in propidium iodide (2.5 μg/mL) for 5 minutes at room temperature and analyzed with a flow cytometer. When necessary, N-acetyl-L-cysteine (NAC), specific PARP inhibitors (50 μmol/L and DIQ (50 μmol/L), or caspase-3 inhibitor z-DEVD-fmk (100 μmol/L) and caspase-3 inhibitor z-DEVD-fmk (100 μmol/L) were applied 1 hour before As2O3 treatment and kept in the medium during and after As2O3 treatment until the cells were analyzed.

Measurement of Mitochondrial Membrane Potential and ROS Generation. The measurement of ΔΨm and ROS generation were performed as described by Marchetti et al. (32). Briefly, cells (5 × 107/mL) were exposed to 2 μmol/L As2O3 for the indicated times. After exposure, cells were incubated in 30 mmol/L 3,3′-dihexyloxacarbocyanine iodide (DiOC6(3)) and 30 μmol/L 2′,7′-dichlorodihydrofluorescein diacetate DCHF-DA (Molecular Probes, Eugene, OR) at 37°C for 30 minutes and harvested by trypsinization and washed with cold PBS solution three times. ROS and ΔΨm were determined by fluorescence-activated cell-sorting analysis.

Confocal Microscopy. HeLa cells treated with As2O3 were washed twice with ice-cold PBS before fixation with ice-cold methanol. After blocking with 2% bovine serum albumin in PBS containing 0.2% Triton X-100, cells were incubated with the primary antibody against AIF for 1 hour. Cells were washed with blocking solution three times and incubated with the secondary antibody conjugated with FITC (Molecular Probes) for 1 hour. Nuclei were stained with propidium iodide (Sigma) for 10 minutes after secondary antibody incubation and two rinses with PBS. After washing three times with PBS, coverslips were mounted onto microscope slides using ProLong antifade mounting reagent (Molecular Probes). The slides were analyzed using a confocal laser-scanning microscope (Leica Microsystems, Bannockburn, IL).

Western Blot Analysis. Western blot analysis was performed as described previously (33). Briefly, cell lysates were prepared by extracting proteins with lysis buffer [250 mmol/L Tris-Cl (pH 8.0), 120 mmol/L NaCl, and 0.1% NP40] supplemented with protease inhibitors. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline and then incubated with primary antibodies for 1 hour at room temperature. Blots were developed by peroxidase-conjugated secondary antibody, and proteins were visualized by enhanced chemiluminescence procedures (Amersham Biosciences, Piscataway, NJ) according to the manufacturer’s recommendations.

Preparation of Nuclear and Mitochondrial Fractions. The cells were washed with ice-cold PBS, left on ice for 10 minutes, and then resuspended in isotonic homogenization buffer [250 mmol/L sucrose, 10 mmol/L KCl, 1.5 mmol/L MgCl2, 1 mmol/L Na-EDTA, 1 mmol/L Na-EGTA, 1 mmol/L diithiothreitol, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 10 mmol/L Tris-HCl (pH 7.4)] containing a proteinase inhibitor mixture (Roche, Basel, Switzerland). After 80 strokes in a Dounce homogenizer, the unbroken cells were spun down at 30 × g for 5 minutes. The nuclei and heavy mitochondria fractions were fractionated at 750 × g for 10 minutes and 14,000 × g for 20 minutes, respectively, from the supernatant. The nuclear fraction was washed three times with homogenization buffer containing 0.1% NP40.

RESULTS

To investigate kinetics of the apoptotic cell death induced by As2O3 in human cervical cancer cells, we treated three different human cervical cancer cell lines (HeLa, CaSki, and SiHa) with different doses of As2O3 for various amount of time and analyzed induction of the cell death by flow cytometry. Fig. 1A shows that there is a dose- and time-dependent increase of cells with sub-G1 contents of DNA, reaching ~40% of HeLa cells after 36 hours of the treatment with the final concentration of 2 μmol/L. Compared with HeLa cells, CaSki and SiHa cells were less sensitive to As2O3. However, the CaSki and SiHa cells treated with 10 μmol/L of As2O3 resulted in a time-dependent increase of apoptotic cells. These results show that As2O3 induces apoptotic cell death in several human cervical cancer cells in a dose- and time-dependent manner.

