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Arsenic trioxide-induced apoptosis is independent of stress-responsive signaling pathways but sensitive to inhibition of inducible nitric oxide synthase in HepG2 cells

Shin-Hae Kang[2], Ji-Hoon Song[2], Hee-Kyoung Kang[1], Ji-Hoon Kang[1], Se-Jae Kim[2], Hyun-Wook Kang[1], Young-Ki Lee[1] and Deok-Bae Park[1,3]

[1]Department of Medicine, College of Medicine and
[2]Department of Life Science, College of Natural Science
Cheju National University, Ara-1, Jeju 690-756, Korea
[3]Corresponding author: Tel, 82-64-754-3827;
Fax, 82-64-725-2593; E-mail, parkdb@cheju.ac.kr

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Abbreviations: APL, acute promyelocytic leukemia; ATO, arsenic trioxide; DPI, diphenyleneiodonium; ERK, extracellular signal-regulated protein kinase; ETU, 2-ethyl-2-thiourea; GED, guanidinoethyldisulfide dihydrochloride; JNK, c-jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; NAC, *N*-acetylcysteine; ROS, reactive oxygen species

Abstract

Arsenic trioxide (As_2O_3) has been found to be remarkably effective in the treatment of patients with acute promyelocytic leukemia (APL). Although evidences for the proapoptotic activity of As_2O_3 have been suggested in leukemic and other solid cancer cells, the nature of intracellular mechanisms is far from clear. In the present study, we investigated As_2O_3 affect on the stress-responsive signaling pathways and pretreatment with antioxidants using HepG2 cells. When treated with micromolar concentrations of As_2O_3, HepG2 cells became highly apoptotic paralleled with activation of caspase-3 and members of mitogen-activated protein kinases (MAPKs) including extracellular signal-regulated kinase (ERK) and c-jun NH_2-terminal kinase (JNK) but not p38 MAP kinase. However, inhibition of each kinase activity failed to inhibit apoptosis by As_2O_3. Addition of n-acetyl cysteine (NAC) or diphenyleneiodonium (DPI) effectively protected cells from apoptosis and significantly lowered As_2O_3-induced activation of caspase-3. However, neither NAC nor DPI was able to effect ERK or JNK activation induced by As_2O_3. Guanidinoethyldisulfide dihydrochloride (GED) and 2-ethyl-2-thiopseudourea (ETU), known inhibitors of the inducible nitric oxide synthase (iNOS), also suppressed the apoptotic activity of As_2O_3. These results suggest that As_2O_3 induces caspase-mediated apoptosis involving a mechanism generating oxidative stress. However, activation of some stress-responsive signaling pathways by As_2O_3 may not be the major determinant in the course of apoptotic processes.

Keywords: antioxidant; apoptosis; arsenicals; extracellular signal-regulated kinase; mitogen-activated protein kinase; nitric oxide synthase

Introduction

Arsenic trioxide (As_2O_3) has been used as a therapeutic agent for centuries to treat several diseases like as anaemia, dyspepsia and some tumors (Huang *et al.*, 1995). Recently, As_2O_3 was found to be a very potent anti leukemic reagent, especially against acute promyelocytic leukemia (APL) (Shen *et al.*, 1997) by inducing apoptosis (Soignet *el al.*, 1998). As_2O_3 induced apoptosis in different myeloid leukemia cell lines such as HL60, U937 and KG-1 (Wang *et al.*, 1998). Although the precise mechanism of As_2O_3 action is still unclear, a variety of *in vitro* studies suggest that several mechanisms may be involved in the remission of leukemic cancer cells. Studies of the APL cell line NB4 and other myeloid leukemia cell lines treated with As_2O_3 showed down-regulation of the Bcl-2 protein (Thomas *et al.*, 2000) and activation of caspases (Akao *et al.*, 1998). Number of reports on arsenic compounds treated different malignant cell lines indicate proapoptotic actions may dependent on p53. In gastric cancer cells and in MBC-1, a B-cell lymphoma line, As_2O_3 exposure causes up-regulation of p53 gene expression, resulting in caspase activation leading to apoptosis (Shen *et al.*, 2000; Jiang *et al.*, 2001). In human fibroblasts, arsenite causes breakage of DNA strand, which leads to accumulation and phosphorylation of p53 (Yih and Lee, 2000). Roles of the cell cycle inhibitor $p27^{KIP1}$ and Cip1/p21

