Arsenic trioxide concentration determines the fate of Ewing’s sarcoma family tumors and neuroblastoma cells in vitro

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Abstract Arsenic trioxide (As\textsubscript{2}O\textsubscript{3}) induces both the differentiation and apoptosis of acute promyelocytic leukemia cells in a concentration dependent manner. We assessed the effects of As\textsubscript{2}O\textsubscript{3} in CADO-ES Ewing’s sarcoma (ES), JK-GMS peripheral primitive neuroectodermal tumor (PNET), and SH-SY5Y neuroblastoma cells, as they share common histogenetic backgrounds. As\textsubscript{2}O\textsubscript{3} at low concentrations (0.1–1 \mu M) induced SH-SY5Y differentiation, and whereas PNET cells acquired a slightly differentiated phenotype, change was minimal in ES cells. Extracellular signal-regulated kinase 2 (ERK2) was activated at low As\textsubscript{2}O\textsubscript{3} concentrations, and PD98059, an inhibitor of MEK-1, blocked SH-SY5Y cell differentiation by As\textsubscript{2}O\textsubscript{3}. High concentrations (2–10 \mu M) of As\textsubscript{2}O\textsubscript{3} induced the apoptosis in all three cell lines, and this was accompanied by the activation of c-jun N-terminal kinase. The generation of 

H\textsubscript{2}O\textsubscript{2} and activation of caspase 3 were identified as critical components of As\textsubscript{2}O\textsubscript{3}-induced apoptosis in all of the above cell lines. Fibroblast growth factor 2 (FGF2), an extracellular signal-regulated kinase 2 (ERK2) activator, enhanced the effect of As\textsubscript{2}O\textsubscript{3} in vivo. Moreover, As\textsubscript{2}O\textsubscript{3} induces the apoptosis of various NB cell lines by reducing the level of intracellular GSH (glutathione) and enhances the effect of SH-SY5Y cells treated with 1–2 \mu M As\textsubscript{2}O\textsubscript{3} undergoing apoptosis by both PML-RAR\alpha-dependent and -independent mechanisms. Moreover, the downregulation of bcl-2 and the activation of caspase 3 are associated with As\textsubscript{2}O\textsubscript{3}-induced PNET cell apoptosis [4–7], and ascorbic acid is known to enhance the effect of As\textsubscript{2}O\textsubscript{3} in vivo [8].

Neuroblastoma (NB) is one of the most common extracranial solid tumors of childhood, and shares some similarity with APL in that some of the tumors are responsive to retinoic acid (RA). Moreover, As\textsubscript{2}O\textsubscript{3} induces the apoptosis of various NB cell lines by reducing the level of intracellular GSH (glutathione) and by activating caspase 3 [1,9,10]. Ewing’s sarcoma (ES) and peripheral primitive neuroectodermal tumor (PNET) are small round-cell tumors that predominantly affect bone and soft tissues in children and adolescents. Despite the developments of therapeutic modalities, ES/PNET patients with advanced disease have a poor prognosis as compared with those with other solid tumors of childhood. Although the histogenesis of ES/PNET remains debatable, the current consensus is that they are derived from neural crest and have primitive neural characteristics.

As\textsubscript{2}O\textsubscript{3} at high doses induces the apoptosis of NB cells, and this is accompanied by the activation of caspase 3. Moreover, sensitivity to As\textsubscript{2}O\textsubscript{3} is inversely proportional to the intracellular level of reduced GSH [9]. Although ES/PNET cells have features in common with NB cells, the effects of As\textsubscript{2}O\textsubscript{3} have not been investigated in these cells. Therefore, in the present study, we compared the effects of As\textsubscript{2}O\textsubscript{3} in ES/PNET and NB cells. High and low concentrations of As\textsubscript{2}O\textsubscript{3} were found to differentially modulate the fates of these cells, i.e. they induced differentiation and apoptosis, respectively, as is the case for APL cells. Extracellular signal-regulated kinase 2 (ERK2) activation by low dose As\textsubscript{2}O\textsubscript{3} was found to be linked with the differentiation of SH-SY5Y cells, whereas reactive oxygen species (ROS) generation by high dose As\textsubscript{2}O\textsubscript{3} induced apoptosis in all cell lines. As\textsubscript{2}O\textsubscript{3} appears to have potential as a treatment for ES/PNET and NB by inducing either the differentiation or apoptosis of cells.

