Caspase-Independent Cell Death by Arsenic Trioxide in Human Cervical Cancer Cells: Reactive Oxygen Species-Mediated Poly(ADP-ribose) Polymerase-1

ABSTRACT

Although mechanisms of arsenic trioxide (As$_2$O$_3$)-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 As$_2$O$_3$-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 As$_2$O$_3$ induces dissipation of mitochondrial membrane potential ($\Delta\Psi_m$), translocation of AIF from mitochondria to the nucleus, and subsequent cell death. Small interfering RNA targeting of AIF effectively induces an increase of intracellular ROS level and a marked activation of /H9004/H9274 apoptotic effects of arsenic trioxide (As$_2$O$_3$) are not restricted to APL cells but also can be observed in other malignant cells in vitro, including non-APL acute myeloid leukemia cells, myeloma cells, and chronic myeloid leukemia cells, as well as various solid tumor cells, such as esophageal, prostate, and ovarian carcinomas and neuroblastoma cells (4–6).

The nature of apoptotic signals from mitochondria has been well documented (7–9). Cytochrome c and apoptosis-inducing factor (AIF) are well known as proapoptotic molecules released from the mitochondria (10, 11). Cytochrome c usually is released from the mitochondrial intermembrane space into the cytosol as consequence of the mitochondrial membrane potential ($\Delta\Psi_m$) loss (10, 12, 13). Cytochrome c released from the mitochondria forms a complex with procaspase-9 and apoptotic protease-activating factor-1 (Apaf-1), resulting in activation of procaspase-9. Contrarily, AIF condenses chromatin to induce apoptosis without involvement of caspases. Although mechanism of the AIF release from the mitochondria has not been fully understood, the regulatory role of AIF during programmed cell death has been well documented. AIF is a putative caspase-independent effector of cell death, and it recently has been characterized as proapoptotic mitochondrial intermembrane flavoprotein (11, 13). Similar to cytochrome c, AIF is released from the mitochondria in response to death stimuli. On induction of apoptosis, AIF is translocated to the nucleus and causes large-scale DNA fragmentation and chromatin condensation in a caspase-independent manner (11, 14, 15).

Arsenic disturbs the natural oxidation and reduction equilibrium in cells via various mechanisms, which involve complex redox (reduction/oxidation) reactions with endogenous oxidants and cellular anti-oxidant systems. Arsenic exposure leads to an increase of reactive oxygen species (ROS) by a variety of redox enzymes, including flavoprotein-dependent superoxide-producing enzyme such as NADPH oxidase (16–18). It recently has been shown that As$_2$O$_3$ decreases glutathione (GSH) and increases intracellular ROS level in certain APL cells (19). The intracellular GSH redox system has been proposed to be responsible for the As$_2$O$_3$ sensitivity. Compared with other leukemia cells that are less sensitive to As$_2$O$_3$, APL-derived NB4 cells contain lower levels of the GSH peroxidase and catalse and relatively higher levels of the intracellular hydrogen peroxide ($H_2O_2$; ref. 20). In vitro investigations of NB4 cells showed that clinically achievable concentration of As$_2$O$_3$ (1 to 2 $\mu$mol/L) induces apoptosis through a ROS-dependent pathway: Accumulation of intracellular ROS led to disruption of the $\Delta\Psi_m$, release of cytochrome c, followed by the activation of the caspase cascade, and ultimately to programmed cell death (20, 21). Although the mechanisms of As$_2$O$_3$-induced cell death have been studied extensively in hematologic cancers, those in solid cancers have yet to be clearly defined.

