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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_2O_3) induces both the differentiation and apoptosis of acute promyelocytic leukemia cells in a concentration dependent manner. We assessed the effects of As_2O_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_2O_3 at low concentrations (0.1–1 μM) 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_2O_3 concentrations, and PD98059, an inhibitor of MEK-1, blocked SH-SY5Y cell differentiation by As_2O_3. High concentrations (2–10 μM) of As_2O_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_2O_2 and activation of caspase 3 were identified as critical components of As_2O_3-induced apoptosis in all of the above cell lines. Fibroblast growth factor 2 enhanced As_2O_3-induced apoptosis in JK-GMS cells. The overall effects of As_2O_3 strongly suggest that it has therapeutic potential for the treatment of ES/PNET.**

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

1. Introduction

Arsenic trioxide (As_2O_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 μM) induce APL cell differentiation by degrading promyelocytic leukemia protein (PML)-retinoic acid receptor α (RARα) fusion protein, which is specific for APL cells with the chromosomal translocation t(15;17), whereas cells treated with 1–2 μM As_2O_3 undergo apoptosis by both PML-RARα-dependent and -independent mechanisms. Moreover, the downregulation of bcl-2 and the activation of caspase 3 are associated with As_2O_3-induced APL cell apoptosis [4–7], and ascorbic acid is known to enhance the effect of As_2O_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_2O_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_2O_3 at high doses induces the apoptosis of NB cells, and this is accompanied by the activation of caspase 3. Moreover, sensitivity to As_2O_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_2O_3 have not been investigated in these cells. Therefore, in the present study, we compared the effects of As_2O_3 in ES/PNET and NB cells. High and low concentrations of As_2O_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_2O_3 was found to be linked with the differentiation of SH-SY5Y cells, whereas reactive oxygen species (ROS) generation by high dose As_2O_3 induced apoptosis in all cell lines. As_2O_3 appears to have potential as a treatment for ES/PNET and NB by inducing either the differentiation or apoptosis of cells.

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Abbreviations: As_2O_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% CO_2/95% air atmosphere. As_2O_3 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. Fibroblast 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 As_2O_3 were grown, and MTT (500 μg/mL) was added after As_2O_3 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, 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. H_2O_2 production

Cells were treated with 10 mM NAC an hour prior to treatment with 5 μM As_2O_3. After 6 h of As_2O_3 treatment, cells were labeled with 20 μM of 2′,7′-DCFH-DA (dichlorofluorescein 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 $As_2O_3$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 14000 × *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 As_2O_3 for 16 h in the presence or absence of z-VAD-fmk, SP600125, or NAC, and then resuspended in RIPA buffer. After centrifugation at 14000 × *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 As_2O_3 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 As_2O_3 over 72 h by MTT assays (Fig. 1). The low (0.5–1 μM) and high (⩾ 2 μM) levels of As_2O_3 used were based on the findings of previous studies [1,11,12]. Treatment of cells with As_2O_3 at 2, 5, or 10 μM led to a dose-dependent decrease in cell viability for all cell lines. At 2 μM As_2O_3, 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 As_2O_3 significant difference was not noted between the viabilities of SH-SY5Y cells and CADO-ES/JK-GMS cells (20% and 10%, respectively).

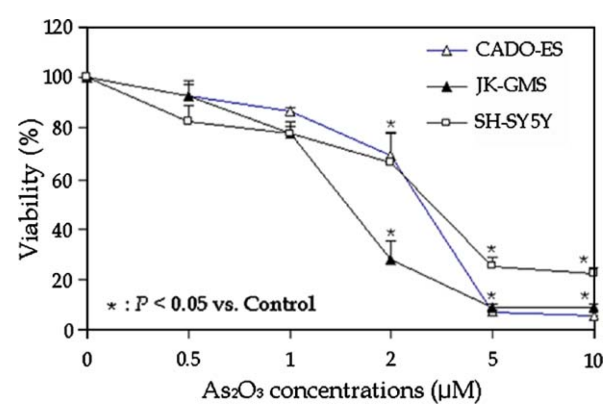


Fig. 1. The growth inhibitory effect of As_2O_3. ES/PNET and NB were treated with the indicated concentrations of As_2O_3. After 3 days, cell viabilities were determined by MTT assay. Exposure of these cells to As_2O_3 led to concentration-dependent decreases in cell viabilities. Results are presented as the means ± S.D. of percentages of treated versus non-treated cells ($n = 3$).

