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Inhibition of Cell Proliferation and the Action Mechanisms of Arsenic Trioxide (As_2O_3) on Human Breast Cancer Cells

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Abstract Arsenic trioxide (As_2O_3) is one of the arsenic compounds found in nature. As_2O_3 has recently been used to treat patients suffering from retinoic acid receptor (AML). It is of clinical interest to investigate whether As_2O_3 is also effective in treating solid tumors. Here, we report that As_2O_3 exhibited inhibitory effects on the proliferation of human breast cancer MCF-7 cells in a dose- and time-dependent manner. The 50% inhibitory concentration (IC_{50}) of As_2O_3 in inhibiting proliferation of MCF-7 cells were 8, 1.8, and 1.2 μM upon 1-, 2-, and 3-day treatment, respectively. In elucidating the underlying action mechanisms, the results of experiments concerning DNA fragmentation and externalization indicated that As_2O_3 exerted its action on MCF-7 cells via apoptosis, whereas the result of flow cytometry also indicated that As_2O_3 could induce mitochondrial mediated cell-cycle arrest at G_1 phase. Further studies by Western blot analysis indicated that As_2O_3 regulated apoptosis and the expression of cell-cycle-related proteins as it upregulated p53 protein level and downregulated bcl-2 protein level. Results in present study indicated that As_2O_3 might also be a good candidate for treating breast cancer. J. Cell. Biochem. 93: 173–187, 2004.

Key words: arsenic trioxide; MCF-7; apoptosis

Arsenic trioxide (As_2O_3) has been used as therapeutic agent since 15th century in the Western world. It was not until 1970 that As_2O_3 was found to be effective in the treatment of a type of leukemia called acute promyelocytic leukemia (APL). Since then, As_2O_3 has been used in clinical trial of APL patients worldwide and its mechanisms were examined in both in vitro and in vivo studies. APL accounts for 10–15% of all acute myeloid leukemia (AML) in adults [Soignet et al., 1998]. The molecular pathologies of APL are associated with the reciprocal translocation of retinoic acid receptor α gene (RARα) on chromosome 17 to promyelocytic leukemia gene (PML) on chromosome 15 resulting in the fusion of PML and RARα proteins [Mu et al., 1994]. PML mediates apoptosis and suppress growth [Le Goff et al., 1994] while RARα is responsible for the differentiation signal transduction [Labrecque et al., 1998]. The resulting PML/RARα fusion protein interferes myeloid differentiation and antagonizes growth suppression by PML, leading to deregulated cell growth [Warrell et al., 1993].

In vitro studies of APL cell line, NB4, and in vivo studies have shown that all-trans-retinoic-acid (ATRA) induces re-localization of the PML and restores the normal structure of PML oncogenic domains (POD) early in the differentiation [Weis et al., 1994]. Nevertheless,

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adverse effects are also observed. Secondary ATRA resistance occurs in all patients upon ATRA treatment. APL patients under ATRA treatment usually relapse within 6 months and become refractory to the treatment for 6–12 months [Degos and Wang, 2001]. The side effects of ATRA treatment prompt researchers to modify the treatment. The first clinical trial on APL by As_2O_3 was conducted in 1971. In general, As_2O_3 achieved complete remission rate (CR) of 57–98% in both de novo and relapsed APL patients.

Besides APL patients, As_2O_3 was also administrated intravenously in patients with different cancers. Therapeutic effects were observed in chronic myeloid leukemia (CML), lymphoma, esophageal cancer, and APL. The effective treatment of As_2O_3 on APL patients encouraged the elucidation of its action mechanisms. Over the past 10 years, numerous studies have been performed to investigate the action mechanisms of As_2O_3 on APL. These in vitro studies were done by using NB4 cells, an APL cell line with chromosome translocation t(15;17) from a relapsed APL patient [Lanotte et al., 1991]. In NB4 cells, As_2O_3 was shown to exert dose-dependent dual effects. At low concentration (0.1–0.5 μM), As_2O_3 induces differentiation while at high concentration (0.5–2 μM), it induces apoptosis [Chen et al., 1997]. The therapeutic efficacy of As_2O_3 in APL patients and the studies on APL cell line, NB4, have prompted the investigation of As_2O_3 in other cell lines and further elucidation of the mechanisms in mediating these effects.

In the present study, the anti-tumor effects of As_2O_3 on human breast cancer cells were investigated. Breast cancer is the most common cancer in Hong Kong in 2000. The early phase of breast cancer involved the expression of estrogen receptor (ER). Human breast adenocarcinoma cell line MCF-7, an ER positive breast cancer cell line, was examined as cellular model for As_2O_3 treatment to mimic the early phase of breast cancer. Meanwhile, the effect of As_2O_3 on the proliferation of a normal cell line, Hs68, at the effective dosage was also examined to study the non-specific toxicity. In addition, the action mechanisms mediating the anti-tumor effects were elucidated. Several action mechanisms that were found commonly involved in the chemotherapy of solid tumors or cancer cell lines were studied. Here, the study mainly focused on cell death apoptotic pathway. Other mechanisms, such as cell-cycle regulation, free radicals induction, were also studied.

MATERIALS AND METHODS

Cell Culture

MCF-7 cell line and Hs68 cell line were purchased from American Type Culture Collection. MCF-7 is human breast tumor cell line. The cells are subcultured in phenol red free Roswell Park Memorial Institute tissue culture medium 1640 (RPMI 1640) supplemented with 10% dextran-coated charcoal stripped fetal calf serum (v/v) and 1% antibiotics. Hs68 cells are human normal fibroblasts, which are subcultured in phenol red Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% antibiotics (v/v), penicillin/streptomycin (10,000 U/ml). Both cell lines are maintained at 37°C in a humidified atmosphere of 5% carbon dioxide.

Preparation of As_2O_3

The stock solution of As_2O_3 was prepared by dissolving As_2O_3 powder (Sigma) in PBS at a concentration of 10 mM.

Inhibition of Cell Proliferation by As_2O_3

Effects of As_2O_3 on the proliferation of MCF-7 cells and Hs68 cells were performed using methylthiazoletetrazolium (MTT) conversion assay. MCF-7 cells and Hs68 cells were seeded at 1×10^4 cells/well in 96-well plate. After treated with appropriate concentrations of As_2O_3 (cells without drug treatment was used as control), suspension medium was removed and 30 μl MTT solution (5 mg/ml, Sigma) was added to each well and incubated at 37°C for 2–4 h. After that, 100-μl DMSO was added to each well and further incubated for 15 min. Optical density at 540 nm was measured. Percentage of cell survival was calculated as follows:

$$\%\ \text{Survival} = 100\% \times (\text{OD}_{540\,\text{nm}}\ \text{of test sample} / \text{OD}_{540\,\text{nm}}\ \text{of control})$$

[Methyl-^{3}H]-Thymidine Incorporation Into DNA

Cells were seeded at 1×10^4 cells/well in 96-well plate. After treatment with appropriate concentrations of As_2O_3, 0.5 μl [methyl-^{3}H]-thymidine solution (1 μCi/μl, Amersham Bio-

science) was added to each well and the cells were incubated at 37°C for 6 h. The cell suspension was then transferred to glass microfibre filters (Whatman) by using cell harvester. The nucleic acids incorporated with [methyl-^{3}H]-thymidine were trapped on the filter paper. Radioactivity was measured by scintillation counter. The percentage [methyl-^{3}H] thymidine incorporation was calculated as follows:

$$\% \text{ Incorporation} = 100\% \times [(\text{cpm of tested sample})/(\text{cpm of control})].$$

Detection of DNA Fragmentation

To determine DNA fragmentation induced by As_2O_3, Cell Death Detection ELISA^{PLUS} kit (Roche) was used. Cells were seeded at 1×10^4 cells/well in 96-well plate. After treatment with appropriate concentrations of As_2O_3, the cells were centrifuged at 200*g* for 10 min. All other steps were referred to the manual supplied by the manufacturer. Calculation was done by measuring the absorbance at 405 nm against 490 nm. Enrichment factor was calculated after normalization of protein amount in each treatment. The enrichment factor was calculated as follows:

$$\frac{\text{OD of test sample (incubation medium/cell lysate)/mg protein}}{\text{OD of the control/mg protein}}.$$

The enrichment factor of control was defined as 1.

