

# Arsenic Trioxide Induces Apoptosis in Human Colorectal Adenocarcinoma HT-29 Cells Through ROS

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**Purpose:** Treatment with arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) results in a wide range of cellular effects that includes induction of apoptosis, inhibition of cell growth, promotion or inhibition of cellular differentiation, and inhibition of angiogenesis through a variety of mechanisms. The mechanisms of As<sub>2</sub>O<sub>3</sub>-induced cell death have been mainly studied in hematological cancers, and those mechanisms in solid cancers have yet to be clearly defined. In this study, the mechanisms by which As<sub>2</sub>O<sub>3</sub> induces apoptosis in human colorectal adenocarcinoma HT-29 cells were investigated.

**Materials and Methods:** To examine the levels of apoptosis, HT-29 cells were treated with As<sub>2</sub>O<sub>3</sub> and then we measured the percentage of Annexin V binding cells, the amount of ROS production and the mitochondrial membrane potential. Western blot analysis was performed to identify the activated caspases after As<sub>2</sub>O<sub>3</sub> exposure, and we compared the possible target molecules of apoptosis. As<sub>2</sub>O<sub>3</sub> treatment induced the loss of the mitochondrial membrane potential and an increase

of ROS, as well as activation of caspase-3, -7, -9 and -10.

**Results:** As<sub>2</sub>O<sub>3</sub> induced apoptosis via the production of reactive oxygen species and the loss of the mitochondrial membrane potential. As<sub>2</sub>O<sub>3</sub> induced the activation of caspase-3, -7, -9 and -10. Furthermore, As<sub>2</sub>O<sub>3</sub> treatment downregulates the Mcl-1 and Bcl-2 expressions, and the release of cytochrome c and an apoptosis-inducing factor (AIF). Pretreating the HT-29 cells with *N*-acetyl-L-cysteine, which is a thiol-containing antioxidant, inhibited the As<sub>2</sub>O<sub>3</sub>-induced apoptosis and caspase activation.

**Conclusion:** Taken together, these results suggest that the generation of reactive oxygen species (ROS) by As<sub>2</sub>O<sub>3</sub> might play an important role in the regulation of As<sub>2</sub>O<sub>3</sub>-induced apoptosis. This cytotoxicity is mediated through a mitochondria-dependent apoptotic signal pathway in HT-29 cells. (*Cancer Res Treat.* 2006;38:54-60)

**Key Words:** Apoptosis, Arsenic trioxide, Mitochondria, Reactive oxygen species

## INTRODUCTION

Arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) has been used as a therapeutic agent for treating hypertension, bleeding gastric ulcers, heartburn, chronic rheumatism and cancer in the Orient countries (1). As<sub>2</sub>O<sub>3</sub> has a long history of use for the treatment of leukemia, and it was recently approved for the treatment of relapsed or refractory acute promyelocytic leukemia (APL) by the US Food

and Drug Administration (2). Treating APL patients with As<sub>2</sub>O<sub>3</sub> is associated with the disappearance of the promyelocytic leukemia-retinoic acid receptor  $\alpha$  (PML-RAR $\alpha$ ) fusion transcript; this is the gene product of the chromosomal translocation t (15;17), which is characteristic of APL (3). In addition, several in vitro studies revealed that As<sub>2</sub>O<sub>3</sub> induces apoptosis in APL cells, multiple myeloma cells and some solid tumor cells such as prostate, ovarian carcinoma and neuroblastoma cells (4). Despite of the extensive investigations that have been conducted, the molecular mechanism of As<sub>2</sub>O<sub>3</sub>-induced apoptosis in solid tumors is not yet fully understood.

Programmed cell death or apoptosis is a physiological process that plays an important role in the maintenance of cellular homeostasis via the selective elimination of excessive cells (5). Apoptotic cells show the characteristic morphological changes that include cell shrinkage, chromatin condensation, nuclear fragmentation, membrane blebbing, caspase activation and the formation of membrane bound vesicles that are called apoptotic bodies (6). Aberrant regulation of apoptosis has been observed in many forms of human disease including cancers, AIDS and many neurodegenerative diseases (7). Therefore, understanding

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the mechanism of apoptosis has important implications for the prevention and treatment of many diseases.