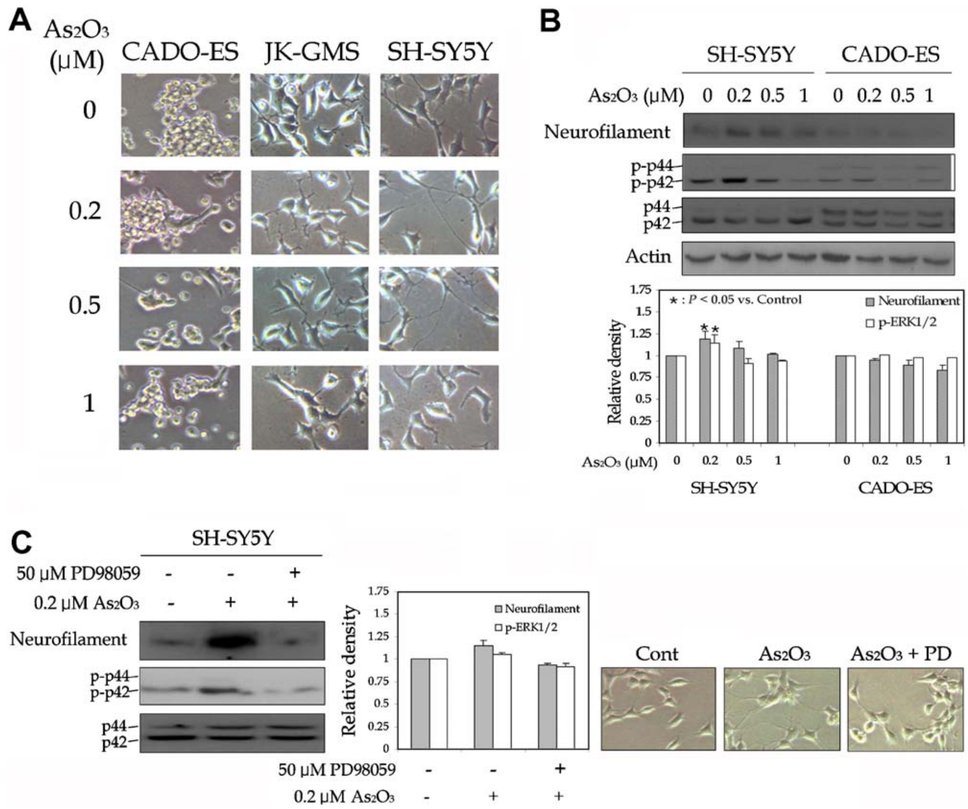


Fig. 2. Neuronal differentiation by low dose As_2O_3. (A) Treatment with 0.2 or 0.5 μM As_2O_3 for 3 days induced neuronal differentiation characterized by neurite extension, especially in SH-SY5Y cells. (B) Immunoblot for neurofilament and phospho-ERK1/2 in SH-SY5Y and CADO-ES cell lines at the indicated doses. Cellular proteins were resolved in 6–10% SDS–polyacrylamide gels. Membranes were probed with anti-neurofilament, phospho-ERK1/2 and ERK1/2 primary antibodies. (C) SH-SY5Y cells were treated with 0.2 μM As_2O_3 alone or combination with 50 μM PD98059 for 3 days and then immunoblotted. ERK2 activation by low dose As_2O_3 induced NB cells differentiation. However, PNET and ES cells showed hardly any neurite extension changes or ERK1/2 activation. Results are presented as relative fold activations versus control levels and are expressed as means ± S.D. (n = 3).