Flow Cytometry With Propidium Iodide (PI) Staining

Cells at 3×10^5 cells/well were seeded in 6-well plate. After treatment, both floating and trypsinized adherent cells were collected, washed with PBS twice, and fixed with 70% ethanol for 30 min. After washing with PBS, cells were stained with PI staining solution (PI, 2 mg/ml; RNase A, 10 mg/ml in PBS) for 30 min at 37°C. Stained cells were analyzed using FACSort flow cytometer (Becton Dickinson). The fluorescence signal of PI was collected at channels of FL-2.

Flow Cytometry With Annexin V-PI Staining

Cells at 3×10^5 cells/well were seeded in 6-well plate. After treatment, both floating and trypsinized adherent cells were collected by centrifugation at 250*g* for 5 min. The number of cells in each sample was adjusted to 5×10^5 cells. Cells were pelleted and incubated with 100 μl Annexin V Incubation Reagent (Trevigen) containing 10 μl 10× binding buffer, 10 μl PI, 2 μl Annexin V-Conjugate, and 78-μl distilled water for 15 min in dark at room temperature. After incubation, 400 μl of 1× binding buffer was added to each sample. The fluorescent signals of Annexin V-conjugate and PI were detected at channels of fluorescence intensity (FL1) and FL2 by using FACSort flow cytometer (Becton Dickinson).

Flow Cytometry With JC-1 Staining

Cells at 3×10^5 cells/well were seeded in 6-well plate. After treatment, both floating and trypsinized adherent cells were collected and resuspended in 400 μl of serum and phenol red free RPMI. Hundred microliter of JC-1 staining solution (50 μM) was added to the cell suspension and incubated for 15 min at 37°C in the dark. Stained cells were analyzed using FACSort flow cytometer (Becton Dickinson) at channel of FL-2.

Flow Cytometry With Hydroethidine (HE) Staining

Cells at 3×10^5 cells/well were seeded in 6-well plate. After treatment, cells were collected and resupended in 400-μl serum and phenol red free RPMI. Hundred microliter of HE staining solution (10 mM) was added to the cell suspension and incubated for 15 min at 37°C in the dark. Stained cells were analyzed using FACSort flow cytometer (Becton Dickinson) at channel of FL-2.

Western Blot Analysis of Bcl-2 and p53 Protein

Cells were seeded at 1×10^6 cells/well in 100-mm culture dish. After various time treatments, both floating and trypsinized adherent cells were collected by centrifugation at 250*g* for 5 min. The pellet was washed with cold PBS twice and lysed in lysis buffer. The supernatant was then collected by centrifugation at 12,000*g* for 20 min. Different samples were normalized by measuring total protein amount using BCA protein assay (Sigma). For gel electrophoresis, 10% and 15% SDS–PAGE was used for analysis of p53 and bcl-2, respectively. The

electrophoresed protein was then transferred to PVDF membrane by using Semi-dry Transfer Cell device (Trans-Blot SD semi-dry transfer cell, BIO-RAD). After electroblotting, the PVDF transfer membrane was soaked in 10% non-fat milk for 2 h at room temperature. Then, the membrane was probed with mouse anti-human Bcl-2 antibody or human p53 antibody (Santa Curz Biotechnology) for 2 h at room temperature with gentle rotation. After washing, it was incubated with HRP-conjugated anti-mouse IgG for further 1 h. The signal was detected by enhanced chemiluminescence (ECL) assay kit according to the manual supplied by the manufacturer.

Statistical Analyses

Data are expressed as means ± SD for three repeated experiments. The Student's *t*-test was used for statistical analyses. $P < 0.05$ indicated that the difference between the tested and control groups was considered statistically significant.

RESULTS

Effect of As_2O_3 on Cell Survival of MCF-7 Cells

Figure 1 shows the dose- and time-response curves of As_2O_3 on the viability of MCF-7 cells. MCF-7 cells were incubated with 1–16 μM of As_2O_3 for 24, 48, and 72 h, respectively. The curves indicate the reduction of cell viability of As_2O_3 on MCF-7 cells at increasing concentrations and longer treatment durations. The 50% inhibitory concentration (IC_{50}) of As_2O_3 on MCF-7 cells was 8, 1.8, and 1.2 μM upon treatment for 24, 48, and 72 h, respectively.

Effect of As_2O_3 on Cell Survival of Hs68 Cells

Figure 2 shows the dose- and time-response curves of As_2O_3 on the cell viability of Hs68 cells. Hs68 cells were incubated with 1–16 μM As_2O_3 for 24, 48, and 72 h, respectively. Exposure to concentrations above 10 μM inhibited cell survival by 20–50%. At lower concentrations, below 8 μM, most cells were viable and less than

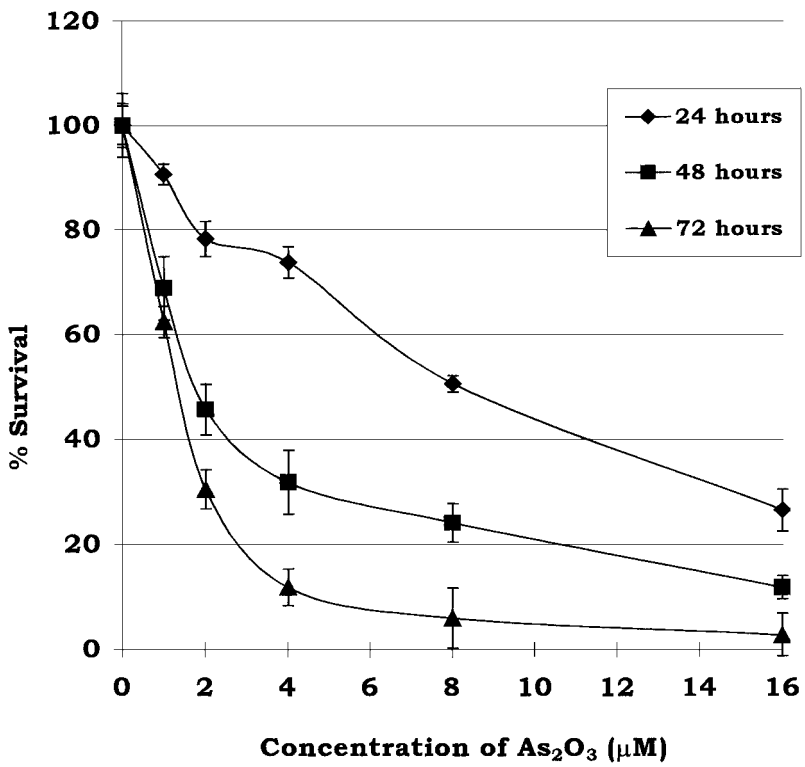

Fig. 1. Cell survival of MCF-7 cells upon As_2O_3 treatment for various time courses. Cells were treated with different concentrations (1–16 μM) of As_2O_3 for 24, 48, and 72 h. Data are presented as means ± SD for six replicate measurements. The percentage survival is expressed relative to control, which is defined as 100%.