in As_2O_3 induced apoptosis also have been suggested (Ishitsuka *et al*., 2000). On the other hand, evidences from different model systems have suggested the ability of arsenic compounds to affect signaling molecules, especially, members of mitogen-activated protein kinases (MAPKs). c-Jun NH_2-ternimal kinase (JNK) as well as p38, not extrasignal-regulated protein kinase (ERK), are activated by arsenic compounds (Cavigelli *et al*., 1996; Porter *et al*., 1999) whereas ERK is activated by arsenic in bronchial epithelial cells (Wu *et al*., 1999). Barchowsky *et al*. (1999) observed activations of ERK and p38 in arsenite- induced apoptosis in primary vascular cells. Chronic exposure to As_2O_3 has resulted in numerous pathogenesis such as lung and skin cancers, noncancerous skin lesions, peripheral nerve effects, and cardiovascular changes (Snow, 1992).

Homeostasis of natural oxidation and reduction equilibrium within cells is crucial for maintaining cellular viability. Arsenic compounds are known to imbalance such equilibrium, thereby generating oxidative stress resulting in sensitizing a number of signaling molecules including AP-1, NF-κB, IκB, p53, $p21^{ras}$ (Simeonova and Luster, 2000). The cellular effects of complex redox reactions with endogenous oxidants and antioxidant systems in response to arsenic chemicals have been reported (Miller *et al*., 2002). However, it is far from clear to comprehend all activities of different signaling molecules affected by arsenic chemicals in different cellular systems. Thus, it is important to determine the sensitivity of different tumors against As_2O_3 and action mechanism of its anti-tumor activity. In the present study, we examined whether As_2O_3 can induce apoptosis in HepG2 cells and by which stimulations of stress-responsive signaling proteins are participating. Moreover, the effects of different antioxidant systems against the apoptotic activity of As_2O_3 were also investigated.

Materials and Methods

Materials

PD98059, SB202190, the fluorogenic caspase-3 substrate, Ac-DEVD-AMC, and the caspase inhibitor, z-DEVD-fmk, propidium iodide, guanidinoethyldisulfide dihydrochloride (GED), 2-ethyl-2-thiopseudourea (ETU) were obtained from Calbiochem (La Jolla, CA). Arsenic trioxide (As_2O_3), wortmannin, Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA solution, n-acetylcysteine (NAC), diphenyleneiodonium (DPI) were obtained from Sigma Chemical Corp. (St. Louis, MO), and FBS from Life Technologies Inc. (Rockville, MD). Monoclonal antibodies against phospho-ERK1/2 (E-4), phospho-JNK (G-7), phospho-p38MapK (D-8), ERK2 (D-2) and polyclonal antibodies against phospho-Akt1 (ser473), PARP (H- 250) were from Santa Cruz Biotechnology (Santa Cruz, CA). Electrophoresis reagents, such as polyacrylamide, Tris-glycine SDS running buffer, and poly (vinylidene difluoride) (PVDF) membrane were from Novex Corp. (San Diego, CA).

Cell culture

HepG2 cells were obtained from Korean Cell Line Bank (Seoul, Korea) and grown in DMEM containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FBS, and maintained in a humidified atmosphere of 5% CO_2 in air at 37°C. Two days after plating in 35-mm tissue culture dishes, cells were serum-starved for 24 h and then treated with different reagents. Cells were quickly frozen in liquid nitrogen and stored at -70°C until analysis.

SDS-PAGE and immunoblotting

Unless otherwise indicated, cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl, 1% nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μM aprotinin, 1 μM leupeptin, 1 μM pepstatin A). Same amount of proteins were separated by SDS-PAGE on 4-20% polyacrylamide gel and electrotransferred onto PVDF membrane. The membrane was incubated in blocking buffer [5% nonfat dry milk in Tris-buffered saline (TBS)-0.1% Tween-20 (TBS-T)] for 1 h at room temperature and then probed with different primary antibodies (1: 1,000-1:5,000). After a series of washes, the membrane was further incubated with different horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2,000-1:10,000). The signal was detected with enhanced chemiluminescence (ECL) detection system (Intron, Seoul, Korea).

Determination of caspase-3 activity

After treatments with reagents, cells were collected and lysed in ice-cold 0.5 ml caspase assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA) for 15 min. After centrifugation at 12,000 *g* for 15 min at 4°C, aliquots of supernatant were incubated with 10 mM Ac-DEVD-AMC for 3 h at 37°C. The fluorescence from the cleaved product was detected with Spectrafluor multiwell fluorescence reader (Tecan, Salzburg, Austria) at 360 nm and 465 nm wavelengths for excitation and emission, respectively.