Reactive oxygen species (ROS) play important roles in a variety of normal cellular biochemical functions and abnormal pathological processes. The biological functions of ROS and their potential roles in the development of cancers and disease progression have been investigated for decades (8~9). Evidence from recent studies suggests that cancer cells, as compared to normal cells, are under increased oxidative stress that is associated with the increased generation of ROS (10). Because ROS are chemically active and they can inflict severe cellular damage, the possibility that cancer cells are under increased intrinsic ROS stress may provide a unique opportunity to destroy malignant cells based on their vulnerability to further ROS insults (11).

Although the mechanisms of As<sub>2</sub>O<sub>3</sub>-induced cell death have mainly been studied in hematologic cancers, those studies have not clearly defined a role for As<sub>2</sub>O<sub>3</sub> in solid tumors. In the present study, we have investigated As<sub>2</sub>O<sub>3</sub>'s induction of apoptosis in human colorectal adenocarcinoma cells, and we have elucidated the regulatory mechanism of apoptosis via As<sub>2</sub>O<sub>3</sub>, by focusing on the generation of ROS.

## MATERIALS AND METHODS

### 1) Materials

As<sub>2</sub>O<sub>3</sub>, MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazoliumbromide) and *N*-acetyl-*L*-cysteine (NAC) were obtained from Sigma (St. Louis, MO). Annexin V-FITC was purchased from Becton Dickson (San Diego, CA). The enhanced chemiluminescence (ECL) detection system was purchased from Amersham Biosciences (Buckinghamshire, UK). RPMI 1640 was purchased from Life Technologies, Inc. (Gaithersburg, MD), and fetal bovine serum was purchased from Hyclone (Logan, Utah). Antibodies against caspase-3, -7, -9 and -10 were obtained from Cell Signaling Technology (Beverly, MA) and the antibodies against Mcl-1, Bcl-2 and apoptosis-inducing factor (AIF) were from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody against cytochrome *c* was purchased from Pharmingen (San Diego, CA), and antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was purchased from Chemicon (Temecula, CA).

### 2) Cell culture

HT-29 human colorectal adenocarcinoma cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were maintained in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat inactivated fetal bovine serum, and they were grown at 37°C in 5% CO<sub>2</sub> fully humidified air; they were subcultured twice weekly.

### 3) Cell viability assay

Cells (5×10<sup>3</sup> cells/well) were cultured in 96-well microplates and treated with As<sub>2</sub>O<sub>3</sub> in the presence or absence of various concentrations of inhibitors. After incubating the plates for 48 h, 50 µl of MTT (2 mg/ml) was added and the plates were incubated for an additional 4 h under the same conditions.

During the final 4 h of incubation, the yellow MTT tetrazolium salt is reduced to purple-blue formazan crystals in only the viable cells. Formazan crystals were dissolved by adding 250 µl DMSO and the absorbance, which is linearly related to the number of viable cells, was measured at 570 nm on a BioRad M450 microplate reader. The cell death rate was expressed as a percentage of untreated control cells.

### 4) Annexin V-FITC staining

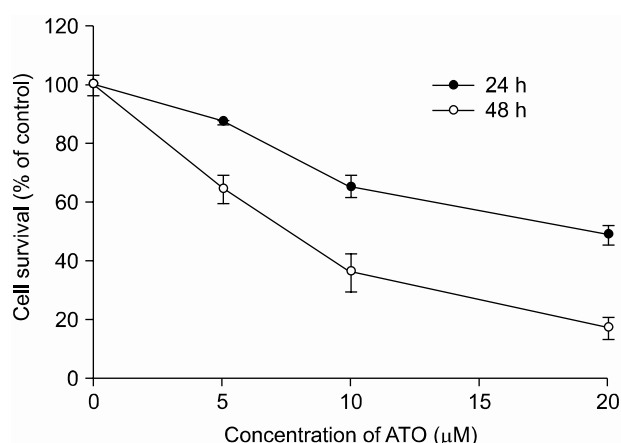
To detect early apoptosis, the cells were stained with FITC-conjugated Annexin V. After treatment with or without As<sub>2</sub>O<sub>3</sub>, the cells were washed with cold PBS before being resuspended in a 100 µl cold binding buffer. Five microliters of Annexin V-FITC were added and the incubation was continued for 15 min at room temperature and in the dark. A further 400 µl of binding buffer were added to terminate the reaction; flow cytometry analysis was then performed immediately for at least 10,000 cells by using a fluorescence-activated cell-sorting (FACS) analysis system (Becton Dickinson, San Jose, CA).