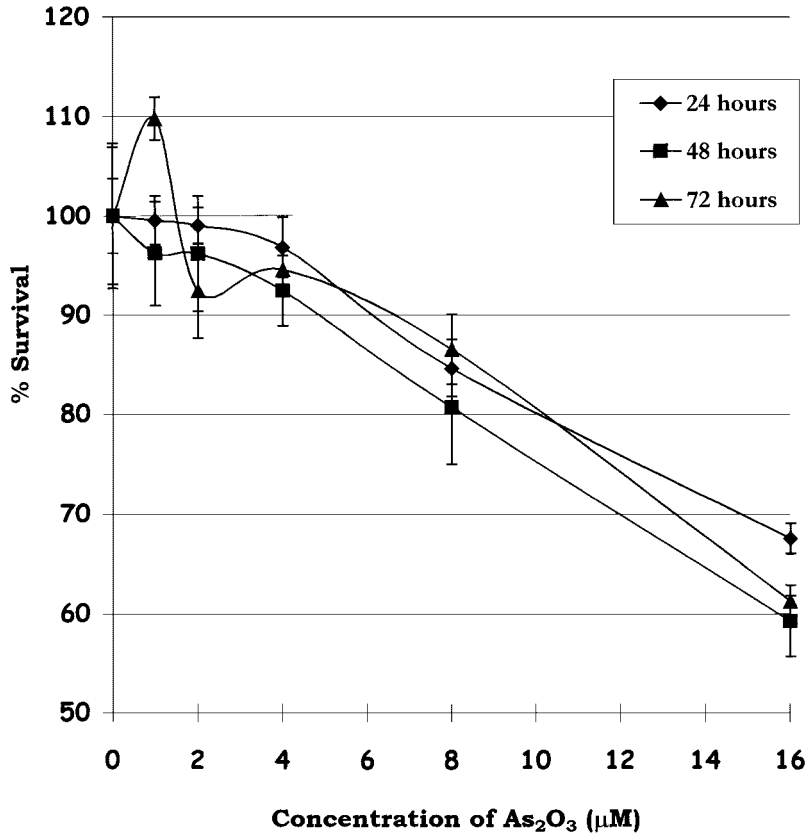

Fig. 2. Cell survival of Hs68 cells upon As_2O_3 treatment for various time courses. Cells were treated with various concentrations (1–16 μM) of As_2O_3 for 24, 48, and 72 h. Data are presented as means ± SD for six replicate measurements. The % survival is presented relative to untreated control, which is defined as 100%.

20% of cell proliferation was inhibited. As_2O_3 at 2 μM exhibited no inhibition on cell survival.

Effect of As_2O_3 on DNA Synthesis and Cell Proliferation of MCF-7 Cells by [Methyl-^{3}H] Thymidine Incorporation Into DNA Strand

Incorporation of thymidine indicated the active DNA synthesis and thus the cell proliferation. MCF-7 cells were incubated with 0.125–32 μM of As_2O_3 for 24, 48, and 72 h. Figure 3 showed the percentage of [methyl-^{3}H]-thymidine incorporation into MCF-7 cells after 24-, 48-, and 72-h treatment. When incubated with high concentrations of As_2O_3 (above 8 μM), over 50% reduction of [methyl-^{3}H]-thymidine incorporation was observed. The inhibition increased with treatment time. When incubated with low concentrations of As_2O_3 (below 2 μM) for 24 and 48 h, no significant inhibition was observed in the test samples comparing to the control. When the incubation was increased to 72 h, over 50% of inhibition was observed.

Detection of DNA Fragmentation

Cell Death Detection ELISA Plus kit allowed the detection of DNA fragments together with the detection of necrosis. The random sized DNA fragments resulted from necrosis can be detected by analyzing the supernatant (culture medium) after treatment. For apoptotic cells, DNA fragments including high molecular weight DNA fragments remain in the cell cytoplasm, which can only be detected after the disruption of cell membrane by cell lysis. After treatment of As_2O_3, the supernatants and the cell lysate were analyzed separately by ELISA. DNA fragmentation resulted from apoptotic MCF-7 cells was shown in Figure 4. Upon higher concentration of As_2O_3 and longer treatment time, more DNA fragments were detected. DNA fragmentation was significantly induced by twofolds as compared with control after 72-h treatment with 1 μM As_2O_3 or 48 h with 2 μM of As_2O_3. Further incubation with 2 μM As_2O_3 to

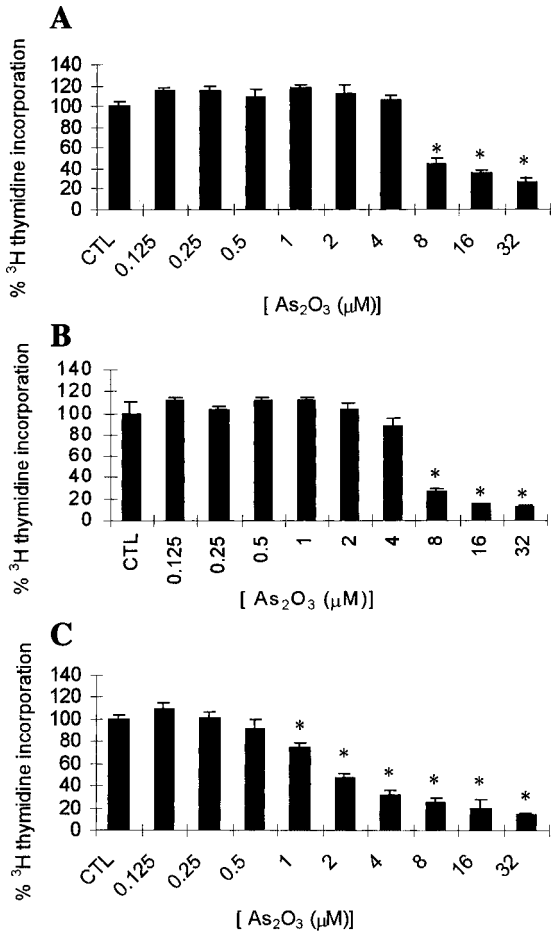

Fig. 3. Effect of As_2O_3 on DNA synthesis and cell proliferation of MCF-7 cells upon various time courses. Cells were treated with various concentrations (0.125–32 μM) of As_2O_3 for (**A**) 24, (**B**) 48, (**C**) 72 h. Data are presented as means ± SD for six replicate experiments. The percentage is expressed relative to untreated control, which is defined as 100%. *$P < 0.05$ indicated that the difference between the test sample and control was considered statistically significant.

72 h enhanced DNA fragmentation to 3.5-folds. When analyzing DNA fragmentation of supernatants (incubation media), no significant increase in DNA fragments was induced by various concentrations of As_2O_3 at all incubation time as compared to the untreated control.

Detection of Phosphatidylserine (PS) Externalization by Flow Cytometry With Annexin V-PI Staining

After treatments for 24, 48, and 72 h, MCF-7 cells were collected. In each time point, a control was prepared which acted as a reference for comparison. As shown in Figure 5, the majority of cells were localized in the lower left quadrant after 24-h treatment indicating the presence of viable cells without apoptotic or necrotic stimulation. As the treatment time was increased to 48 h, cells were observed in both lower and upper left quadrants, indicating the presence of both apoptotic and necrotic cells, respectively. Upon treatment for 72 h, there was a shift of cells from lower left quadrant to lower and upper right quadrants. When comparing the percentage of apoptotic cells induced by As_2O_3 with the untreated control, 0.7- to 3-folds increases were observed upon 1-μM As_2O_3 treatment for 24–72 h, respectively. Whereas, two- to fourfolds increases were observed upon 2-μM As_2O_3 treatments for 24–72 h (Table I).