MTT assay

This measure of mitochondrial function was performed as described previously (Parrizas *et al*., 1997) with

cells seeded on 24-well plates. Following treatments, the medium was removed from the wells, and 200 μl of MTT reagent (Sigma, St. Louis, MO) at a concentration of 1 mg/ml in RPMI-1640 medium without phenol red was added to each well. After 1 h incubation at 37°C, the cells were lysed by addition of 1 volume of 2-propanol and shaken for 20 min. Absorbance of converted dye was measured at a wavelength of 570-690 nm.

Detection of apoptotic cells with flow cytometric analysis and H33342 staining

The degree of apoptosis was determined by measuring the number of cells showing below the G1 DNA content from flow cytometric analysis after staining of cells with propidium iodide as originally described by Crissman and Steinkamp (1993). The samples were analyzed with a Coulter EpicsTM cytometer (Beckman, Fullerton, CA). Ten thousands of events were collected for each sample. An excitation wavelength of 488 nm and a fluorescence emission of 580 nm were used. Otherwise, cells were stained with a DNA-specific fluorescent dye (H33342) then observed under a fluorescent microscope equipped with a Cool-SNAP-Pro color digital camera (Media Cybernetics, Silver Spring, MD) to examine the degree of nuclear condensation.

Determination of DNA fragmentation

Cellular DNA was extracted from whole cultured cells using genomic DNA isolation reagents (PureGene DNA isolation kit, Gentra, Minneapolis, MN) by a manufacturer's protocol. Electrophoresis was performed on a 6% polyacrylamide gel in Tris-borate-EDTA (TBE) buffer. After electrophoresis, DNA was visualized by silver staining kit (Intron, Seoul, Korea).

Statistical analysis

Statistical analysis was performed using an analysis program, StatView^R (Abacus Concepts, Berkely, CA). The Student's *t*-test was used to analyze the difference between control and experimental groups. $P < 0.05$ was considered to be significant.

Results

Induction of apoptosis by As_2O_3

HepG2 cells were treated with varying concentration of As_2O_3 up to 20 μM and cellular viability by MTT assay and apoptosis by the degrees of internucleosomal DNA fragmentation and nuclear chromatin condensation were carried out. When confluent cells were incubated for 24 h in normal culture medium containing As_2O_3 (1-20 μM), MTT activity was linearly declined in a dose-dependent manner (Figure 1A). However, at low concentrations of As_2O_3 less than 2 μM, MTT activity was still comparable to that of control group. The degree of DNA fragmentation or nuclear condensation was not affected by treatments with low concentrations of As_2O_3 (Figure 1B and C). However, higher concentrations of As_2O_3 greater than 10 μM sharply decreased MTT activity and induced DNA fragmentation and nuclear condensation. Interestingly, the number of cells with chromosomes at mitotic metaphase as well as cells with condensed- or blebbed nuclei was increased at 24 h after 10-20 μM As_2O_3 treatment (Figure 1C). Results from cell cycle analysis also showed an increase of the number of cells at metaphase as well as that of apoptotic