To determine whether mitochondrial pathway is involved in induction of the apoptotic cell death seen after As2O3 treatment, we examined changes in ΔΨm and cytochrome c release from the mitochondria into the cytosol in the As2O3-treated HeLa cells. As2O3 treatment significantly disrupted ΔΨm (Fig. 1B). At the same time, levels of the cytosolic cytochrome c were markedly increased (Fig. 1C), coinciding with changes in ΔΨm. Treatment of cells with As2O3 also caused activation of caspase-3 and cleavage of PARP (Fig. 1C).
Requirement of caspase activities for As$_2$O$_3$-induced apoptosis was examined by using a broad-spectrum caspase inhibitor, z-VAD-fmk, and a caspase-3–specific inhibitor, z-DEVD-fmk. These caspase inhibitors were able to prevent activation of caspases (data not shown) but failed to attenuate As$_2$O$_3$-induced apoptotic cell death (Fig. 1D). These results indicate that As$_2$O$_3$-induced apoptotic cell death occurs in a caspase-independent fashion.

Because AIF is known to be involved in induction of apoptotic cell death via a caspase-independent pathway, we next examined whether AIF plays a role in As$_2$O$_3$-induced apoptotic cell death (11, 13). AIF is a mitochondria-localized flavoprotein that is released from the mitochondria on death stimuli, subsequently translocates into the nucleus, and causes nuclear condensation (11, 14). Subcellular fractionation showed that treatment of cells with As$_2$O$_3$ dramatically redistributed AIF from the mitochondria to the nucleus (Fig. 2A). Analysis by confocal microscopy clearly revealed that AIF was translocated into the nucleus and caused nuclear condensation after treatment with As$_2$O$_3$ (Fig. 2B). Moreover, siRNA targeting of the AIF effectively attenuated As$_2$O$_3$-induced apoptosis (Fig. 2C and D). These results suggest that AIF translocation into the nucleus is required in As$_2$O$_3$-induced cell death of human cervical cancer cells.

Several recent reports have suggested that AIF is involved in a caspase-dependent cell death pathway (34, 35), and we next investigated whether AIF translocation induced by the As$_2$O$_3$ treatment is caspase dependent. As shown in Fig. 3A and B, the caspase inhibitors z-VAD-fmk and z-DEVD-fmk did not affect AIF translocation into the nucleus and nuclear condensation seen after the As$_2$O$_3$ treatment. These results indicate that As$_2$O$_3$-induced apoptotic cell death in human cervical cancer cells is mediated by the AIF translocation from mitochondria into the nucleus via a caspase-independent pathway.

Because oxidative damage plays an important role in anticaner effects of arsenic compounds (36), we subsequently examined the level of intracellular ROS in HeLa cells after treatment with As$_2$O$_3$ using an oxidation-sensitive fluorescent probe DCFH-DA, which is oxidized to 2',7'-dichlorofluorescein (DCF) in the presence of ROS. Fig. 4A shows that treatment of cells with As$_2$O$_3$ (2 μM) led to a 2.5-fold increase of mean DCF fluorescence. We next investigated whether generation of ROS induced by the As$_2$O$_3$ treatment is accompanied by the loss of $\Delta$ψ$_{mt}$, AIF release, and apoptotic cell death seen after As$_2$O$_3$ treatment. To determine a link between elevation of the intracellular ROS level and apoptotic cell death in As$_2$O$_3$-treated cells, HeLa cells were preincubated with thiol-containing antioxidant NAC before treatment with As$_2$O$_3$. Inhibition of ROS by NAC prevented loss of $\Delta$ψ$_{mt}$ (Fig. 4B), AIF release, and apoptotic cell death seen after As$_2$O$_3$ treatment. Moreover, As$_2$O$_3$ treatment failed to induce cell death in cells pretreated with NAC (Fig. 4E). These observations suggest that increase in the...
intracellular ROS level after As$_2$O$_3$ treatment is required in the cell death pathway accompanied by the H$_9004$/H$_9274$ loss and AIF nuclear translocation.