1. Introduction

Arsenic trioxide (As\textsubscript{2}O\textsubscript{3}) is toxic to various human cancers [1–5], and has been primarily used in the treatment of acute promyelocytic leukemia (APL). Moreover, its therapeutic effects are also under investigation in other malignancies [6]. Arsenic compounds at low doses (0.1–1 \mu M) induce APL cell differentiation by degrading promyelocytic leukemia protein (PML)-retinoic acid receptor \alpha (RAR\alpha) fusion protein, which is specific for APL cells with the chromosomal translocation t(15;17), whereas cells treated with 1–2 \mu M As\textsubscript{2}O\textsubscript{3} undergo apoptosis by both PML-RAR\alpha-dependent and -independent mechanisms. Moreover, the downregulation of bcl-2 and the activation of caspase 3 are associated with As\textsubscript{2}O\textsubscript{3}-induced PNET cell apoptosis [4–7], and ascorbic acid is known to enhance the effect of As\textsubscript{2}O\textsubscript{3} in vivo [8].

Keywords: Arsenic trioxide; Ewing’s sarcoma/peripheral primitive neuroectodermal tumor

Abbreviations: As\textsubscript{2}O\textsubscript{3}, arsenic trioxide; APL, acute promyelocytic leukemia; NB, neuroblastoma; ES, Ewing’s sarcoma; PNET, peripheral primitive neuroectodermal tumor; MAPKs, mitogen activated protein kinases; ERK1/2, extracellular signal-regulated kinase 1/2; JNK, c-jun N-terminal kinase; FGF2, fibroblast growth factor 2; ROS, reactive oxygen species

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2. Materials and methods

2.1. Cell lines and culture methods

ES (CADO-ES)/PNET (JK-GMS) and NB (SH-SY5Y) cell lines were used in the study. Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics, at 37 °C in a humidified 5% CO₂/95% air atmosphere. As₂O₃ was dissolved in 1 N NaOH, then diluted to 1 mM with phosphate-buffered saline (PBS), and this was used as stock solution. PD98059 (Cell Signaling Technology, Beverly, MA), an inhibitor of MEK-1, and SP600125 [anthra(1,9-cd)pyrazol-6(2H)-one; 1,9-pyrazoloanthrone] (Calbiochem, La Jolla, CA, USA), a c-jun N-terminal kinase (JNK) specific inhibitor, were used. The concentration of antioxidant NAC ([N-acetylcysteine] was 5 or 10 mM. Broad caspase inhibitor z-VAD-fmk (fluoromethyl ketone) was obtained from R&D systems (Minneapolis, MN) and used at a final concentration of 20 μM. Fibrinoblast growth factor 2 (FGF2) purchased from Sigma was dissolved in sterile PBS and treated at concentrations of 50 or 100 ng/mL.

2.2. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay

Cells were treated with various concentrations of As₂O₃ were grown, and MTT (500 μg/mL) was added after As₂O₃ treatment for 24, 48 or 72 h. After incubation for 3 h with MTT, absorbance was measured at 490 nm using an ELISA microplate reader.

2.3. Immunoblotting

Cell lysates were prepared using RIPA buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, 0.1% SDS, 2 mM EDTA, 2 mM PMSF and protease inhibitors). Extracts were electrophoresed in 10% SDS–polyacrylamide gel and electrotransferred to nitrocellulose membranes. Membranes were then blocked with TBST (tris-buffered saline supplemented with 1% Tween-20) containing 3% non-fat skim milk at room temperature for 1 h. The membranes were then incubated with anti-neurofilament (Zymed, San Francisco, CA), anti-phospho-JNK (Cell Signaling Technology, Beverly, MA), anti-JNK (Cell Signaling Technology), anti-phospho-ERK1/2 (Cell Signaling Technology), anti-ERK1/2 (Cell Signaling Technology), anti-caspase 3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-actin (Santa Cruz Biotechnology) antibodies at 4°C overnight. After incubation with HRP-conjugated secondary antibody (Amersham, Piscataway, NJ), signals were detected with ECL (enhanced chemiluminescence).