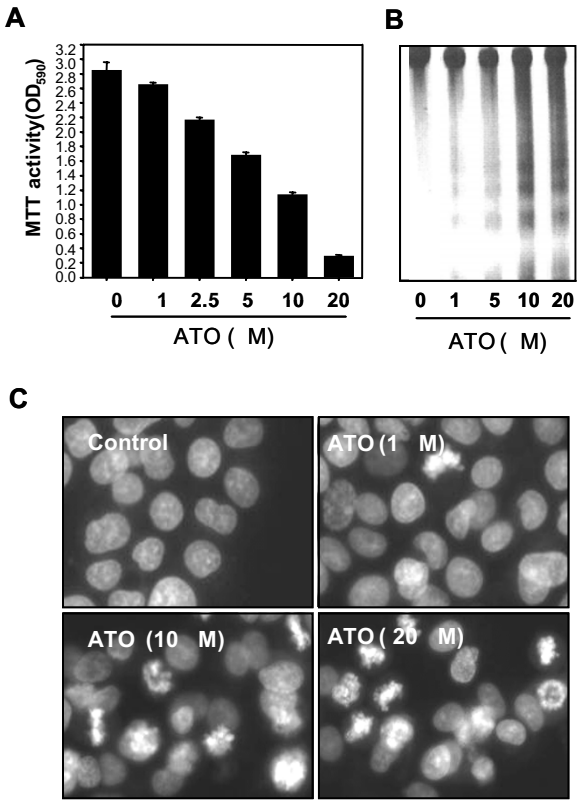

Figure 1. As_2O_3 (ATO)-induced apoptosis. HepG2 cells were plated in 35 mm culture dishes 24 h before As_2O_3 treatment. After replacing with fresh medium containing 10% FBS, cells were treated with different doses of As_2O_3 as indicated for an additional 24 h. Viability of each group was assessed by a MTT assay. Each bar represents the mean±SE of three independent experiments (n = 3) (A). A representative of internucleosomal DNA fragmentation is shown in (B). Cellular DNA was isolated, separated on a 6% polyacrylamide gel and visualized with silver staining. H33342 (10 μg/ml) was added directly into the cultured cells after As_2O_3 treatment for 24 h and maintained for 20 min. The fluorescent images were captured with the inverted fluorescent microscope at 200× magnification (C).

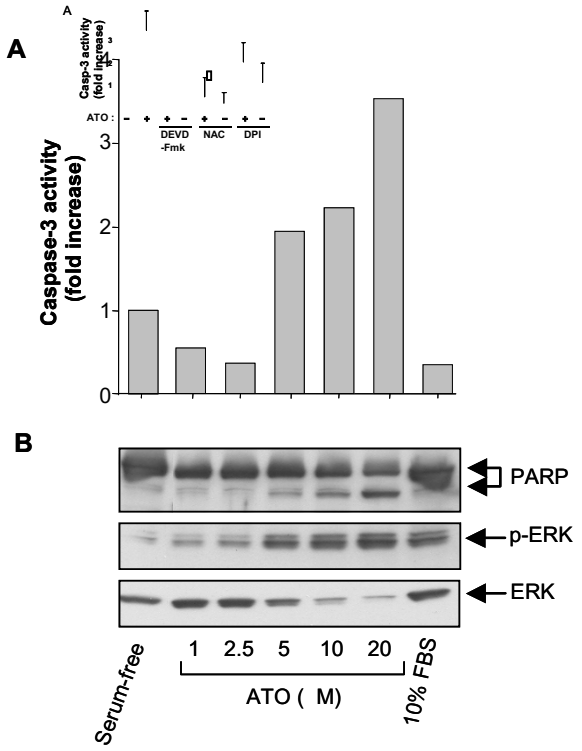

Figure 2. As_2O_3 induces caspase-3 stimulation and PARP hydrolysis. HepG2 cells in 35 mm dishes were serum-starved for 24 h before treatment and replaced with fresh serum-free medium containing different doses of As_2O_3 or normal culture medium containing 10% FBS. (A) After an additional incubation for 24 h, cells were homogenized in 200 μl lysis buffer without protease inhibitors and aliquots of supernatant were incubated with Ac-DEVD-AMC (10 μM) for 3 h at 37°C. Caspase-3 activities were presented as fold increases over control (serum-free). Each bar represents a mean of two independent experiments. (B) After treatments, cells were lysed in ice-cold lysis buffer (200 μl). Same amounts of proteins were separated by SDS-PAGE on 4-20% polyacrylamide gel and subjected to immunoblot analysis using an antibody to PARP (1:1,000) as described in Materials and Methods. Blots were repeatedly stripped and reprobed with different antibodies to phospho-ERK (P-ERK), and ERK as an internal standard. The panel is a representative of two separate experiments.

cells at 24 h after 10-20 μM As_2O_3 treatment, however, most of cells became apoptotic after 48 h incubation (data not shown).

Caspase-3 activity and the degree of hydrolysis of poly (ADP-ribose) polymerase (PARP), common indicators of the occurrence of apoptosis measurement showed a small occurrence of cell apoptosis at concentrations less than 2 μM As_2O_3 (Figure 1) whereas caspase-3 activity and the degree of PARP hydrolysis were markedly elevated at higher concentrations of As_2O_3 after 24 h treatment of cells (Figure 2). Interestingly, HepG2 cells were found to be less apoptotic in serum-free conditions for 24 h indicated by a weak caspase-3 activation or the absence of PARP hydrolysis.