3.2. Biological effects of As_2O_3 at low concentrations

To investigate whether As_2O_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_2O_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).

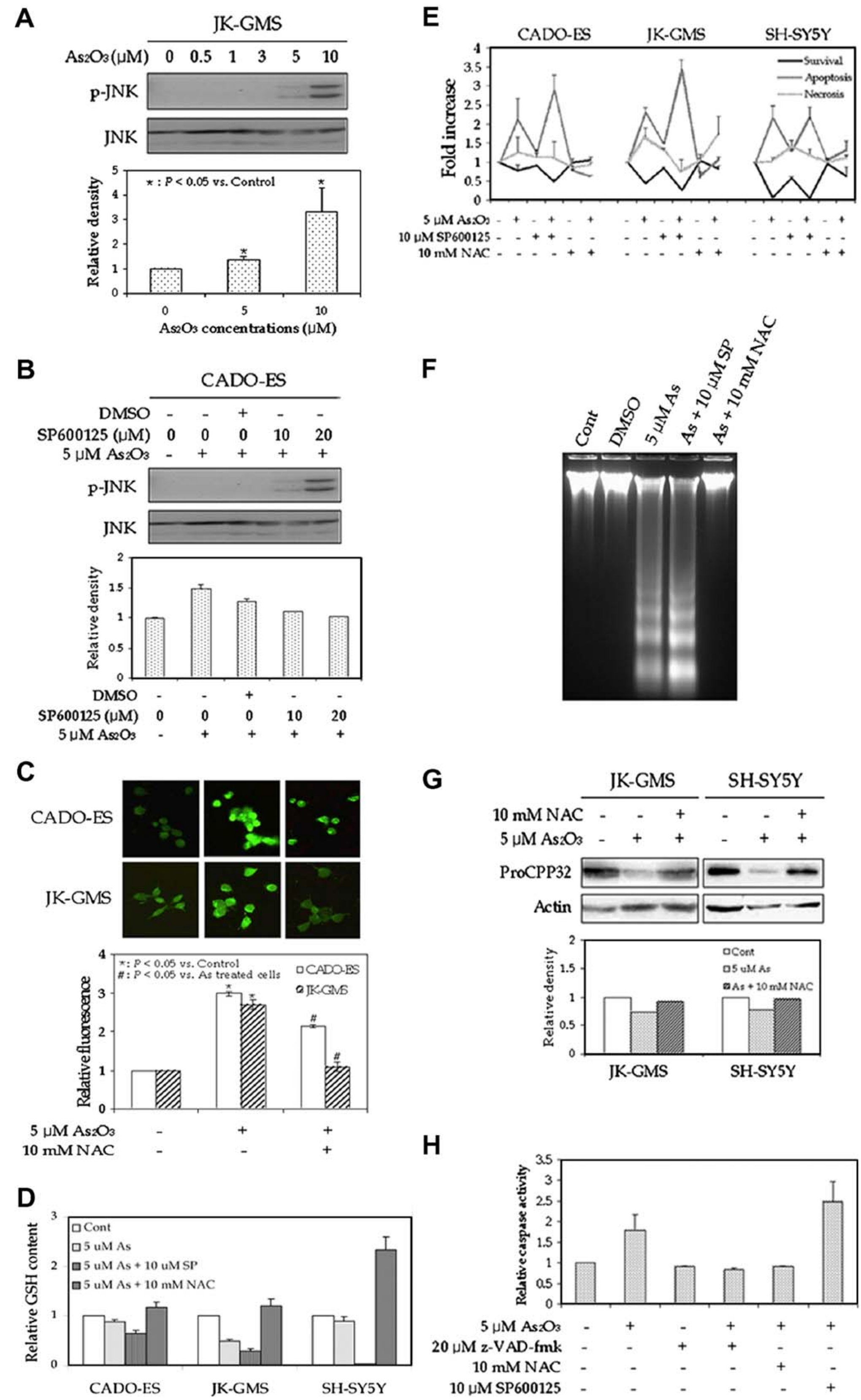
As As_2O_3-induced neuronal differentiation was accompanied by p42 phosphorylation, we tested whether ERK1/2 inhibition blocked this differentiation. Pre-treatment of cells with 50 μM PD98059, a specific inhibitor of MEK-1, blocked both the phosphorylation of p42 and the neuronal differentiation induced by 0.2 μM As_2O_3, demonstrating that p42 activation is critically required for neuronal differentiation (Fig. 2C).