### 5) Measurement of ROS production

Cells were treated with the indicated concentrations of As<sub>2</sub>O<sub>3</sub> for 48 h. Cells were incubated in 10 µM 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCF-DA) (Molecular Probes, Eugene, OR) at 37°C for 20 min. The cells were harvested by trypsinization and washed twice with cold PBS. The ROS were determined by FACS analysis.

### 6) Determination of mitochondrial membrane potentials

The mitochondrial membrane potentials were measured using JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; Molecular Probes, Eugene, OR), which is based on staining live cells in a membrane potential-dependent fashion: green fluorescence (λ<sub>em</sub>=527 nm) was the results of the JC-1 monomer and red fluorescence (λ<sub>em</sub>=590 nm) was the



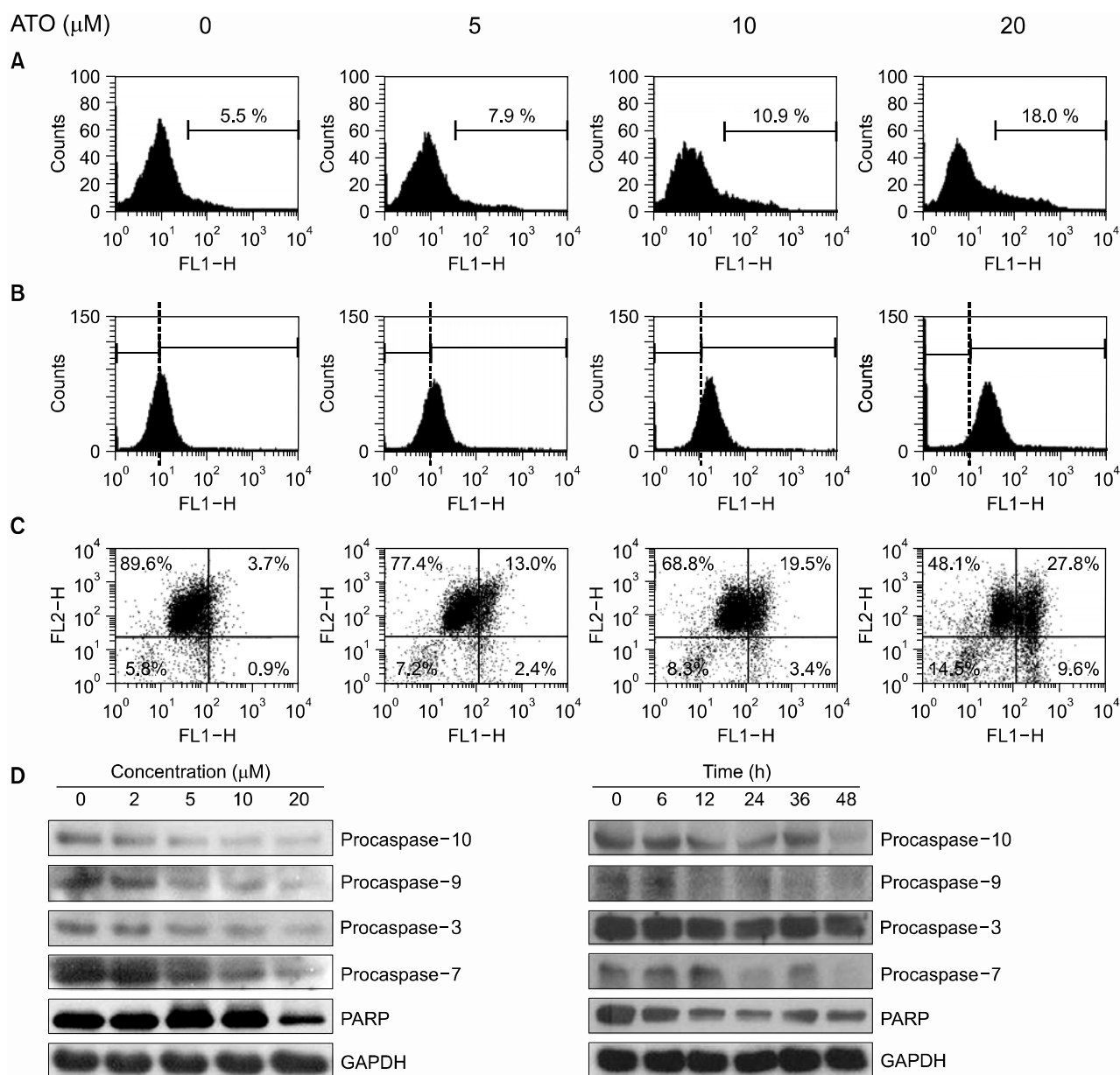
**Fig. 1.** Effects of As<sub>2</sub>O<sub>3</sub> on cell survival in human colorectal adenocarcinoma HT-29 cells. The cells were treated with increasing concentrations of As<sub>2</sub>O<sub>3</sub>. Cell survival was measured by the MTT assay. The values are the mean±SD of three independent experiments.