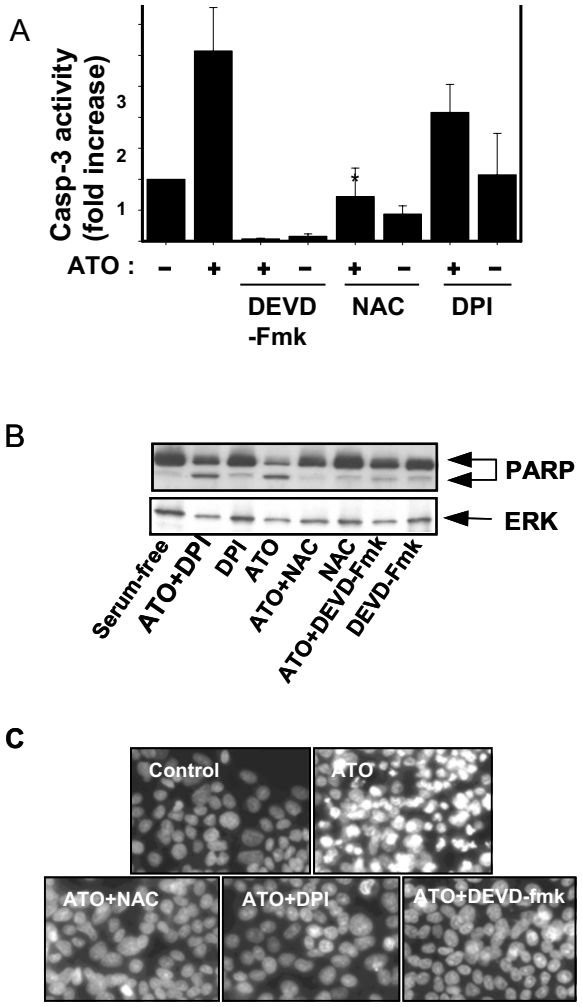

Figure 3. Antioxidants suppress caspase-3 stimulation, PARP hydrolysis and nuclear condensation. Serum-starved (24 h) cells were pretreated with 10 μM z-DEVD-fmk, 2 mM NAC or 1 μM DPI for 30 min and further incubated for 24 h in the absence or presence of 10 μM As_2O_3. (A) Caspase-3 activity was assayed as described in Figure 2. Each bar represents the mean±SE of three independent experiments ($n = 3$). *$P < 0.05$ compared to the control (As_2O_3-alone). Immunoblotting for PARP and ERK (B) and observation of nuclear condensation (C) were performed using cells treated for 24 h. Note that HepG2 cells are less apoptotic even incubated in the serum-free medium shown in (A)-(C). Each panel (B) or (C) is a representative of three independent experiments.

Effects of antioxidants on the apoptotic activity of As_2O_3

The roles of reactive oxygen species (ROS) in As_2O_3-induced apoptosis are well documented. The study was carried to examine the effects of known antioxidants, NAC, an effective scavenger molecule of intracellular ROS and DPI, a reagent widely used to inhibit iNOS on the As_2O_3-treated cells. NAC completely protected cells treated with 10 μM As_2O_3 from apoptosis, suppressed caspase-3 stimulation, and

PARP hydrolysis induced by (Figure 3), suggesting the mediation of As_2O_3-induced apoptosis by ROS and caspase cascades. Caspase-3 stimulation or PARP hydrolysis in the cells treated by As_2O_3 was not significantly suppressed by the addition of DPI whereas nuclear condensation was suppressed. DPI is known to irreversibly inhibit not only iNOS in macrophages but also endothelial NOS (eNOS) (Stuehr and Griffith, 1992). Moreover, DPI is also known to inhibit mitochondrial NADPH oxido-reductase (Majander *et al*., 1994). A specific inhibitors against iNOS was selected to examine possible dependence of iNOS stimulation in As_2O_3 induced apoptosis. GED and ETU are highly specific and potent inhibitors of iNOS over both eNOS or neuronal NOS (nNOS). Inhibition of iNOS by GED and ETU effectively suppressed PARP hydrolysis and nuclear condensation in cells treated with As_2O_3 (Figure 4). Again, serum-free condition did not affect cell's viability at least within 24 h, like as shown in Figure 2 and 3.