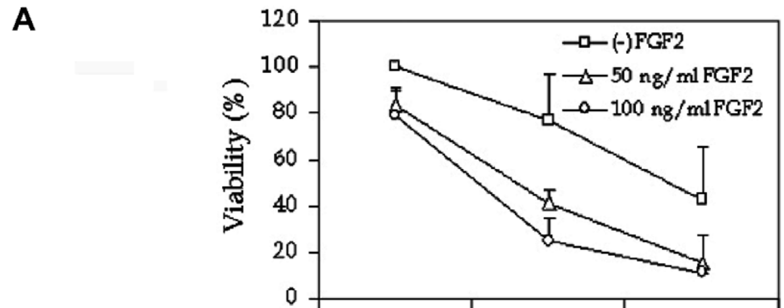
3.3. Biological effects of As_2O_3 at high concentrations

Analyses of MAPK phosphorylation showed that JNK was activated in ES/PNET and NB cells treated with high concentrations of As_2O_3 (5 or 10 μM). Moreover, pretreatment with SP600125, a specific JNK inhibitor, one hour prior to As_2O_3 treatment, abrogated JNK phosphorylation, but total JNK levels were unaffected (Fig. 3A and B).

Because As_2O_3 is known to generate ROS in tumor cell lines [11,13,14]. We assessed H_2O_2production 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_2O_3 treatment in CADO-ES and JK-GMS cells, respectively. Moreover, H_2O_2 generation was found to be associated with increased apoptosis by As_2O_3, which was markedly suppressed by pre-treatment with 10 mM NAC, a ROS inhibitor, an hour prior to As_2O_3 treatment (Fig. 3C). In addition, and analysis of intracellular GSH contents showed that treatment with 5 μM As_2O_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_2O_2 generation on the biological effects of As_2O_3. ES/PNET and NB cells were cultured with 5 μM As_2O_3 for 24 h in the presence of SP600125 or NAC, and cellular viabilities and


A
JK-GMS
As2O3 (μM) 0 0.5 1 3 5 10
p-JNK
JNK
*: P < 0.05 vs. Control
Relative density
As2O3 concentrations (μM)
B
CADO-ES
DMSO
SP600125 (μM)
5 μM As2O3
p-JNK
JNK
Relative density
C
CADO-ES
JK-GMS
*: P < 0.05 vs. Control
#: P < 0.05 vs. As treated cells
Relative fluorescence
5 μM As2O3
10 mM NAC
D
Cont
5 uM As
5 uM As + 10 uM SP
5 uM As + 10 mM NAC
Relative GSH content
CADO-ES
JK-GMS
SH-SY5Y
E
CADO-ES
JK-GMS
SH-SY5Y
Survival
Apoptosis
Necrosis
Fold increase
5 μM As2O3
10 μM SP600125
10 mM NAC
F
Cont
DMSO
5 μM As
As + 10 μM SP
As + 10 mM NAC
G
JK-GMS
SH-SY5Y
10 mM NAC
5 μM As2O3
ProCPP32
Actin
Cont
5 uM As
As + 10 mM NAC
Relative density
H
Relative caspase activity
5 μM As2O3
20 μM z-VAD-fmk
10 mM NAC
10 μM SP600125