Regulation of Cell-Cycle Distribution of MCF-7 Cells Upon As_2O_3 Treatment

By comparing the percentage of different cell-cycle phases of MCF-7 cells, the regulation of As_2O_3 on cell-cycle progression was studied. Figure 6 shows the cell-cycle distribution of MCF-7 cells after treatment with 1 and 2 μM of As_2O_3 for 24, 48, and 72 h. Sub-G_1 population increased upon treatment with higher concentrations of As_2O_3 throughout the experimental time frame. With respect to the percentage of sub-G_1 peak of control, greater increase was observed upon longer time treatment. Apart from induction of sub-G_1 peak, As_2O_3 was also shown to regulate cell-cycle distribution of MCF-7 cells. The change of phase distribution was most significant upon 2 μM of As_2O_3 treatment for 72 h. After treatment, the percentages of cells in G_1, S, and G_2/M phases of untreated MCF-7 cells were changed from 47.76%, 24.48%, and 27.17% to 67.53%, 9.26%, and 23.21%, respectively. As_2O_3 induced a shift of cells partly from G_2/M phase and mostly from S phase to G_1 phase of cell cycle (Fig. 6).

Effect of As_2O_3 on Mitochondrial Membrane Potential of MCF-7 Cells

During early apoptosis, the mitochondria permeability transition pores (PTP) opens resulting in the free distribution of ions between both sides of the membrane. As a consequence, the mitochondrial transmembrane potential is disrupted ($\Delta\psi_m$). The collapse of mitochondrial transmembrane potential can be detected by flow cytometry with JC-1 staining. JC-1 is a fluorochrome that emitted fluorescence upon excitation. At low membrane potentials, JC-1 exists as monomer and emits green fluorescence. At high membrane potentials, JC-1 forms

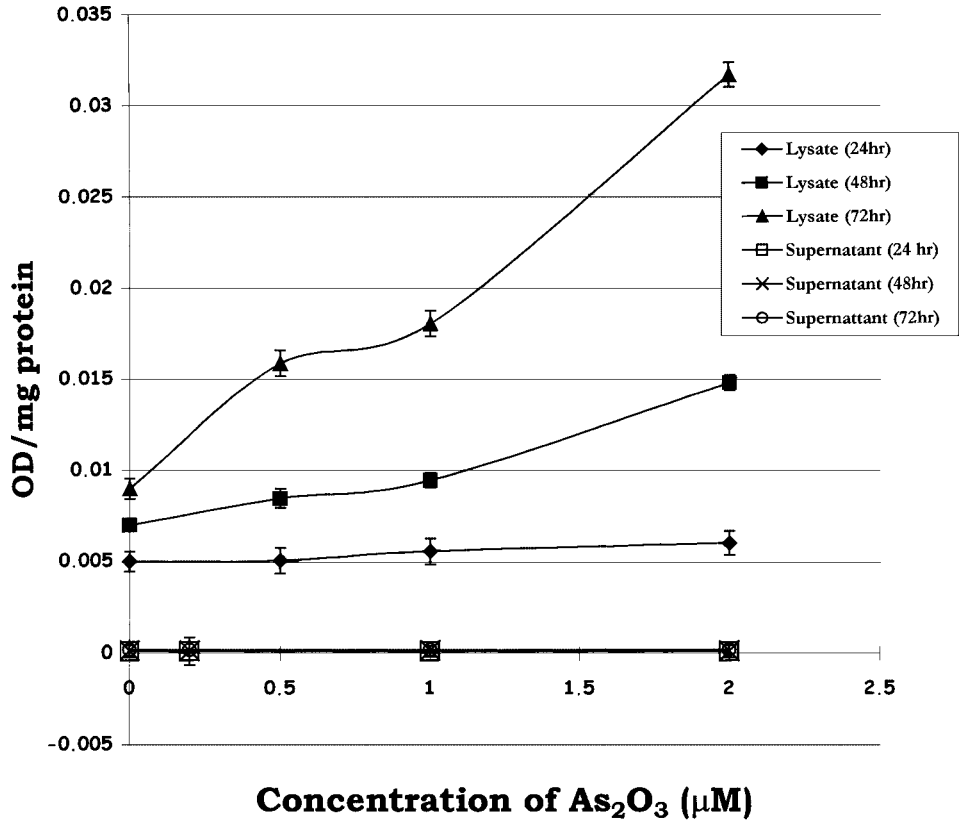

Fig. 4. Detection of DNA fragmentation in the incubation medium of MCF-7 cells upon As_2O_3 treatment for various time courses. After treatments for 24, 48, and 72 h with various concentrations (0.25–2.0 μM) of As_2O_3, the supernatants (incubation media) and cell lysate were incubated with immunoreagents for 2 h. DNA fragmentation was measured spectrophotomically by microplate reader at 405-nm wavelength against 490-nm reference wavelength. Data are the means ± SD SD for five separate experiments. Data is expressed as absorbance per milligram of protein in each sample.

dimmer (J-aggregates) and emits red fluorescence. By measuring emitted green and red fluorescent intensities, the state of mitochondrial transmembrane potential can be detected. With respect to the control, treatment with both 1- and 2-μM As_2O_3 induced a shift of cell population to right after both 48- and 72-h treatment. As shown in Figure 7, the increments were directly proportional to the concentrations of As_2O_3. Greater shift was observed upon 72-h treatment than that of 48-h treatment. This implies that the induction of change in mitochondrial transmembrane potential was time-dependent. Valinomycin treatment was used as a positive control to illustrate the disruption of mitochondrial membrane potential.

Analysis of Superoxide Production in MCF-7 Cells Upon As_2O_3 Treatment by Flow Cytometry With HE Staining

The change in cellular reactive oxygen species (ROS) in MCF-7 cells after As_2O_3 treatment was measured by flow cytometry with HE staining. In the presence of superoxide, blue fluorescence emitting HE is oxidized to ethidium bromide and intercalates with cellular DNA. When excited during analysis, red fluorescence is emitted with intensity proportional to the amount of ethidium bromide and superoxide presence in treated or untreated cells. After treatment with various concentrations of As_2O_3 over 48 and 72 h, the cells were stained with HE. Results are analyzed in histogram of events plotted against FL1. Elevation of superoxide will result in histogram move to right. Positive control was performed by incubating MCF-7 cells with valinomycin. Valinomycin disrupts mitochondrial membrane potential and causes release of superoxide to cytosol. Thus the fluorescence intensity after treatment increased and showed in histogram as moving to right. As shown in Figure 8, no obvious shift of histograms was observed with respect to untreated control after treatment with 0.5–2-μM As_2O_3 for 48 and 72 h.