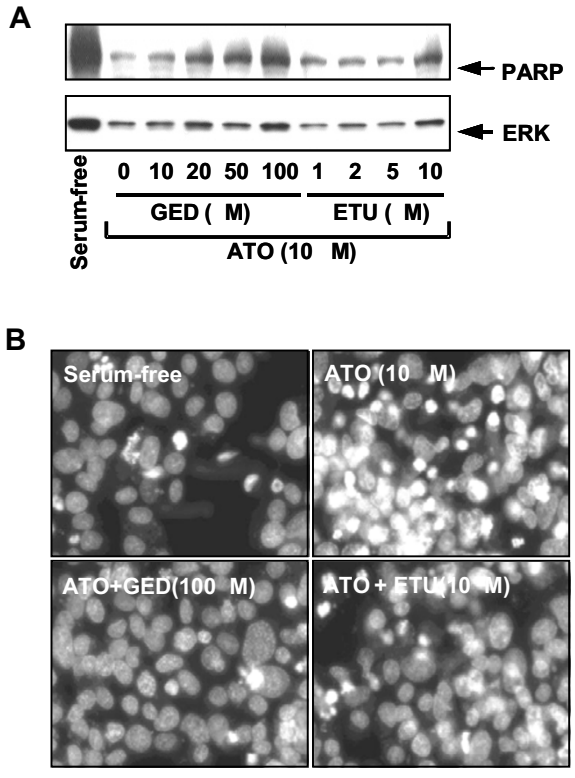

Figure 4. Inhibition of iNOS can protect HepG2 cells from apoptosis induced by As_2O_3. Serum-starved (24 h) cells were pretreated with different doses of GED (0-100 μM) or ETU (0-10 μM) for 30 min and further incubated for 24 h in the absence or presence of 10 μM As_2O_3. Immunoblotting for PARP and ERK (A) and observation of nuclear condensation (B) were performed as described in the previous experimets. Note that HepG2 cells are less apoptotic even incubated in the serum-free medium. Each panel is a representative of three independent experiments.

Roles of stress-responsive signaling proteins in As_2O_3-induced apoptosis

Members of MAPK families including ERK, JNK and p38 are activated in response to stimuli by growth factors as well as extracellular stresses. Stimulations of MAPKs are generally known to promote cell proliferation, but occasionally lead to apoptosis of severely effected cells by a number of physical and chemical stresses (Barchowsky *et al*., 1999; Bernstam and Nriagu, 2000). Here we examined whether As_2O_3 can stimulate activities of members of MAPK family. When cells were treated with 10 μM As_2O_3 for 60 min, remarkable stimulations of JNK and ERK but not p38 were observed from immunoblots using phospho-specific antibodies against signaling proteins (Figure 5A). PD98059, an inhibitor of MEK, completely blocked ERK stimulation by As_2O_3 but failed to inhibit proteolytic cleavage of procaspase-3 to be activated (Figure 5B). Protein contents of intact ERK, JNK or p38 were not changed in any groups (only ERK-panel was shown in Figure 5). Failure of PD98059 to block As_2O_3-induced apoptosis also demonstrated in separate experiments measuring intracellular DNA content by flow cytometric analysis (Figure 6A) and the degree of nuclear condensation (Figure 6B). These results suggest that As_2O_3-induced ERK activation is not crucial, at least, in the course of caspase-3-

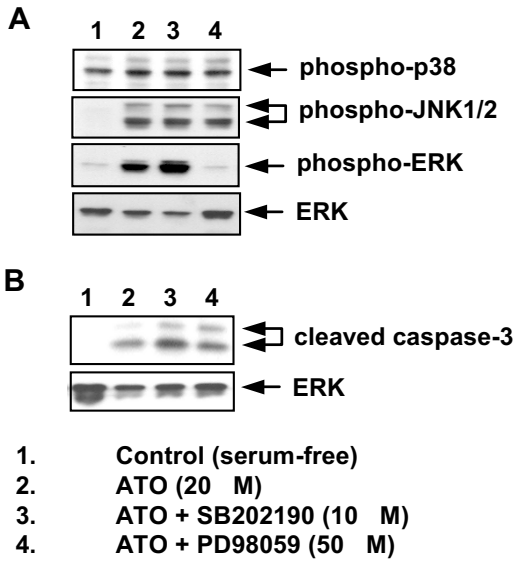

Figure 5. As_2O_3 stimulates ERK and JNK but not p38 MAPK and inhibition of ERK does not suppress PARP hydrolysis induced by As_2O_3. Serum-starved (24 h) cells were pretreated with inhibitors against ERK (PD98059, 50 μM) or p38 MAPK (SB202190, 10 μM) for 30 min and further incubated for 60 min for the detection of activities of p38 MAPK, ERK and JNK (A), or for 24 h for the detection of cleavage of inactive procaspase-3 into active caspase-3 (B) in the absence or presence of 10 μM As_2O_3. Each panel is a representative of three independent experiments.