	(-) As	2 uM As	3 uM As
(-) FGF2	100	76.61	42.61
50 ng/ml FGF2	83.67	40.92	15.07
100 ng/ml FGF2	79.18	24.66	10.80

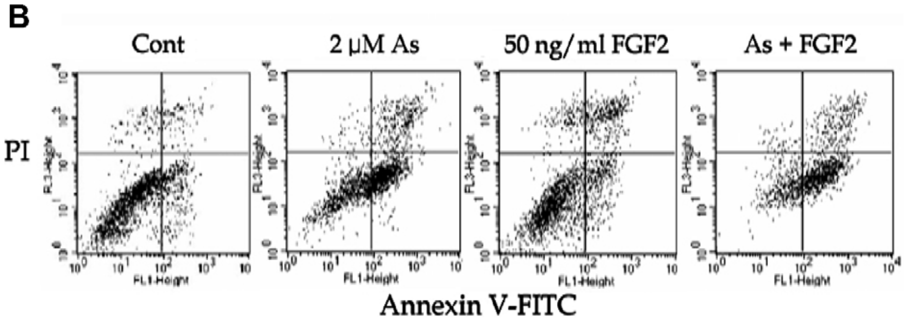


Fig. 4. Effects of FGF2 on As_2O_3-induced apoptosis in the JK-GMS cell line. (A) Cells were treated with multiple combinations of concentrations of As_2O_3 and FGF2. After 2 days of combined FGF2 + As_2O_3 treatment increased numbers of dead and floating cells were observed. Cell viabilities were determined using MTT assays. Results are shown as means ± S.D. ($n = 3$). (B) JK-GMS cells were treated with As_2O_3 alone or in combination with 50 ng/mL FGF2. Cell death types were determined by double staining with Annexin V-FITC and PI. FGF2 + As_2O_3 treatment was found to be more effective at causing apoptosis than As_2O_3 or FGF2 alone.

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 As_2O_3-induced growth inhibition and apoptosis, and 10 mM NAC effectively blocked the effects of As_2O_3 on cell growth and apoptosis. However, SP600125 or NAC alone did not affect cell growth or apoptosis. Flow cytometric analysis showed that 5 μM As_2O_3 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 As_2O_3-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 As_2O_3, 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 As_2O_3 for 8 or 16 h induced JNK phosphorylation (data not shown) and caspase 3 activation,

Fig. 3. Biological effects due to high dose As_2O_3. (A) JK-GMS cells were treated at the indicated doses. Cellular proteins were resolved in 10% SDS–polyacrylamide gels. Membranes were probed with anti-phospho-JNK and JNK primary antibodies. (B) CADO-ES cells were treated with 5 μM As_2O_3 alone or combination with 10 or 20 μM SP600125 for 8 h and then immunoblotted. Higher concentrations of As_2O_3 induced JNK phosphorylation, which was efficiently blocked by SP600125. Results are presented as the means ± S.D. of relative fold activations versus control levels ($n = 3$). (C) Increased generation of H_2O_2 following As_2O_3 treatment in ES/PNET cells. Cells were treated with 5 μM As_2O_3 alone or in combination with 10 mM NAC for 6 h. The cells were then labeled with 20 μM DCFH-DA. H_2O_2 generation was increased by As_2O_3 treatment, and this was markedly suppressed by pre-treatment with 10 mM NAC. The graphs represent changes in relative fluorescence intensities. (D) Changes in intracellular GSH content. ES/PNET and NB cells were treated with As_2O_3 for 8 h, and intracellular GSH contents were measured using a Glutathione Assay Kit, as described in Section 2. As_2O_3-induced H_2O_2 generation was found to be inversely correlated with intracellular GSH contents. GSH contents are represented relative to untreated controls. Measurements of As_2O_3-induced apoptosis. (E) Flow cytometric analysis by staining with Annexin V-FITC/PI. The cells were treated with the indicated concentrations of As_2O_3, SP600125 and NAC. Results are presented as the means ± S.D. of fold activations versus controls ($n = 3$). As_2O_3 treatment induced the apoptosis of ES/PNET and NB cell lines. (F) DNA fragmentation caused by treating cells with the indicated doses of various reagents for 24 h in JK-GMS. Extracted genomic DNA was electrophoresed in 1% agarose gels. (G) Relationship between ROS generation and the activation of caspase 3. Cells were treated with 5 μM As_2O_3 alone or combined with 10 mM NAC for 16 h. Cellular proteins were resolved in 10% SDS–polyacrylamide gels. Membranes were probed with anti-ProCPP32 and actin primary antibodies. (H) Cells were treated with 5 μM As_2O_3 alone or in combination with these reagents for 8 h. Cell extracts were prepared and incubated for 2 h at 37 °C with Ac-DEVD-pNA, and then DEVDase activity was determined colorimetrically. As_2O_3 was found to induce a caspase dependent mode of apoptosis. Activities are presented as fold increases of values treated versus non-treated controls.