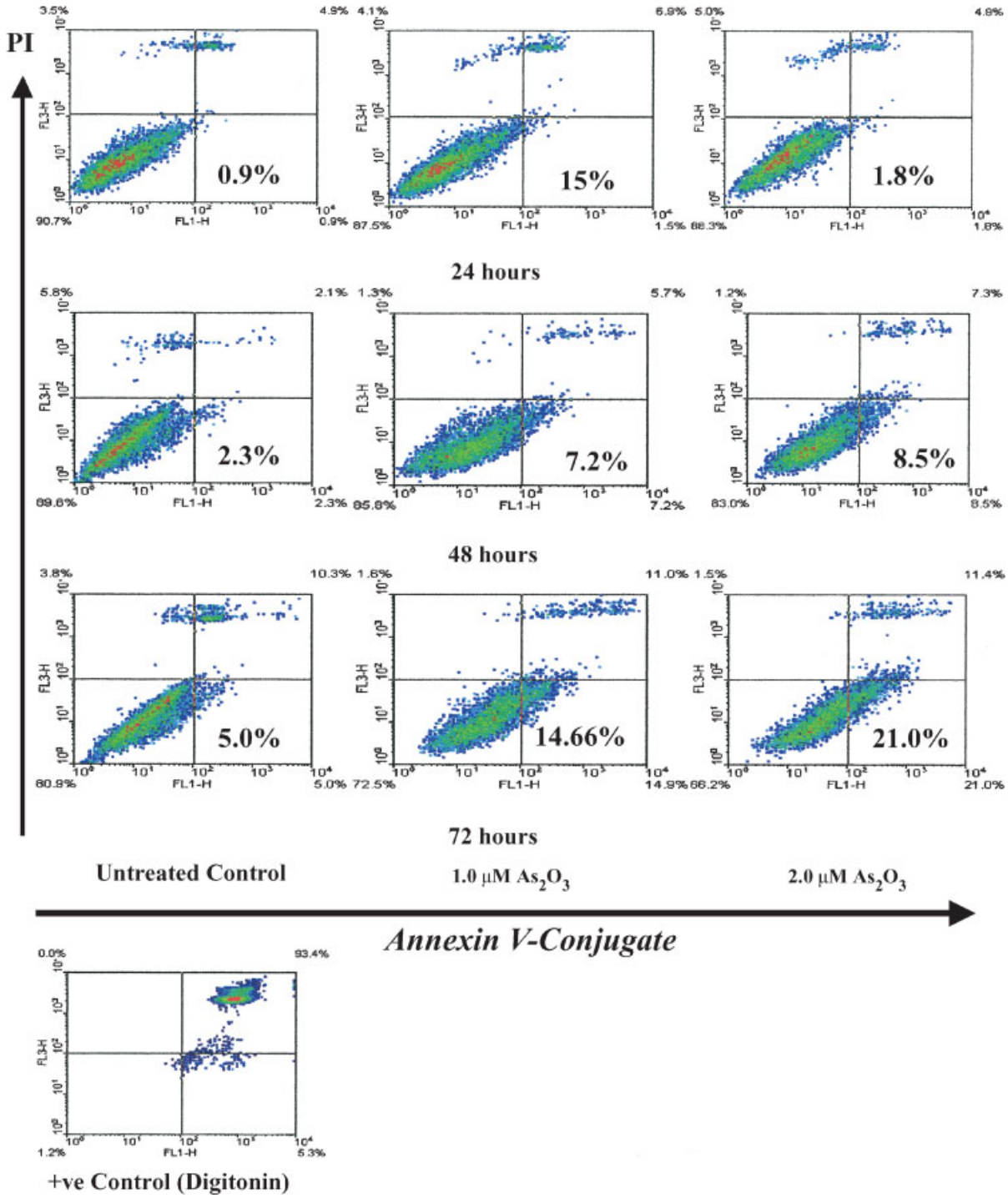

Fig. 5. Detection of phosphatidylserine (PS) externalization by flow cytometry. Dot plots of PI fluorescent intensity plotted against Annexin V-conjugate fluorescent intensity. Ditgitonin was used as positive control and representing the condition at which late apoptosis and necrosis took place. The percentage shown at the lower right quadrant indicated the percentage of apoptotic cells. Data are from a representative of three independent experiments.

TABLE I. Comparison of Percentage Apoptotic Cells and Percentage Necrotic Cells Induced in MCF-7 Cells Treated With Various Concentrations of As_2O_3 for 24, 48, and 72 h

		% Cells			% Cells relative to control	
Time incubation (h)	Treatment	Annexin V−/PI− (viable)	Annexin V+/PI− (early apoptosis)	Annexin V+/PI+ (late apoptosis and necrosis)	% Apoptotic cells	% Late apoptotic and necrotic cells
24	Control	90.7	0.9	4.9	100	100
	1 μM As_2O_3	87.5	1.5	6.9	167	141
	2 μM As_2O_3	88.3	1.8	4.9	200	100
48	Control	89.9	2.3	2.1	100	100
	1 μM As_2O_3	85.8	7.2	5.7	313	271
	2 μM As_2O_3	83	8.5	7.3	400	348
72	Control	80.9	5	10.3	100	100
	1 μM As_2O_3	72.5	14.66	11	292	107
	2 μM As_2O_3	66.2	21	11.4	420	111

Regulation of Bcl-2 and p53 Protein Level in MCF-7 Cells Upon As_2O_3 Treatment

Bcl-2 is a pro-oncogene protein. Extensive studies demonstrated that bcl-2 acts as a negative regulator of apoptosis [Kluck et al., 1997]. In the present study, bcl-2 protein level in MCF-7 cells upon As_2O_3 treatment was determined. After the first 24 h of treatment, no significant alternation in Bcl-2 protein level was observed. At 48 h, 2 μM As_2O_3 induced a decrease in Bcl-2 protein level by approximately 30% relative to control (Fig. 9). p53 tumor suppressor protein plays a crucial role in many cellular functions such as cell proliferation. Comparing to the untreated control, 2 μM As_2O_3 upregulated p53 protein level in MCF-7 cells over 50% after 48-h treatment. It further upregulated the protein level by 90% upon 72-h treatment (Fig. 9).

DISCUSSION

Growing studies have revealed the effectiveness of As_2O_3 in combating against solid tumors since its pilot treatment in APL patients. In view of the promising effectiveness of As_2O_3 as chemotherapeutic drug, the potential application of As_2O_3 in breast cancer treatment was explored in this study. The cytotoxicity of As_2O_3 on human breast tumor MCF-7 cells was assessed by several parameters. Result of MTT conversion assay (Fig. 1) showed a dose- and time-dependent survival suppression on MCF-7 cells by As_2O_3. The IC_{50} were 8, 1.8, and 1.2 μM at 24-, 48-, and 72-h treatment, respectively. At these concentrations, As_2O_3 exhibited no cytotoxic effect on normal cells as indicated from the high percentage survival of human fibroblasts Hs68 cells upon As_2O_3 treatment for 24–72 h (Fig. 2). So, the results suggested that the concentration of As_2O_3, which induced cytotoxic effect on MCF-7 cells, was not cytotoxic to normal human cells. The cytotoxicity of As_2O_3 at concentration below 2 μM was selected in further study.

As_2O_3 elicits apoptosis in mediating antitumor effects on APL cell lines and other solid human tumors. In our studies, As_2O_3 at concentrations between 0.5 μM and 2 μM were found to induce apoptosis in MCF-7 cells over 72-h treatment in dose- and time-dependent manner. It was supported from the evidence that DNA fragmentation was detected in cytoplasm of MCF-7 cells after 1-μM As_2O_3 treatment for 72 h (Fig. 4). DNA fragments were also induced upon 48-h treatment of 2 μM As_2O_3. It is widely accepted that DNA fragments of 180 base pairs (bp) detected in conventional agarose gel electrophoresis as DNA ladder is a hallmark feature of apoptosis [Wyllie et al., 1980]. Our results did not indicate any DNA ladder formation after As_2O_3 treatment (data not shown). The result in the detection of DNA fragmentation is controversial. Some studies reported the absence of detectable internucleosomes DNA in MCF-7 cells owing to initial cleavage of DNA fragments of approximately 300 kb and then 50 kb in chromatin instead of typical 180-bp fragments observed as DNA ladder [Oberhammer et al., 1993]. A recent study showed that the absence of DNA fragmentation and other typical biochemical features of apoptosis were due to the absence of functional caspase 3, which is responsible for internucleosomal DNA