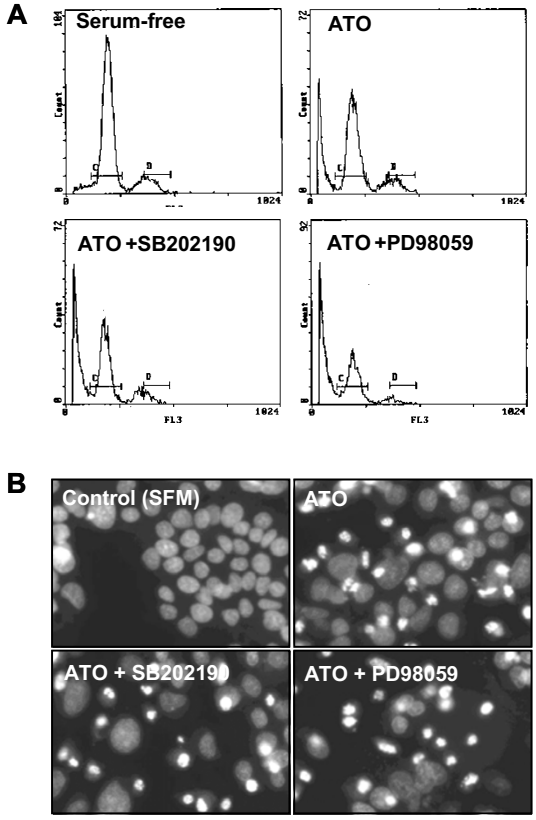

Figure 6. Inhibition of As_2O_3-induced ERK stimulation cannot protect cells from apoptosis. Serum-starved (24 h) cells were pretreated with inhibitors against ERK (PD98059, 50 μM) or p38 MAPK (SB202190, 10 μM) for 30 min and further incubated for 24 h. Cells were trypsinized, fixed and stained with propidium iodide (10 μg/ml in PBS) for the flow cytometric analysis. Population of apoptotic cells is represented as the first peak (sub G1) in each panel (A). Observation of nuclear condensation (B) was performed as described before. Note that HepG2 cells are less apoptotic even incubated in the serum-free medium. Each panel is a representative of three independent experiments.

mediated apoptotic processes by As_2O_3. The importance of JNK stimulation in As_2O_3-induced apoptosis was not clear at present due to the limited availability of inhibitors against JNK. As shown in Figure 3C, antioxidants like as NAC and DPI suppressed apoptosis in the As_2O_3 treated cells. Experiment was carried out to examine the effect on MAPK proteins in the cells treated with different antioxidants together with As_2O_3. As shown in Figure 7, treatments of cells with antioxidants; NAC, DPI or even PDTC (1-pyrrolidinecarbodithioic acid, an inhibitor of superoxide dismutase) did not suppress activities of ERK or JNK stimulated by As_2O_3. Thus, activations of JNK as well as ERK by As_2O_3 do not seem to play crucial roles to lead HepG2 cells to apoptosis.

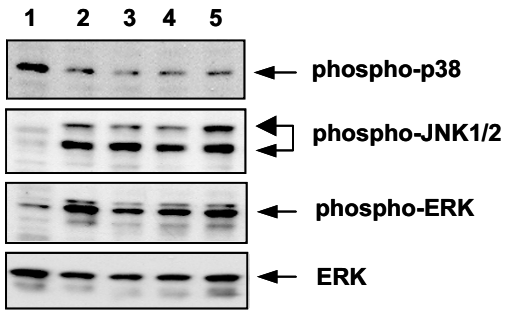

Figure 7. Antioxidants do not inhibit ERK- or JNK-stimulation by As_2O_3. Serum-starved (24 h) cells were pretreated with 2 mM NAC, 1 μM DPI or 1 mM PDTC for 30 min and further incubated for 60 min in the absence or presence of 10 μM As_2O_3. Immunoblotting for the detection of activated p38 Map kinase, ERK, JNK or ERK (internal standard) were performed as described in previous experiments. Lanes: 1, control (serum-free); 2, ATO (10 μM); 3, ATO+DPI (1 μM); 4, ATO+ NAC (1 mM); 5, ATO+PDTC (1 mM). The panel is a representative of three independent experiments.