result of the JC-1 aggregates (12). Briefly, the cells ( $3 \times 10^5$  cells/well) were treated with  $\text{As}_2\text{O}_3$  for 48 h and then washed twice with PBS. The cell suspension was incubated with JC-1 (10  $\mu\text{g}/\text{ml}$ ) dye for 30 min at  $37^\circ\text{C}$ ; the green (FL-1) and red (FL-2) fluorescence was then measured by FACS analysis.

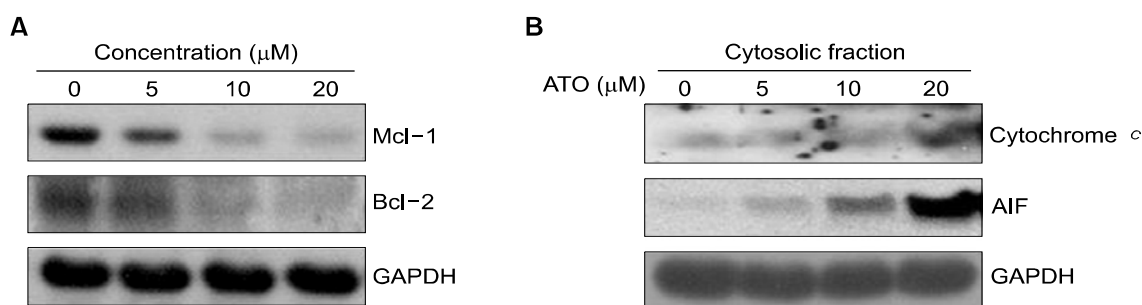
### 7) Isolation of the cytosolic and mitochondrial fractions

The cells were washed once with cold PBS and then they were resuspended with 5 volumes of an isolation buffer (250 mM sucrose containing 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM  $\text{MgCl}_2$ , 1 mM EDTA, 1 mM EGTA, 1 mM

dithiothreitol and 0.1 mM PMSF). After chilling on ice for at least 20 min, the cells were homogenized with 30~40 strokes of a Dounce homogenizer (70~80% cell breakage), and the homogenates were centrifuged at 750 g for 10 min at  $4^\circ\text{C}$  to remove the unbroken cells and nuclei. The supernatants were harvested and centrifuged at 12,000 g for 30 min at  $4^\circ\text{C}$  (the cytosolic fraction). The mitochondria-enriched pellets were resuspended in isolation buffer. The cytosolic and mitochondrial fractions were then subjected to electrophoresis and Western blot analysis.