Poly(ADP-ribose) polymerase-1 (PARP-1) is a nuclear enzyme that catalyzes covalent attachment of long branched chains of poly(ADP-ribose) (PAR), with NAD$^{+}$ (NAD) as its substrate to a variety of nuclear DNA binding proteins, including PARP-1 itself, and facilitates DNA repair in response to genomic DNA damage (22, 23). In several pathologic situations that involve massive DNA damage, excessive activation of PARP-1 depletes cellular reserve of NAD and its precursor ATP, leading to cell death via energy failure (24, 25). It recently has been shown that ROS-mediated DNA damage triggers activation of the 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 the cell death (27, 30). The data in these studies suggested that translocation of AIF from
Treatment of HeLa cells with As2O3 increased intracellular ROS through a ROS-dependent pathway 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-dihydroxy-5-(1-piperidinyl)butoxy-1-(2H)-isoquinolinoline (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-VAD-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 Roswell Park Memorial Institute 1640 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 GGAAUAUUGGAAAGAUCGCGTdT. A nonrelated control siRNA was GGAAUAUUGGAAAGAUCGCGTdT. The nonrelated control siRNA that targeted the green fluorescent protein DNA sequence CCACCTACTGAGCCACCCAG 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 $1 \times 10^5$ 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 mg/mL) for 5 minutes at room temperature and analyzed with a flow cytometer. When necessary, N-acetyl-L-cystein (NAC), specific PARP inhibitors DPQ (50 μmol/L) and DQ (50 μmol/L), or pancaspase inhibitor z-VAD-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. Briefly, cells (5 × 10^5/mL) were exposed to 2 μmol/L As2O3 for the indicated times. After exposure, cells were incubated in 30 nmol/L 3,3′-dihexyloxycarbocyanine iodide (DiOC6(3)) and 10 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. 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 using enhanced chemiluminescence procedures (Amersham Biosciences, Piscataway, NY) 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 diithio-teritol, 0.1 mmol/L phenylmethylsulfonylfluoride, 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 nuclei 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, the level of the cytosolic cytochrome c was 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 \( \text{As}_2\text{O}_3 \)-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 \( \text{As}_2\text{O}_3 \)-induced apoptotic cell death (Fig. 1D). These results indicate that \( \text{As}_2\text{O}_3 \)-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 \( \text{As}_2\text{O}_3 \)-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 \( \text{As}_2\text{O}_3 \) 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 \( \text{As}_2\text{O}_3 \) (Fig. 2B). Moreover, siRNA targeting of the AIF effectively attenuated \( \text{As}_2\text{O}_3 \)-induced AIF nuclear translocation and nuclear condensation (Fig. 2C and D). These results suggest that AIF translocation into the nucleus is required in \( \text{As}_2\text{O}_3 \)-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 \( \text{As}_2\text{O}_3 \) 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 \( \text{As}_2\text{O}_3 \) treatment. These results indicate that \( \text{As}_2\text{O}_3 \)-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 anticancer effects of arsenic compounds (36), we subsequently examined the level of intracellular ROS in HeLa cells after treatment with \( \text{As}_2\text{O}_3 \) using an oxidation-sensitive fluorescent probe DCFH-DA, which is oxidized to \( \text{DCF} \) in the presence of ROS. Fig. 4A shows that treatment of cells with \( \text{As}_2\text{O}_3 \) led to a 2.5-fold increase of mean DCF fluorescence. We next investigated whether generation of ROS induced by the \( \text{As}_2\text{O}_3 \) treatment is accompanied by the loss of \( \Delta\psi_{m} \), AIF release, and apoptotic cell death seen after \( \text{As}_2\text{O}_3 \) treatment. To determine a link between elevation of the intracellular ROS level and apoptotic cell death in \( \text{As}_2\text{O}_3 \)-treated cells, HeLa cells were preincubated with thiol-containing antioxidant NAC before treatment with \( \text{As}_2\text{O}_3 \). Inhibition of ROS by NAC prevented loss of the \( \Delta\psi_{m} \), AIF release, and apoptotic cell death seen after \( \text{As}_2\text{O}_3 \) treatment. Moreover, \( \text{As}_2\text{O}_3 \) 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
/H$_9262$/ 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 excluding 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-
totic cell death induced by \( \text{As}_2\text{O}_3 \) seems to be caspase independent because caspase inhibitors failed to attenuate cell death induced by the \( \text{As}_2\text{O}_3 \) 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 \( \text{As}_2\text{O}_3 \) treatment of HeLa cells. AIF translocation and nuclear condensation were detectable within 24 hours of \( \text{As}_2\text{O}_3 \) treatment. Furthermore, siRNA knockdown of the AIF protein in cervical cancer cells effectively attenuated \( \text{As}_2\text{O}_3 \)-induced cell death and AIF translocation and nuclear condensation. Attenuation of the \( \text{As}_2\text{O}_3 \)-induced cell death by siRNA targeting of the AIF protein in cervical cancer cells effectively attenuated \( \text{As}_2\text{O}_3 \)-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 \( \text{As}_2\text{O}_3 \) 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 \( \Delta \psi_m \) 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
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 $\Delta\psi_{in}$, 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 $\Delta\psi_{in}$ 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 $\Delta\psi_{in}$. 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 $\Delta\psi_{in}$, 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-
indicating that PARP-1 activation is mediated by ROS in As$_2$O$_3$-treated cells. These results suggest that As$_2$O$_3$ 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 As$_2$O$_3$.

In summary, we report here that translocation of AIF from mitochondria to the nucleus is required for As$_2$O$_3$-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 As$_2$O$_3$ 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 As$_2$O$_3$.

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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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