Discussion

Although As_2O_3 has been successful in inducing apoptosis in APL and other leukemic cancer cells, there is little evidence against solid tumor cells. The present study showed an effectiveness of As_2O_3 to lead HepG2 cells into apoptosis at concentrations higher than 10 μM. These results imply that HepG2 cells are more resistant to As_2O_3 in comparison with APL cells and other leukemic cancer cells sensitive to lower concentrations (<5 μM) (Akao *et al.*, 1999; Gurr *et al.*, 1999). Interestingly, HepG2 cells incubated in serum-free medium for 24 h were not respondent to As_2O_3. These results show a remarkable survival potential of HepG2 cells even in nutrient-deficient conditions because most of immortalized cell lines are apoptotic in serum-free conditions at least within 24 h. In addition, a delayed onset of apoptosis after accumulations of cells at mitotic metaphase was observed for 48 h treatment with As_2O_3. Recently, As_2O_3 (0.5-6 μM) was shown to induce G2/M growth arrest which was associated with changes in the amount of cyclin B and cyclin B-dependent kinase and apoptosis in promonocytic U937 cells after 24 h treatment (Park *et al.*, 2001), suggesting As_2O_3's growth-inhibitory effects by modulating expressions and/or activities of several key G2/M regulatory proteins. However, in MC/CAR myeloma cells, As_2O_3 induced G1 arrest as well as G2/M arrest, binding Cip/p21 to cyclin E and cyclin A, but did not change the steady-state levels of cyclin B1, cyclin E and cyclin D1 (Park *et al.*, 2000). In head and neck cancer cell line PCI-1, As_2O_3 induced G2/M arrest whereas it did not change the steady-state levels of

cyclin D1, cyclin E and cyclin A but decreased protein levels of cyclin B1 (Seol *et al*., 1999). Therefore, As_2O_3-induced cell cycle arrest might be associated with different changes in the amount and activity of even a same cell cycle-modulating protein in different cell models. The present study did not measure expression levels or activities of G2/M regulatory proteins after As_2O_3 treatment in HepG2 cells. However, our results provide an evidence to support a hypothesis that As_2O_3 leads to cell cycle arrest at G2/M phase and subsequent onset of apoptosis. Stimuations of caspase-3 and hydrolysis of poly (ADP-ribose) polymerase (PARP) by As_2O_3 (10-20 μM) support the notion that As_2O_3 leads HepG2 cell to apoptosis by inducing typical, stepwise stimulations of apoptotic biochemical changes. These results are paralleled with other studies showing the classic morphologic and biochemical changes of apoptosis induced by As_2O_3.

Recently, the role of reactive oxygen species (ROS) in As_2O_3-induced apoptosis was extensively studied. As_2O_3 inhibits glutathione peroxidase (GPx) activity and increases cellular hydrogen peroxide (H_2O_2) content in NB4 but not U937 cells, thereby induces cytochromc c release, caspase-3 activation and DNA fragmentation (Jing *et al*., 1999). Our data suggests the mediating role of ROS in the course of As_2O_3-induced apoptosis. Other studies also have reported similar results regarding the involvement of ROS in As_2O_3-induced apoptosis in different cellular models (Barchowsky *et al*., 1999; Akao *et al*., 2000). From these results, the mediating role of ROS in inducing apoptosis by As_2O_3 in HepG2 cells should also be considered.

The involvement of members of MAPK families in As_2O_3-induced apoptosis is still controversial. Barchowsky *et al*. (1999) observed activations of ERK and p38 in arsenite-induced apoptosis in primary vascular cells. Oxidative stress by arsenic compounds induced stimulations of ERK, JNK and p38 MAPK activities in different cell models (Bernstam and Nriagu, 2000). In the meanwhile, Maeda *et al*. (2001) showed activations of JNK and p38 after As_2O_3 treatment in prostate cancer cells, however, pharmacological inhibition of p38 and over-expression of dominant-negative JNK failed to protect cells from apoptosis by As_2O_3. In the meanwhile, As_2O_3 leads to inactivation of ERK, and activation of p38 that plays a role in inducing apoptosis by As_2O_3 in human leukemia U937 cells (Iwama *et al*., 2001). These diverse observations about signaling nature in As_2O_3-induced apoptosis represent the unusual characteristics of the mechanism of apoptosis by As_2O_3 in different cellular systems.

In summary, moderately higher concentrations of As_2O_3 (not less than 2 μM) induce apoptosis and antioxidants can protect cells from apoptosis by As_2O_3 in HepG2 cells. Although ERK and JNK, not p38 are stimulated in response to As_2O_3, they might not play important roles in executing proapoptotic activity of As_2O_3. In addition, inhibition of iNOS can protect HepG2 cells from apoptosis induced by As_2O_3, suggesting the stimulation of ROS production by As_2O_3 as one of its apoptotic processes.

Acknowledgement

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