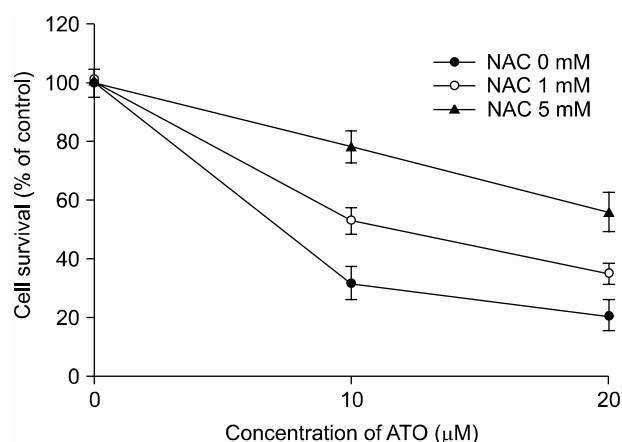
**Fig. 2.** Induction of apoptosis by  $\text{As}_2\text{O}_3$ -mediated cytotoxicity. The cells were treated with increasing concentrations of  $\text{As}_2\text{O}_3$  for 48 h. The cells were incubated with Annexin V (A),  $\text{H}_2\text{DCF-DA}$  (B), or JC-1 (C), and then they were analyzed by flow cytometry. The cell lysates were further analyzed by Western blotting with using anti-caspase and anti-PARP antibodies (D). The GAPDH level was used to demonstrate equal loading of proteins on each lane.



**Fig. 3.** Effects of As<sub>2</sub>O<sub>3</sub> on the levels of Mcl-1, Bcl-2, cytochrome *c* and AIF. The cells were treated with increasing concentrations of As<sub>2</sub>O<sub>3</sub> for 48 h. The cell lysates were analyzed by Western blotting using anti-Mcl-1, anti-Bcl-2, anti-cytochrome *c* and anti-AIF antibodies. The GAPDH level was used to demonstrate equal loading of proteins on each lane.

### 8) SDS-PAGE and Western blot analysis

The cells were washed twice in cold PBS and lysed on ice with lysis solution (1% Triton X-100, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM PMSF and a protease inhibitor cocktail). The protein concentrations were determined using the BioRad protein assay according to the manufacturer's recommendations. The protein samples (30 μg of each) were separated on 10% SDS-polyacrylamide gel and electrotransferred to nitrocellulose membranes. The membranes were blocked for at least 30 minutes at room temperature in Tris-buffered saline plus 0.05% Tween-20 (TTBS) that contained 5% nonfat dry milk, and then the membranes were incubated with TTBS that contained a primary antibody. For immunoblotting, incubation was performed for 4 h at room temperature. After five washes within a 30 minute period, the membranes were incubated with a peroxidase-conjugated secondary antibody for 1 h. After five washes within a 30 minute period in TTBS, the membranes were developed using an ECL detection system and then they were exposed to X-ray films.



**Fig. 4.** The effects of NAC on the As<sub>2</sub>O<sub>3</sub>-mediated cytotoxicity. The cells were treated with As<sub>2</sub>O<sub>3</sub> in the presence or absence of NAC. After 48 h, cell survival was determined by the MTT assay. The values are the mean ± SD of three independent experiments.

## RESULTS

### 1) Effects of As<sub>2</sub>O<sub>3</sub> on the apoptotic cell death of HT-29 cells

We examined the cytotoxic effect of As<sub>2</sub>O<sub>3</sub> on human colorectal adenocarcinoma HT-29 cells. Treatment with As<sub>2</sub>O<sub>3</sub> caused decreases in cell survival in a dose- and time-dependent manner (Fig. 1). To investigate whether the As<sub>2</sub>O<sub>3</sub>-induced cytotoxicity is mediated by apoptosis, we measured the Annexin V staining, ROS production, the mitochondrial membrane potential ( $\Delta\psi_m$ ) changes and the caspase cleavages in the cells that were treated with As<sub>2</sub>O<sub>3</sub>. Annexin V binds to the membrane phospholipid phosphatidylserine that is located within the plasma membrane of apoptotic cells. The percentage of cells stained with Annexin V was increased with the increasing concentrations of As<sub>2</sub>O<sub>3</sub> (Fig. 2A). Since oxidative damage plays an important role in the anticancer effects of arsenic compounds (13), the intracellular generation of ROS in the HT-29 cells treated with As<sub>2</sub>O<sub>3</sub> was investigated using an oxidation-sensitive fluorescent probe (H<sub>2</sub>DCF-DA), which is