Because it has been shown that ROS-mediated DNA damage triggers activation of PARP-1 and subsequent cell death (26, 27), we examined whether PARP-1 is involved in As$_2$O$_3$-induced apoptotic cell death. Exposure of HeLa cells to 2 mol/L of As$_2$O$_3$ for 36 hours induces a marked activation of PARP-1 in these cells (Fig. 5A). Pretreatment of these cells with the PARP-1–specific inhibitors DIQ or DPQ attenuated As$_2$O$_3$-induced cell death and PARP-1 activity (Fig. 5B and C). Furthermore, PARP-1 inhibitors efficiently blocked loss of the H$_9004$/H$_9274$, AIF nuclear translocation, and nuclear condensation induced by As$_2$O$_3$ (Fig. 5D and E). Confocal microscopy revealed that cells treated with DIQ or DPQ maintained punctuated immunostaining pattern of the AIF throughout the cytoplasm excluded from the nucleus, indicating that this death effector still resides in mitochondria. This result suggests that PARP-1 activation triggers AIF nuclear translocation. Interestingly, inhibition of PARP-1 by DIQ did not affect generation of the As$_2$O$_3$-induced ROS (Fig. 6A), whereas thiol-containing antioxidant NAC completely blocked PARP-1 activation (Fig. 6B), indicating that PARP-1 activation is downstream of the increased ROS generation after As$_2$O$_3$ treatment.

Collectively, these results suggest that ROS-mediated PARP-1 activation signals AIF release from the mitochondria and subsequent nuclear translocation of this protein, resulting in caspase-independent pathway of cell death in the As$_2$O$_3$-treated human cervical cancer cells.

**DISCUSSION**

In recent years, our understanding how As$_2$O$_3$ induces cell death in hematologic cancers has greatly improved. However, mechanisms of the As$_2$O$_3$-mediated cell death in solid cancers are not well understood. The aim of our investigation was to elucidate molecular mechanisms of the caspase-independent cell death triggered by As$_2$O$_3$ in human cervical cancer cells. We showed that translocation of AIF from the mitochondria to the nucleus is a critical step for As$_2$O$_3$-induced caspase-independent cell death in these cells. We also showed that ROS-mediated PARP-1 activation is necessary for AIF release from the mitochondria during As$_2$O$_3$-induced apoptotic cell death. Caspase activation is thought to be important for As$_2$O$_3$-induced cell death in vitro and in vivo (18, 37–40); however, our findings suggest that cell death induced by As$_2$O$_3$ in human cervical cancer cells is caspase independent. Although treatment of these cells with As$_2$O$_3$ caused cytochrome c release and caspase-3 activation, apop-