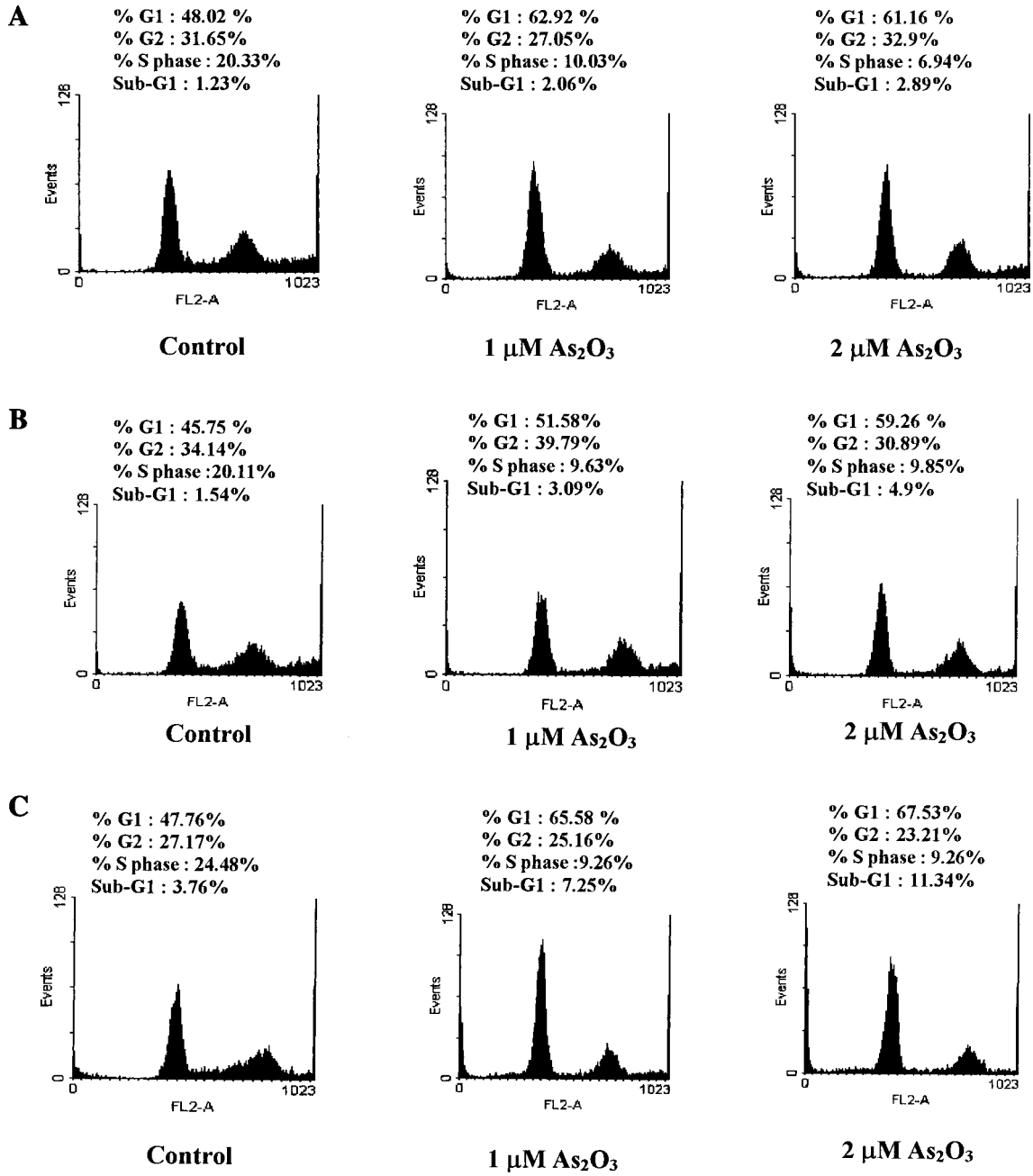

Fig. 6. Effects of 1- and 2-μM As_2O_3 on cell-cycle distribution of MCF-7 cells after 24- (**A**), 48- (**B**), and 72-h (**C**) treatments. The cells were stained with PI solution at 37°C for 30 min. The populations of cells in G_1, S, $G_2 + M$, and sub-G_1 phases were determined by FACSort flow cytometer and analyzed by Modfit LT. Cell populations are expressed as percentages shown in the histograms. Data are from a representative of five separate experiments.

fragmentation [Wolf et al., 1999]. In contrary, some studies of cytotoxic agents induced apoptosis showed DNA fragments of 180-bp detectable in agarose gel electrophoresis [Sokolova et al., 1995]. This may be explained by strain-specific difference in MCF-7 cells [Gooch and Yee, 1999] and separate endonuclease activity responsible for high molecular and internucleosomal DNA fragmentation [Pandey et al., 1994]. So, in the present study, ELISA method was adopted in which DNA binding POD bound to DNA fragments of higher molecular weights to detect whether DNA fragments were produced after As_2O_3 treatment.