respectively, and these were completely blocked by 10 mM NAC pretreatment an hour prior to As_2O_3 treatment (Fig. 3G). Caspase activities after treating the cells with As_2O_3 alone or with As_2O_3 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 As_2O_3, a 2-fold increase in caspase activity was observed compared to untreated cells, but caspase activities remained at the control level with As_2O_3 was treated in the presence of z-VAD-fmk or NAC. Moreover, the combination As_2O_3 and SP600125 markedly enhanced caspase activity versus As_2O_3 alone, demonstrating that the apoptosis is caspase 3 dependent (Fig. 3H).

3.4. Effects of FGF2 on As_2O_3-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 As_2O_3 (Fig. 4). JK-GMS cells were treated with 2 or 3 μM As_2O_3, 50 or 100 ng/mL of FGF2, or a combination of FGF2 and As_2O_3. After 2 days of exposure to As_2O_3, 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 As_2O_3 or FGF2 alone, and this occurred in a dose dependent manner. Moreover, the decrease in cell viability observed at 48 h after treatment with As_2O_3 and FGF2 was much more profound than that caused by either of the reagents alone. Taken together, it is thought that the treatment of As_2O_3 and FGF2 combination induces the synergistic effect in JK-GMS cells.

4. Discussion

We investigated the effects of As_2O_3 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 As_2O_3, these changes were minimal or absent in similarly treated PNET and ES cells. Moreover, As_2O_3 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 As_2O_3 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 As_2O_3-induced neuronal differentiation. MAPKs are also involved in the As_2O_3-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 As_2O_3-induced apoptosis of ES/PNET and NB cells by treating cells with a JNK inhibitor. Unexpectedly, combined treatment with SP600125 slightly enhanced As_2O_3-induced apoptosis. Although JNK is generally believed to cause As_2O_3-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 As_2O_3induced 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 O^{2-} (superoxide radical), H_2O_2 (hydrogen peroxide), OH (hydroxyl free radical) and $^1O_2$ (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 [poly (ADP-ribose) polymerase] and DNA into nucleosomal fragments [15,18,19]. Moreover, intracellular GSH is a major antioxidant and protects cells from As_2O_3-induced ROS generation, and H_2O_2 is detoxified by GSH peroxidase (which requires GSH as a substrate), or by catalase [9,14]. The inverse correlation between As_2O_3-induced H_2O_2 generation and intracellular GSH content demonstrates that the cytotoxicity of As_2O_3 is largely dependent on the generation of ROS. The present study also shows that ROS seem to play a key role in the As_2O_3-induced apoptosis of the cell lines tested, and that pre-treatment with the antioxidant NAC significantly blocks As_2O_3-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 As_2O_3-induced apoptosis in JK-GMS cells.

In summary, the present study describes the biological effects of As_2O_3 in ES/PNET cells for the first time. As_2O_3 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 As_2O_3 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 As_2O_3 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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