2.4. H₂O₂ production

Cells were treated with 10 mM NAC an hour prior to treatment with 5 μM As₂O₃. After 6 h of As₂O₃ treatment, cells were labeled with 20 μM of 2′,7′-DCFH-DA (dichlorofluorescin diacetate; Sigma–Aldrich Co., St. Louis, MO) for 30 min at 37 °C. After washing with PBS, DCF fluorescence (an ROS oxidized form of DCFH-DA) was measured using fluorescence microscopy with excitation and emission settings of 495 and 525 nm, respectively.

2.5. Annexin V-FITC/PI staining

After cells had been treated with As₂O₃ for 24 h, apoptotic rates were analyzed by flow cytometry using Annexin V-FITC/PI kits (MBL; Medical & Biological Laboratories, Nagoya, Japan). Cells were considered as apoptotic, necrotic, or viable if they showed Annexin V’PI’, PI’, or no staining, respectively. Samples were prepared according to the manufacturer’s instructions and analyzed using a Becton Dickinson FACS Calibur flow cytometer.

2.6. DNA gel electrophoresis

Cells were collected by centrifugation at 2000 × g for 5 min, washed twice with ice-cold PBS, resuspended in lysis buffer [10 mM Tris–HCl (pH 7.4), 100 mM NaCl, 25 mM EDTA, 0.5% SDS, and 0.3 mg/mL proteinase K], and incubated at 48 °C overnight. Cold 5 M NaCl solution was then added to a final concentration of 1 M, and mixtures were vortexed and centrifuged at 14,000 × g for 5 min. After precipitation with isopropanol, cell pellets were resuspended in TE buffer [10 mM Tris–HCl (pH 7.4), 1 mM EDTA] containing 20 μg/mL DNAse free RNase and then incubated at 37 °C for 1 h. DNA samples (20 μg) were subjected to electrophoresis in 1% agarose gels, and visualized by ethidium bromide staining.

2.7. Measurement of intracellular GSH

Intracellular GSH contents were measured using a Glutathione Assay Kit (Calbiochem). Cells were homogenized in 5% metaphosphoric acid and then separated by centrifugation at 6000 × g. Supernatants were used to measure GSH contents, according to the manufacturer’s instructions. Pellets were dissolved in RIPA lysis buffer and analyzed for protein concentrations using the BCA method. GSH content normalized versus untreated controls.

2.8. Caspase 3 activity

Cells were treated with 5 μM As₂O₃ for 16 h in the presence or absence of z-VAD-fmk, SP600125, or NAC, and then resuspended in RIPA buffer. After centrifugation at 14,000 × g for 20 min, supernatants were collected. Assays were performed in 96-well plates by incubating 25 μg of cell lysates in 100 μL of reaction buffer [1% NP-40, 20 mM Tris–HCl (pH 7.4), 137 mM NaCl, 10% glycerol] containing caspase 3 substrate (Ac-DEVD-pNA; Ac-Asp-Glu-Val-Asp-pNA) at 5 μM. Lysates were incubated at 37 °C for 2 h. Absorbances were measured at 405 nm using an ELISA microplate reader.

2.9. Statistical analysis

Data are expressed as means ± standard error of mean (S.E.M.). Different treatments were compared using the Mann–Whitney U test. Statistical analyses were performed using SPSS 11.5 software. For all analyses, P < 0.05 was considered statistically significant. All experiments were performed at least three times.