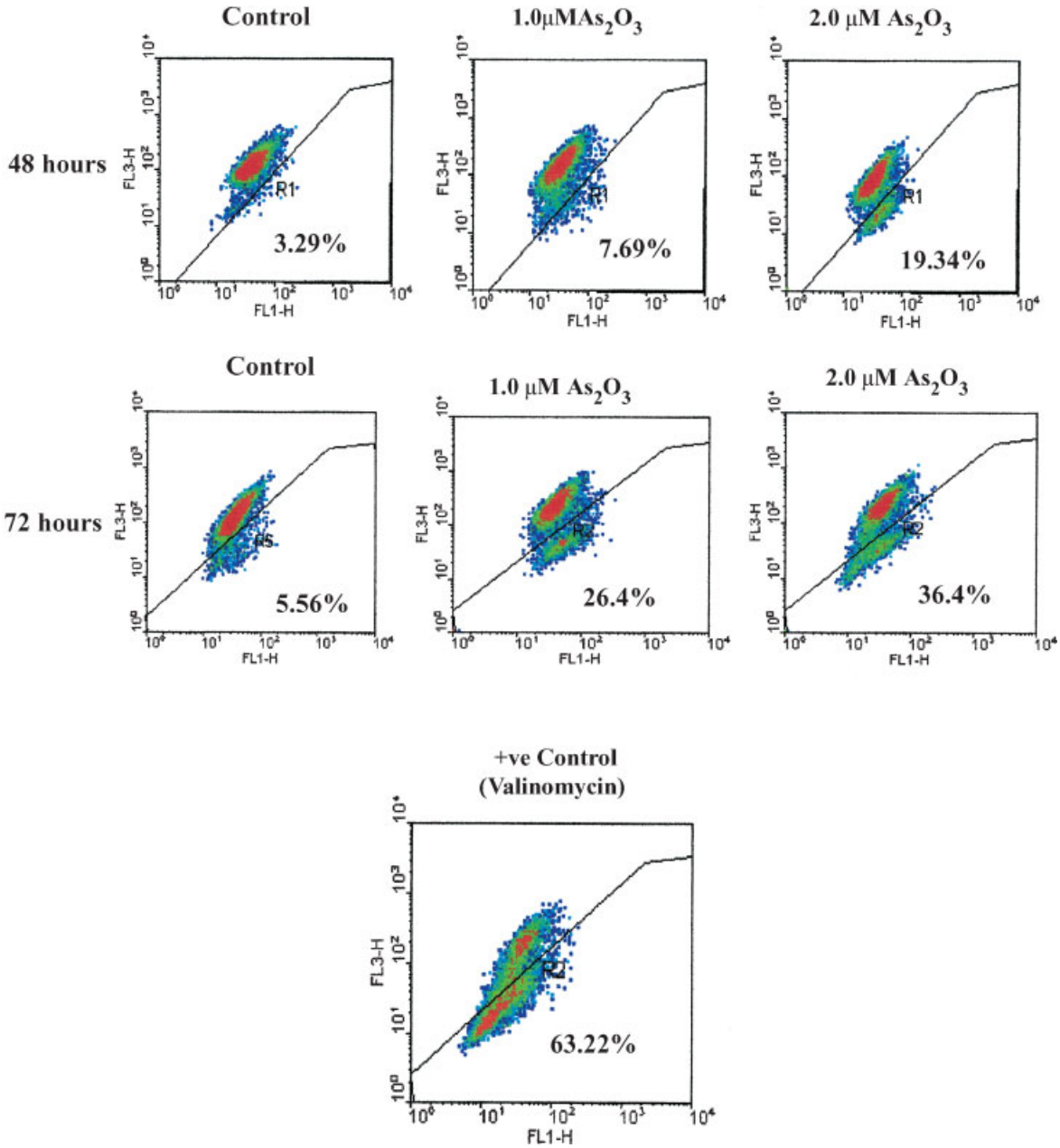

Fig. 7. Effects of As_2O_3 on mitochondrial membrane potential of MCF-7 cells after 48- and 72-h treatments. Cells stained with JC-1 were analyzed using FACSort flow cytometer (Becton Dickinson) at channel of FL-2. Valinomycin was used as positive control, which disrupted mitochondrial membrane potential.

To further confirm the induction of apoptosis by As_2O_3, we examined the externalization of PS by flow cytometry with Annexin V-PI staining. PS externalization was observed in majority of treated cells. Meanwhile, necrotic cells were also shown. However, it contributed to minority of cells as compared to the apoptotic cells (Fig. 5). Also, apparent DNA fragments were detected in culture media showing that necrotic cells were present in insignificant population after treatment (Table I). Together with the detection of sub-G1 peak in cell-cycle analysis of MCF-7 cells after As_2O_3 (Fig. 6), it is obvious that 2-μM As_2O_3 induces apoptosis in inhibiting the growth of MCF-7 cells.

Apoptosis is a complex process regulated by positive and negative regulators in coordinated manner. The downregulation or suppression of apoptotic inactivator and upregulation or activation of apoptotic activator will trigger

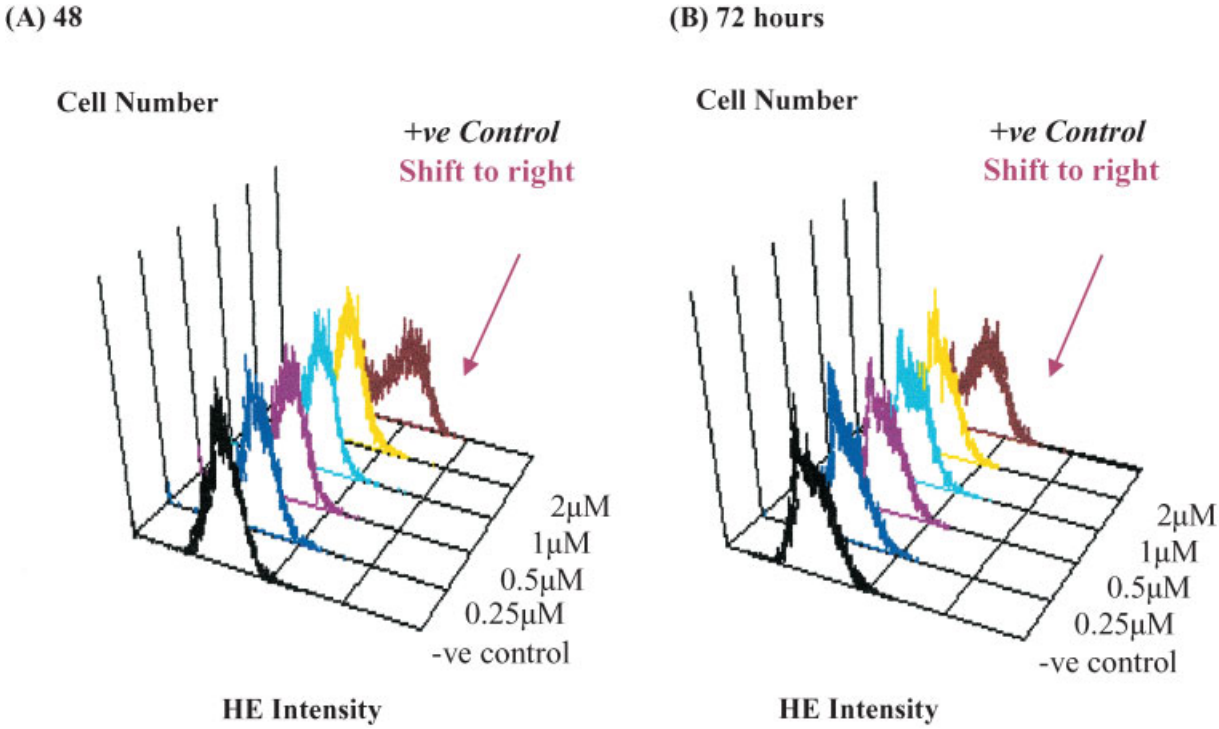

Fig. 8. Detection of superoxide production in MCF-7 cells upon As_2O_3 treatment for 48 and 72 h. After treatment for 48 (**A**) and 72 h (**B**), the cells were harvested and stained with hydroethidine (HE) at 37°C for 30 min. The HE intensity was determined by FACSort flow cytometer and analyzed by Modfit LT. Histograms in black, dark blue, purple, pale blue, and yellow represent untreated control and treatment with As_2O_3 in 0.25, 0.5, 1, and 2 μM, respectively. Dark brown histogram represents positive control, which was prepared by treating valinomycin with MCF-7 cells over same treatment period as those of As_2O_3.

apoptosis. To clarify the downstream pathways proceeding to apoptosis induced by As_2O_3 on MCF-7 cells, mitochondria that plays a critical role in apoptosis was examined. Collapse of mitochondrial membrane potential was detected after 48-h treatment with 1-μM As_2O_3 treatment, further treatment with 2-μM As_2O_3 induced a greater collapse as indicated by the shift of cell populations to right upon JC-1 staining (Fig. 7). p53 is a tumor suppressor protein [Selvakumaran et al., 1994]. It also acts as a transcription factor in regulating target genes expression involving in apoptosis such as Bcl-2 and Bax [Zhao et al., 2000]. In the present study, p53 protein expression in MCF-7 cells was upregulated by twofolds after 48-h treatment of 2-μM As_2O_3. The elevation of p53 protein expression could be detected as early as 24 h after treatment (Fig. 9). Meanwhile, Bcl-2 protein expression was downregulated by 25% after 48-h treatment of 2-μM As_2O_3 (Fig. 9). Bcl-2 acts as a negative regulator in mitochondrial-mediated apoptosis. It counteracts the apoptosis stimulatory effect of Bax and prevents the release of cytochrome-*c* from mitochondria [Kluck et al., 1997]. The suppression of Bcl-2 protein expression by As_2O_3 directly or through the upregulation of p53 will reduce the anti-apoptotic stimulus in MCF-7 cells and in turn enhance the induction of apoptosis. Some studies have indicated that downregulation of Bcl-2 involved in apoptosis induction and increased the sensitive of MCF-7 cells to antitumor drugs [Piche et al., 1998].