oxidized to 2',7'-dichlorofluorescein (DCF) in the presence of ROS. Treatment with As<sub>2</sub>O<sub>3</sub> led to an increase of the DCF fluorescence in a dose-dependent manner (Fig. 2B). Since the production of ROS is usually preceded or accompanied with the loss of  $\Delta\psi_m$ , we also examined whether As<sub>2</sub>O<sub>3</sub> treatment induces the loss of  $\Delta\psi_m$  in the HT-29 cells that had been treated with As<sub>2</sub>O<sub>3</sub> by performing staining with JC-1. As expected, the As<sub>2</sub>O<sub>3</sub> treatment induced the loss of the  $\Delta\psi_m$  in a dose-dependent manner (Fig. 2C). The likely involvement of caspases in the As<sub>2</sub>O<sub>3</sub>-induced apoptosis of HT-29 cells was analyzed by performing Western blot analysis. The treatment of cells with As<sub>2</sub>O<sub>3</sub> caused the activation of caspase-3, -7, -9 and -10 in a dose- and time-dependent manner, as indicated by a reduction in the intensity of the proenzymes (Fig. 2D). In addition, degradation of PARP, which a well-known substrate of caspase-3, increased in the cells treated with As<sub>2</sub>O<sub>3</sub>. However, the expression level of caspase-8 between the As<sub>2</sub>O<sub>3</sub>-treated and As<sub>2</sub>O<sub>3</sub>-untreated cells was not altered (data not shown). These results suggest that As<sub>2</sub>O<sub>3</sub> might induce apoptotic cell death in HT-29 cells.

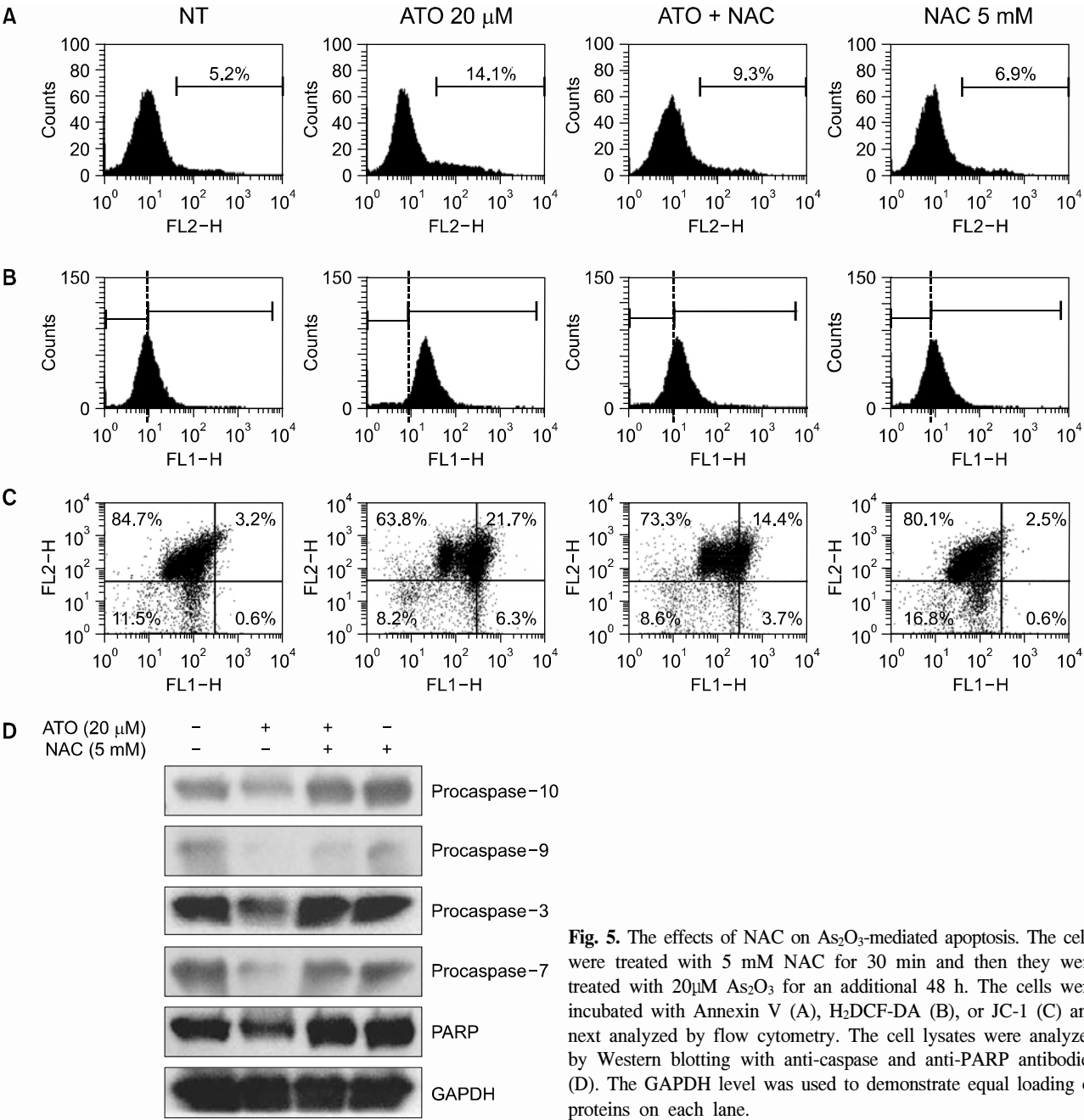
2) Analysis of mitochondrial participation in As<sub>2</sub>O<sub>3</sub>-induced apoptosis