3. Results

3.1. Growth inhibitory effect of As₂O₃ on the ES/PNET and NB cell lines

CADO-ES, JK-GMS, and SH-SY5Y cells were found to be sensitive to increasing concentrations (0.5–10 μM) of As₂O₃ over 72 h by MTT assays (Fig. 1). The low (0.5–1 μM) and high (≥ 2 μM) levels of As₂O₃ used were based on the findings of previous studies [1,11,12]. Treatment of cells with As₂O₃ at 2, 5, or 10 μM led to a dose-dependent decrease in cell viability for all cell lines. At 2 μM As₂O₃, the viable cell number reductions were more prominent in JK-GMS cells than in CADO-ES or SH-SY5Y cells. The cell viabilities of JK-GMS and CADO-ES/SH-SY5Y cells were 30% and 70%, respectively. However, at 10 μM As₂O₃, significant difference was not noted between the viabilities of SH-SY5Y cells and CADO-ES/JK-GMS cells (20% and 10%, respectively).

Fig. 1. The growth inhibitory effect of As₂O₃. ES/PNET and NB were treated with the indicated concentrations of As₂O₃. After 3 days, cell viabilities were determined by MTT assay. Exposure of these cells to As₂O₃ led to concentration-dependent decreases in cell viabilities. Results are presented as the means ± S.D. of percentages of treated versus non-treated cells (n = 3).
3.2. Biological effects of As$_2$O$_3$ at low concentrations

To investigate whether As$_2$O$_3$ at low concentrations induces differentiation, we assessed the expression of neurofilament and also investigated the relationship between differentiation and mitogen activated protein kinase (MAPK) pathways (Fig. 2). Treatment of cells with 0.2 or 0.5 μM As$_2$O$_3$ induced the neuronal differentiations of SH-SY5Y cells, as characterized by neurite extension. JK-GMS cells also showed neurite extension, but to a lesser extent than SH-SY5Y cells, whereas CADO-ES cells did not show distinct morphological changes (Fig. 2A). Neuronal differentiation was accompanied by the increased expression of 160 kDa neurofilament and the phosphorylations of p42, rather than p44, in SH-SY5Y cells. Although both p44 and p42 are readily detectable in JK-GMS PNET or CADO-ES cells, these changes were not as prominent as in SH-SY5Y cells. The ERK1/2 (p44/42) antibody used in our experiments detected differential phosphorylation patterns of p44/42 in CADO-ES and SH-SY5Y cells suggesting that the difference of them is cell type specific (Fig. 2B).

As As$_2$O$_3$-induced neuronal differentiation was accompanied by p42 phosphorylation, we tested whether ERK1/2 inhibition blocked this differentiation. Pre-treatment of cells with 50 μM PD98059, a specific inhibitor of MEK-1, blocked both the phosphorylation of p42 and the neuronal differentiation induced by 0.2 μM As$_2$O$_3$, demonstrating that p42 activation is critically required for neuronal differentiation (Fig. 2C).

3.3. Biological effects of As$_2$O$_3$ at high concentrations

Analyses of MAPK phosphorylation showed that JNK was activated in ES/PNET and NB cells treated with high concentrations of As$_2$O$_3$ (5 or 10 μM). Moreover, pretreatment with SP600125, a specific JNK inhibitor, one hour prior to As$_2$O$_3$ treatment, abrogated JNK phosphorylation, but total JNK levels were unaffected (Fig. 3A and B).

Because As$_2$O$_3$ is known to generate ROS in tumor cell lines [11,13,14]. We assessed H$_2$O$_2$ production using the cell permeable oxidation-dependent fluorescence dye 2',7'-DCFH-DA. It was found that the intensity of the mean oxidized DCF peak was increased by 3- and 2.7-fold compared to controls after 6 h of As$_2$O$_3$ treatment in CADO-ES and JK-GMS cells, respectively. Moreover, H$_2$O$_2$ generation was found to be associated with increased apoptosis by As$_2$O$_3$, which was markedly suppressed by pre-treatment with 10 mM NAC, a ROS inhibitor, an hour prior to As$_2$O$_3$ treatment (Fig. 3C). In addition, and analysis of intracellular GSH contents showed that treatment with 5 μM As$_2$O$_3$ for 8 h reduced GSH contents and that 10 μM SP600125 augmented this decrease. However, intracellular GSH contents were significantly restored by 10 mM NAC (Fig. 3D).