It has been demonstrated that ROS are mediators of PTP opening. p53 transcript, PIG3 was shown to increase ROS production in mitochondria [Polyak et al., 1997]. ROS production is enhanced as a result of early apoptotic processes. Moreover, Bcl-2 is shown to protect cells from oxidative stress and deplete cellular GSH, which is important ROS scavenger [Fernandez-Checa et al., 1998]. The upregulation of p53 protein expression, mitochondria membrane potential disruption, and downregulation of Bcl-2 protein expression observed in our studies suggesting that ROS level may be elevated during As_2O_3 treatment. In contrary, our study of the level of a ROS, namely superoxide, by flow cytometry with HE staining after

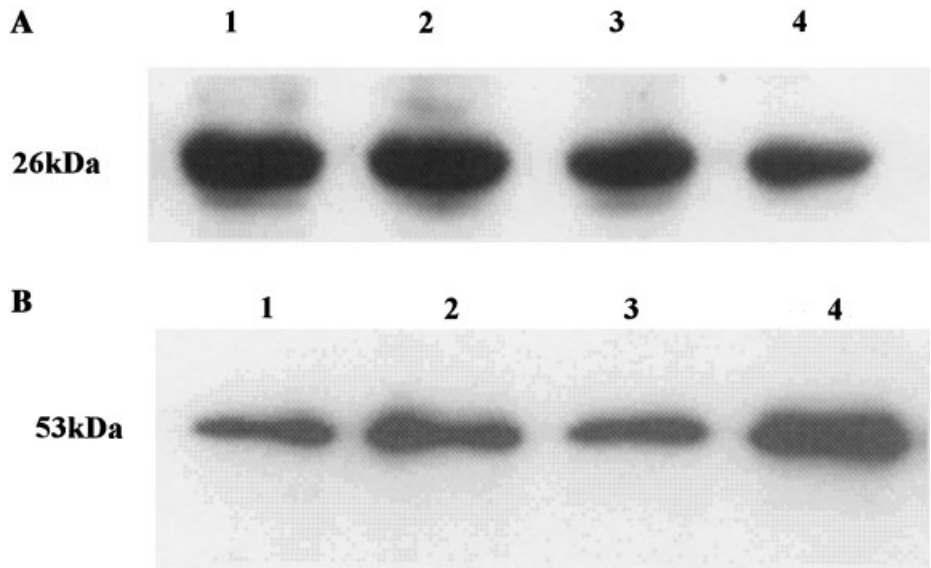

Fig. 9. Regulation of Bcl-2 (**A**) and p53 (**B**) protein levels in MCF-7 cells upon 2-μM As_2O_3 treatment for 24 and 48 h. MCF-7 cells were treated with 2-μM As_2O_3. After 24 and 48 h, cellular proteins were extracted. **Lane 1**: untreated control (24 h); **lane 2**: 2-μM As_2O_3 (24 h); **lane 3**: Untreated control (48 h); **lane 4**: 2-μM As_2O_3 (48 h). Data are from a representative of three independent experiments.

As_2O_3 treatment did not show any enhanced superoxide level (Fig. 8). When incubating MCF-7 cells with As_2O_3 and N-acetyl-cysteine (NAC), the survival inhibitory effect induced by As_2O_3 on MCF-7 cells was not suppressed (data not shown). NAC is a thiol anti-oxidant, which acts as cellular defender to reduce ROS in cells. The reduction of ROS, in turn, enhances apoptosis induction [Castedo et al., 2002; Curtin et al., 2002]. Our observations suggest that ROS may not be responsible for As_2O_3 induced apoptosis.

Apart from apoptosis induction, As_2O_3 was also shown to induce cell proliferation inhibition in MCF-7 cells over 72-h treatment. In assessing new DNA synthesis of MCF-7 cells with [methyl-^{3}H]-thymidine incorporation, an 50% inhibition of DNA synthesis was observed over 72-h treatment with 2-μM As_2O_3 (Fig. 3). As DNA synthesis is essential for cell division and proliferation, As_2O_3 thus inhibits cell proliferation of MCF-7 cells.

G_1 phase arrest is innate cell cycle in response to damage. In normal circumstances, cell cycle proceeds through G_1, S, and G_2/M phase. Upon damage, cell cycle will be suspended so as to allow time for DNA repair, undergo apoptosis, or enter into a permanent G_0 state. G_1 phase arrest induced by As_2O_3 may prevent cells from proceeding to S phase for preparation of cell division such as DNA synthesis. This explains our finding of DNA synthesis reduction in MCF-7 cells upon As_2O_3 treatment. By analyzing the cell-cycle distribution of MCF-7 cells, As_2O_3 was shown to alter the cell-cycle distribution. It was obvious that cell population in G_1 phase increased while that in S phase and G_2/M phase decreased. In other words, 1- and 2-μM As_2O_3 induced cell-cycle arrest in MCF-7 cells at G_1 phase by blocking cell progress to S phase and then G_2/M phase. The change in cell-cycle distribution was dependent in dose- and time-incubation (Fig. 6).

From our studies of p53 protein expression, the cell-cycle arrest at G_1 phase may be attributed to the upregulation of p53 protein expression. The upregulation was observed within 48-h treatment (50% upregulation) and was further enhanced at 72-h treatment (90% upregulation) (Fig. 9). In addition to tumor suppressor protein, p53 acts as transcription factor to regulate target genes expression involving in cell-cycle regulation [Zhao et al., 2000]. Upon upregulation of p53 protein, $p21^{Cip1}$ protein expression is activated [el-Deiry et al., 1998]. $p21^{Cip1}$ will then bind directly to cyclin/Cdk complexes including cyclin D/Cdk4/6 and cyclin E/Cdk2. These complexes promote cell-cycle progression from G_1 phase to S phase by phosphorylating pRb corporately [Harper et al., 1993]. As a consequence, downregulation or inhibition of both cyclin/cdk complexes will lead to

accumulation of cells in G_1 phase. So, As_2O_3 may increase p53 protein level, which in turn regulates cell-cycle regulatory proteins and ultimately induces cell-cycle arrest and reduces cell proliferation of MCF-7 cells. Studies of the effects of As_2O_3 on cell-cycle regulatory proteins will enhance the understanding of the mechanisms induced by As_2O_3 in cell-cycle regulation of MCF-7 cells.

Taken together, treatment with 2-μM As_2O_3 induced anti-tumor effects on MCF-7 cells through the induction of apoptosis and inhibition of cell proliferation. It should be noted that 2-μM As_2O_3 is the clinically achievable concentration in APL patients without severe toxicity [Shen et al., 1997]. At this concentration, As_2O_3 was found effective in inhibiting the growth of non-APL cells such as B-cell leukemia [Zhu et al., 1999], lymphoid leukemia [Zhang et al., 1998], chronic myelogenous leukemia [Lu et al., 1999], multiple myeloma [Prekins et al., 2000], gastric cancer [Zhang et al., 1999], ovarian cancers [Du and Ho, 2001], and hepatoma [Siu et al., 2002]. Here, we also found the non-cytotoxicity of As_2O_3 towards human normal fibroblasts cells at this concentration. Our mechanistic studies suggest that As_2O_3 exhibits its anti-proliferative effect on MCF-7 cells via the upregulation of p53 protein and disruption of mitochondrial membrane potential together with downregulation of apoptosis negative regulator, bcl-2 protein, elicits a mitochondrial-mediated apoptosis, which ultimately resulted in the hallmark features of apoptosis, DNA fragmentation, and PS externalization. Meanwhile, the upregulation of p53 prevents cell entry to S phase and induces cell-cycle arrest at G_1 phase as observed in cell-cycle distribution analysis. By then, DNA synthesis is down-regulated and cell proliferation was suppressed.

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