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**Fig. 2.** As$_2$O$_3$ induces nuclear condensation and cell death in an AIF-dependent but caspase-independent manner. A. Analysis of AIF translocation by subcellular fractionation. Subcellular fractionation was performed with untreated HeLa cells and cells treated with As$_2$O$_3$ for 36 and 48 hours followed by Western blot analysis of the mitochondrial (M) and nuclear (N) fractions. Heat shock protein 60 (HSP60) and Ref-1 were used as mitochondrial and nuclear marker proteins, respectively. B. Representative confocal images for translocation of AIF to the nucleus and nuclear condensation in As$_2$O$_3$-treated HeLa cells. The nuclear translocation of AIF is shown by the overlap of AIF (green) and nuclear staining (red), as noted by yellow color. C. siRNA targeting of the AIF attenuates As$_2$O$_3$-induced AIF translocation. Analysis of the AIF translocation by nuclear fractionation (36 hours) in control siRNA- and AIF siRNA-transfected HeLa cells after As$_2$O$_3$ treatment (2 μmol/L). The coding strand for AIF siRNA was GGAAGAUUAGGAAAGAUCCdTdT. A nonrelated control siRNA that targeted the green fluorescent protein (GFP) DNA sequence CCACCTACCTGAGCACCCAG was used as a control. D. Representative confocal images for AIF translocation and nuclear shrinkage in control siRNA- and AIF siRNA-transfected HeLa cells after As$_2$O$_3$ treatment (2 μmol/L). E. Attenuation of the As$_2$O$_3$-induced cell death by transfection of AIF siRNA. Quantitative analysis of cell death in control siRNA- and AIF siRNA-transfected HeLa cells after As$_2$O$_3$ treatment (2 μmol/L).
totic cell death induced by As2O3 seems to be caspase independent because caspase inhibitors failed to attenuate cell death induced by the As2O3 treatment. It has been reported that AIF mediates cell death through a caspase-independent pathway. Mitochondrial AIF translocates to the nucleus on death stimuli and initiates nuclear condensation (11, 14). Once nucleus condenses, this leads to large-scale chromatin fragmentation, followed by the cell death (11, 14). Consistent with these findings, we found translocation of AIF from the mitochondria to the nucleus after As2O3 treatment of HeLa cells. AIF translocation and nuclear condensation were detectable within 24 hours of As2O3 treatment. Furthermore, siRNA knockdown of the AIF protein in cervical cancer cells effectively attenuated As2O3-induced cell death and AIF translocation and nuclear condensation. Attenuation of the As2O3-induced cell death by siRNA targeting of AIF underscores that activity of AIF is required for As2O3-induced cell death in human cervical cancer cells. Several reports recently advocated the hypothesis that AIF is a caspase-dependent death effector (34, 35), implicating translocation of AIF to the nucleus is caspase dependent. However, we found that caspase inhibitors failed to attenuate AIF translocation and nuclear shrinkage, suggesting that As2O3 induces redistribution of AIF to the nucleus in a caspase-independent manner during progression of apoptosis. Recent studies also showed that cytosolic AIF acts on the mitochondria to collapse ΔΨm and initiates release of cytochrome c, which activates caspases. The late activation of caspases after AIF release may facilitate the dissolution of the cells (11). However, in our study, caspase activation

Fig. 3. The caspase inhibitors fail to block AIF translocation and nuclear shrinkage in HeLa cells treated with As2O3. A, analysis of the AIF translocation by nuclear fractionation in HeLa cells 36 hours after As2O3 treatment (2 μmol/L) in the presence of pan-caspase inhibitor (z-VAD-fmk, 100 μmol/L) or caspase-3-specific inhibitor (z-DEVD-fmk, 100 μmol/L) and control cells. Ref-1 was used as a nuclear marker protein. B, representative confocal images for AIF translocation and nuclear condensation in HeLa cells 36 hours after As2O3 treatment (2 μmol/L) in the presence of pan-caspase inhibitor (z-VAD-fmk, 100 μmol/L) or caspase-3-specific inhibitor (z-DEVD-fmk, 100 μmol/L) and control cells. The nuclear translocation of AIF is shown by the overlap of AIF (green) and nuclear staining (red), as noted by yellow color.

Fig. 4. Increase of intracellular ROS is required for AIF translocation and cell death in cells treated with As2O3. A, elevation of the intracellular ROS level after As2O3 treatment. HeLa cells were loaded with DCFH-DA and further stimulated with As2O3 (2 μmol/L) in the presence or absence of NAC (10 mmol/L). After 36 hours, the DCF fluorescence was visualized using confocal microscope (left), and the amount of fluorescence was measured using flow cytometry (right). B, ROS-dependent ΔΨm loss after As2O3 treatment. Cells were treated with As2O3 (2 μmol/L) in the presence or absence of NAC (10 mmol/L). DiOC6(3) (30 nmol/L) was added to the cultures during the last 30 minutes of treatment. The amount of the retained DiOC6(3) was measured with flow cytometry. C, Increase of ROS is critical for AIF translocation in HeLa cells treated with As2O3. Analysis of AIF translocation by the nuclear fractionation in HeLa cells 36 hours after As2O3 treatment (2 μmol/L) in the presence of NAC (10 mmol/L) and control cells. Ref-1 was used as a nuclear marker protein. D, representative confocal images for AIF translocation and nuclear shrinkage in HeLa cells 36 hours after As2O3 treatment (2 μmol/L) in the presence of NAC (10 mmol/L) and control cells. The nuclear translocation of AIF is shown by the overlap of AIF (green) and nuclear staining (red), as noted by yellow color. E, ROS inhibitor NAC attenuates As2O3-induced cell death. Quantitative analysis of the cell death in HeLa cells 36 hours after As2O3 treatment (2 μmol/L) in the presence of NAC (10 mmol/L) and control cells.
was not required for cell death because caspase inhibitors could not rescue the cells from As$_2$O$_3$-induced cell death.