We further assessed the significance of JNK activation and H$_2$O$_2$ generation on the biological effects of As$_2$O$_3$. ES/PNET and NB cells were cultured with 5 μM As$_2$O$_3$ for 24 h in the presence of SP600125 or NAC, and cellular viabilities and
apoptosis were assessed by using MTT assays (data not shown) and flow cytometry after Annexin V-FITC/PI staining. Pre-treatment with 10 μM SP600125 enhanced As2O3-induced growth inhibition and apoptosis, and 10 mM NAC effectively blocked the effects of As2O3 on cell growth and apoptosis. However, SP600125 or NAC alone did not affect cell growth or apoptosis. Flow cytometric analysis showed that 5 μM As2O3 resulted in a 2-fold increase in the apoptosis compared to control cells in all three cell lines (Fig. 3E). Moreover, the effects of SP600125 and NAC on the nucleosomal DNA fragmentation associated with As2O3-induced apoptosis were also consistent with the results of MTT assay and flow cytometry. Treatment with 10 μM SP600125 was associated with more distinct DNA oligonucleosomal ladders than treatment with As2O3, whereas 10 mM NAC almost completely abrogated DNA ladder formation (Fig. 3F).

We assessed whether ROS generation is related with the activations of JNK and caspase 3. Treatment of the ES/PNET and NB cells with 5 μM As2O3 for 8 or 16 h induced JNK phosphorylation (data not shown) and caspase 3 activation.
respectively, and these were completely blocked by 10 mM NAC pretreatment an hour prior to As₂O₃ treatment (Fig. 3G). Caspase activities after treating the cells with As₂O₃ alone or with As₂O₃ in the presence of z-VAD-fmk, NAC or SP600125 were also examined. When JK-GMS cells were incubated for 16 h with 5 μM As₂O₃, a 2-fold increase in caspase activity was observed compared to untreated cells, but caspase activities remained at the control level with As₂O₃ was treated in the presence of z-VAD-fmk or NAC. Moreover, the combination As₂O₃ and SP600125 markedly enhanced caspase activity versus As₂O₃ alone, demonstrating that the apoptosis is caspase 3 dependent (Fig. 3H).

3.4. Effects of FGF2 on As₂O₃-induced apoptosis
ES/PNET cells undergo apoptosis when treated with FGF2 in a dose-dependent manner [15], and therefore, we investigated the effect of FGF2 in the presence of As₂O₃ (Fig. 4). JK-GMS cells were treated with 2 or 3 μM As₂O₃, 50 or 100 ng/mL of FGF2, or a combination of FGF2 and As₂O₃. After 2 days of exposure to As₂O₃, and FGF2, increases in growth inhibition and apoptosis by MTT assay (Fig. 4A) and Annexin V-FITC/PI staining (Fig. 4B) were observed versus cell treated with As₂O₃ or FGF2 alone, and this occurred in a dose dependent manner. Moreover, the decrease in cell viability observed at 48 h after treatment with As₂O₃ and FGF2 was much more profound than that caused by either of the reagents alone. Taken together, it is thought that the treatment of As₂O₃ and FGF2 combination induces the synergistic effect in JK-GMS cells.

4. Discussion
We investigated the effects of As₂O₃ at different concentrations on ES/PNET cell lines and compared these with its effects in the NB cell line. Whereas NB cells clearly showed morphological and biological characteristics of neuronal differentiation by As₂O₃, these changes were minimal or absent in similarly treated PNET and ES cells. Moreover, As₂O₃ at low concentrations inhibited cellular growth and induced the neuronal differentiation of SH-SY5Y NB cells. This finding is in contrast with the results in a previous study [10], and may be due to differences in the NB cell lines used in terms of their abilities to differentiate. As compared with NB cells, ES/PNET cells have rather primitive neuronal characteristics in vivo and in vitro, yet PNET cells have more differentiated phenotypes than ES cells. The extent of neuronal differentiation induced by As₂O₃ appears to be related to the intrinsic potential of the cells to differentiate.