A key event in the apoptotic process is the opening of the mitochondrial permeability transition pores, and this is an event that's known to be regulated by the Bcl-2 family of proteins. The outcome of this opening is the release of several proteins such as procaspase-2, -3 and -9, cytochrome *c* and AIF from the mitochondrial intermembrane space (14). The expression level of Mcl-1 protein was significantly decreased in the As<sub>2</sub>O<sub>3</sub>-treated cells and the Bcl-2 protein level was slightly decreased (Fig. 3A). The release of cytochrome *c* from the

mitochondria was also increased in the As<sub>2</sub>O<sub>3</sub>-treated cells compared to the untreated cells. The cells treated with As<sub>2</sub>O<sub>3</sub> showed an increased amount of released AIF from the mitochondria into the cytosol (Fig. 3B). These results suggest that As<sub>2</sub>O<sub>3</sub> might induce cellular apoptosis through a mitochondria-dependent apoptotic pathway.

3) Effect of antioxidant on the apoptotic activity of As<sub>2</sub>O<sub>3</sub>

To determine a link between the elevation of the intracellular ROS level and the apoptotic cell death in the As<sub>2</sub>O<sub>3</sub> treated cells, HT-29 cells were incubated with NAC prior to their



**Fig. 5.** The effects of NAC on As<sub>2</sub>O<sub>3</sub>-mediated apoptosis. The cells were treated with 5 mM NAC for 30 min and then they were treated with 20 $\mu$ M As<sub>2</sub>O<sub>3</sub> for an additional 48 h. The cells were incubated with Annexin V (A), H<sub>2</sub>DCF-DA (B), or JC-1 (C) and next analyzed by flow cytometry. The cell lysates were analyzed by Western blotting with anti-caspase and anti-PARP antibodies (D). The GAPDH level was used to demonstrate equal loading of proteins on each lane.

treatment with As<sub>2</sub>O<sub>3</sub>. NAC pretreatment resulted in protection against the As<sub>2</sub>O<sub>3</sub>-induced cytotoxicity (Fig. 4). The As<sub>2</sub>O<sub>3</sub>-induced increases of Annexin V staining, the intracellular ROS levels and the  $\Delta\Psi_m$  were reduced by the NAC pretreatment (Fig. 5A and 5B). Furthermore, NAC inhibited the As<sub>2</sub>O<sub>3</sub>-induced caspase activation and PARP degradation (Fig. 5C). Yet the addition of NAC did not affect the As<sub>2</sub>O<sub>3</sub>-induced down-regulation of Mcl-1 and Bcl-2 (Fig. 5D). These results suggest that an increase in the intracellular ROS level by As<sub>2</sub>O<sub>3</sub> treatment is required for the As<sub>2</sub>O<sub>3</sub>-induced apoptosis that is accompanied by the loss of  $\Delta\Psi_m$ , caspase activation and PARP degradation.