Accumulation of intracellular ROS leads to disruption of the $\Delta \psi_{m}$, release of cytochrome $c$ with subsequent activation of the caspase cascade, and ultimately to programmed cell death through apoptosis (20, 21). It has been proposed that As$_2$O$_3$-induced cell death is caused by ROS generation. Arsenic exposure leads to increase of ROS by a variety of redox enzymes, including the flavoprotein-dependent superoxide-producing enzyme such as NADPH oxidase (16–18). It recently has been shown that As$_2$O$_3$ decreases GSH and increases intracellular ROS level in certain APL cells (19). The intracellular GSH redox system has been proposed as a mechanism to explain As$_2$O$_3$ sensitivity. We previously have shown that increased intracellular ROS played an important role in As$_2$O$_3$-induced cell death in human cervical cancer cells (31). In the present study, we provided additional evidence that increased level of ROS is essential for loss of $\Delta \psi_{m}$ and AIF release from the mitochondria triggered by As$_2$O$_3$. Increased intracellular ROS level appears to coincide with AIF translocation and dissipation of $\Delta \psi_{m}$. Complete inhibition of As$_2$O$_3$-induced AIF translocation, nuclear condensation, and cell death by treatment with NAC suggests that increased intracellular ROS level is critical for As$_2$O$_3$-mediated caspase-independent cell death.

PARP-1 is a nuclear enzyme that facilitates DNA repair in response to DNA damage (22, 23). It recently has been shown that ROS-mediated DNA damage triggers activation of PARP-1 and subsequent cell death (26, 27). Although PARP-1–mediated cell death is thought to be necrotic (28, 29), recent reports have shown that PARP-1–mediated cell death also has many features in common with apoptotic forms of cell death (27, 30). PARP-1 has been implicated in the process of apoptotic cell death following cellular injuries inflicted by the inflammatory response, ROS, and ischemia reperfusion (29, 41, 42). Likewise, in this study, we also showed that PARP-1 activation is necessary for As$_2$O$_3$-induced apoptotic cell death. Pretreatment of cells with PARP-1 inhibitors efficiently attenuated loss of $\Delta \psi_{m}$, AIF translocation, nuclear condensation, and subsequent cell death induced by the As$_2$O$_3$ treatment. However, PARP-1 inhibitors failed to block generation of ROS induced by As$_2$O$_3$. In contrast, thiol-containing antioxidant NAC completely inhibits PARP-1 activation, in-
Previous reports that Bcl-2 can block loss of mitochondria and PARP-1–mediated AIF release in cells treated with As2O3 (data not shown). These results are consistent with previous reports that Bcl-2 can block loss of Δψm and AIF release (43, 44). Interestingly, Bcl-2 overexpression completely blocked elevation of intracellular ROS level and subsequent activation of PARP-1 in As2O3-treated cervical cancer cells (data not shown). These results indicate that Bcl-2 inhibits AIF nuclear translocation and cell death by preventing elevation of intracellular ROS level mediated by As2O3. However, the mechanism by which Bcl-2 blocks increase of intracellular ROS level remains unclear. In some systems, Bcl-2 (or Bcl-xL) appears to influence ROS generation (45, 46), whereas in others it does not affect ROS production but does prevent oxidative damage to cellular constituents (47–49). It also has been proposed that Bcl-2 modulates level of ROS through induction of intracellular antioxidannts (50).

In summary, we report here that translocation of AIF from mitochondria to the nucleus is required for As2O3-induced cell death in human cervical cancer cells and that ROS-mediated PARP-1 activation is necessary for AIF release from mitochondria. These results suggest that As2O3 causes AIF translocation in a PARP-1–dependent manner and that PARP-1 activation is initiated by the increase of intracellular ROS level, providing evidence for the caspase-independent pathway of solid tumor cell death induced by As2O3.

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