Mitogen activated protein kinases (MAPKs) include ERK1/2, JNK and p38, and are family of serine/threonine kinases that participate in signaling pathways activated by various external stimuli [11,12,16–18], and of these ERK1/2 is an important component of As₂O₃-induced neuronal differentiation. MAPKs are also involved in the As₂O₃-induced cell deaths of other cell types, such as, prostate cancer cells and HeLa cells [16,19–21], and these are known to occur via the downregulation of bcl-2 [11,12,18,22]. Thus, we tested whether the phosphorylation of JNK is critical to the As₂O₃-induced apoptosis of ES/PNET and NB cells by treating cells with a JNK inhibitor. Unexpectedly, combined treatment with SP600125 slightly enhanced As₂O₃-induced apoptosis. Although JNK is generally believed to cause As₂O₃-induced apoptosis in other cell lines [12,18], there is evidence that the activation of JNK may be protective in certain cell lines, especially in cases of oxidative stress-related injuries. The activation of JNK was found to have protective effects on cardiac myocytes in a reperfusion injury model and also on the sensory neurons of diabetic rats under oxidative stress [23,24]. It was also found that the JNK inhibitor, SP600125, potentiated As₂O₃-induced activation of p21, and thus caused cell-cycle arrest by inhibiting cyclin-cdk complexes, and cellular cytotoxicity in human epidermoid carcinoma A431 cells [25].

Diverse apoptotic stimuli, such as UV, irradiation, and chemicals, may trigger the mitochondrial apoptotic pathway, which is characterized by the generation of ROS, including O²⁻ (superoxide radical), H₂O₂ (hydrogen peroxide), OH (hydroxyl free radical) and ¹O₂ (singlet oxygen) [9]. ROS damage biological macromolecules and kills cells by oxidizing of lipids in mitochondrial membranes and the subsequent release of cytochrome c. Cytochrome c binds to apoptotic protease activating factor (Apaf-1), recruits initiator caspase 9, which activates caspase 3. Caspase 3 then cleaves DNA repair enzyme PARP (polyl ADP-ribose) polymerase and DNA into nucleosomal fragments [15,18,19]. Moreover, intracellular GSH is a major antioxidant and protects cells from As₂O₃-induced ROS generation, and H₂O₂ is detoxified by GSH peroxidase (which requires GSH as a substrate), or by catalase [9,14]. The inverse correlation between As₂O₃-induced H₂O₂ generation and intracellular GSH content demonstrates that the cytotoxicity of As₂O₃ is largely dependent on the generation of ROS. The present study also shows that ROS seem to play a key role in the As₂O₃-induced apoptosis of the cell lines tested, and that pre-treatment with the antioxidant NAC significantly blocks As₂O₃-induced cell death and the activations of JNK and caspase 3.

FGF2 is a major determinant of neural crest cell fate, and plays a critical role in the differentiation of neural crest and some NB cells [26]. FGF2 induces cell death of ES/PNET cells [15], which is in stark contrast with the neuroprotective role of FGF2 after ischemic, metabolic or traumatic brain injury [27]. Interestingly, FGF2 can induce the neuronal differentiation and apoptosis of PNET cells [28]. In the present study, FGF2 was found to clearly enhance As₂O₃-induced apoptosis in JK-GMS cells.

In summary, the present study describes the biological effects of As₂O₃ in ES/PNET cells for the first time. As₂O₃ was found to differentially affect the biology of both ES/PNET and NB cells by inducing cell differentiation and/or apoptosis depending on its concentration. Recently, Ryu et al. reported that As₂O₃ is well tolerated at concentrations of less than 3 μM by normal lymphocytes, but it inhibits proliferation and/or induces the apoptosis of SH-SY5Y and SK-N-AS NB cells [29]. Our results strongly suggest that As₂O₃ can be used as an effective therapeutic tool for the treatment of neural crest-derived and childhood solid tumors, either independently or in conjunction with other biological response modifiers like growth factors.

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