## DISCUSSION

The aim of the present study was to elucidate the molecular mechanisms of cellular death that are triggered by As<sub>2</sub>O<sub>3</sub> treatment with using human colorectal adenocarcinoma HT-29 cells. Our experiments indicated that ROS have an important role in the As<sub>2</sub>O<sub>3</sub>-induced caspase-dependent cell death of the HT-29 cells.

The caspase cascade is the most-studied and best-understood signaling pathway in apoptosis. These proteases are produced as forms of pro-enzymes, and they can be activated by proteolysis in response to a diverse set of apoptotic stimuli (15). One means of activating the caspase cascade is termed the "death-receptor pathway" and this is initiated at the cell surface by the binding of apoptotic molecules to their receptors (16). Caspase-8 and caspase-10 are the key initiator caspases in the "death-receptor pathway". The mitochondria are another site for apoptotic stimuli to initiate an intracellular signaling process that mediates caspase activation. In this case, caspase-9 functions as an initiator caspase that activates downstream effector caspases such as caspase-3 (17). We found that cells treated with As<sub>2</sub>O<sub>3</sub> show activation of caspase-3, -7, -9 and -10. Degradation of PARP was also observed in the As<sub>2</sub>O<sub>3</sub>-treated cells. Thus, these data suggest that the As<sub>2</sub>O<sub>3</sub>-induced apoptosis of HT-29 cells might be mediated through a mitochondria-dependent pathway.

The mitochondria have been shown to play a pivotal role in the apoptotic process since both the intrinsic and the extrinsic pathways can converge at the mitochondrial level and so trigger changes in the mitochondrial membrane permeability (18,19). Mutations in such apoptosis-regulatory genes as p53, PTEN and Bcl-2 and its homologues are involved in the pathogenesis of most human cancers (20). Similar mutations may also be involved in the development of chemoresistance. Bcl-2 has been shown to be protective against diverse cytotoxic insults, i.e., UV-irradiation and cytokine drugs, while the pro-apoptotic family members may also act as tumor suppressors (21). Mcl-1 also has sequence homology to Bcl-2 and it further resembles Bcl-2 since its expression promotes cell viability (22). It has been reported that AIF mediates mitochondria-related cell death through a caspase-independent pathway wherein the mitochondrial AIF translocates to the cell nucleus in response to death stimuli, and there it initiates nuclear condensation. Once the nucleus condenses, large-scale chromatin fragmentation ensues followed by death of the cell (4). In this study, As<sub>2</sub>O<sub>3</sub> induced

the release of cytochrome *c* and AIF, so it's possible that As<sub>2</sub>O<sub>3</sub> induces mitochondria-mediated apoptosis by both the caspase-dependent and caspase-independent pathways.

Accumulation of intracellular ROS leads to the disruption of the  $\Delta\Psi_m$ , the release of cytochrome *c* with the subsequent activation of the caspase cascade, and ultimately to programmed cell death via apoptosis (23,24). Arsenic exposure leads to increases in the ROS levels by a variety of redox enzymes such as NADPH oxidase (25). In the present study, we showed that increased levels of ROS are essential for the loss of the  $\Delta\Psi_m$  and the caspase activation that's triggered by As<sub>2</sub>O<sub>3</sub>. The significant inhibition of As<sub>2</sub>O<sub>3</sub>-induced caspase activation and cell death after NAC treatment suggests that increased ROS levels are critical for As<sub>2</sub>O<sub>3</sub>-mediated apoptosis in HT-29 cells.

## CONCLUSIONS

The data presented in this study suggest that the generation of ROS might be a critical factor for As<sub>2</sub>O<sub>3</sub>-induced apoptosis in human colorectal adenocarcinoma HT-29 cells, and that the As<sub>2</sub>O<sub>3</sub>-induced apoptosis could be induced by a mitochondria-mediated apoptotic pathway.

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