

# Induction of Autophagic Cell Death in Malignant Glioma Cells by Arsenic Trioxide

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## ABSTRACT

Recent clinical data shows that arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) causes remission in patients with acute promyelocytic leukemia and multiple myeloma without severe side effects. Laboratory data suggest that As<sub>2</sub>O<sub>3</sub> induces apoptosis or cell differentiation of hematopoietic or solid tumor cells. To date, there has been no study on the effects of As<sub>2</sub>O<sub>3</sub> on glioma cells. In this study, we investigated the *in vitro* effect of As<sub>2</sub>O<sub>3</sub> on cell growth inhibition and cell death mechanisms in human glioma cells. As<sub>2</sub>O<sub>3</sub> significantly inhibited the proliferation of all six of the glioma cell lines (U373, U87, U251, GB1, A-172, and T98G) tested in this study in a dose-dependent manner. The IC<sub>50</sub> of As<sub>2</sub>O<sub>3</sub> for all of the tumor cell lines was <2 μM. Previous studies have shown that this is a clinically safe concentration. Treatment with 2 μM As<sub>2</sub>O<sub>3</sub> induced G<sub>2</sub>/M arrest in all of the glioma cell lines. Autophagy (programmed cell death type II), but not apoptosis (programmed cell death type I), was detected by electron microscopy in U-373-MG cells treated with 2 μM As<sub>2</sub>O<sub>3</sub>. Caspase inhibitors did not halt As<sub>2</sub>O<sub>3</sub>-induced cell death. Furthermore, combination of As<sub>2</sub>O<sub>3</sub> with bafilomycin A1 autophagy inhibitor enhanced the antitumor effect of As<sub>2</sub>O<sub>3</sub> through induction of apoptosis. These findings suggest that As<sub>2</sub>O<sub>3</sub> at a clinically safe concentration may be an effective chemotherapeutic agent for malignant gliomas.

## INTRODUCTION

Low concentrations (≤2 μM) of As<sub>2</sub>O<sub>3</sub><sup>2</sup> have been reported recently to induce clinical remission in the patient with APL (1, 2) without severe bone marrow suppression (3). *In vitro* studies showed that low concentration of As<sub>2</sub>O<sub>3</sub> induced apoptosis in APL cell lines via modulation of promyelocytic leukemia proteins (4), Bcl-2 (5–8), modification of the glutathione redox system (9), and caspase activation (10–12). In addition, recent investigations have shown that As<sub>2</sub>O<sub>3</sub> induced apoptosis in MM cells (13, 14) and solid tumors (15). Although the mechanism of As<sub>2</sub>O<sub>3</sub>-induced cell death are still under investigation, it has been suggested that As<sub>2</sub>O<sub>3</sub> induces apoptosis in neuroblastoma cell lines through the activation of caspase 3 *in vitro* (16). Similarly, the alteration of mitochondria is an early event in As<sub>2</sub>O<sub>3</sub>-induced apoptosis in esophageal carcinoma cells (17). Moreover, a recent study showed that As<sub>2</sub>O<sub>3</sub> provides a novel, safe approach for treatment of androgen-independent prostate cancer, activating p38, c-Jun NH<sub>2</sub>-terminal kinase, and caspase-3 (18). These studies suggest that As<sub>2</sub>O<sub>3</sub> is a safe and promising agent for nonhematopoietic cancer treatment. However, the effects of As<sub>2</sub>O<sub>3</sub> on malignant glioma cells have not been described.

Malignant gliomas are resistant to many kinds of treatments including chemotherapy, radiation, and other adjuvant therapies. In addition, glioma cells are prone to acquire drug resistance systems. Up to date, there is still need to identify chemotherapeutic agents with cytotoxic

effects exclusive for malignant glioma cells. In this study, we investigated the effect of As<sub>2</sub>O<sub>3</sub> at low concentration (2 μM) on malignant glioma cells. Our data show that As<sub>2</sub>O<sub>3</sub> inhibited the cell growth of all six of the malignant glioma cell lines and induced cell death. However, apoptosis was not observed in malignant glioma cells. Their cytoplasm was occupied by AVOs before nuclear collapse. These intracellular damages are consistent with autophagy, programmed cell death type II. Thus, As<sub>2</sub>O<sub>3</sub> seems to a promising agent for additional investigations in the treatment of malignant gliomas.

## MATERIALS AND METHODS

**Reagents.** As<sub>2</sub>O<sub>3</sub> solution (0.1%) was kindly supplied by Dr. Samuel Waxman (Mount Sinai Medical Center). Acridine orange and bafilomycin A1 were purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in PBS distilled water or DMSO, respectively. Final DMSO concentration in medium is <0.1% volume.

**Tumor Cell Lines.** Human malignant glioma U87-MG, A172, T98G, and U373-MG cells were purchased from American Type Culture Collection (Rockville, MD). Human malignant glioma GB-1 and U251-MG cells were kind gifts of Dr. Tatsuo Morimura (National Utano Hospital, Kyoto, Japan), and Dr. Akiko Nishiyama (University of Connecticut, Storrs, CT), respectively. Cells were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), 4 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

**Growth Inhibition Assay.** *In vitro* growth effect of As<sub>2</sub>O<sub>3</sub> on glioma cell lines was determined by counting the viable cells with trypan blue staining. Briefly, cells (5 × 10<sup>3</sup> cells/well) were seeded in 96-well plate and preincubated overnight. After exposure of various concentrations of the As<sub>2</sub>O<sub>3</sub> for 78 h, cells were detached by trypsinization, and viable cells were counted with trypan blue dye.

**Cell Cycle Analysis.** For cell cycle analysis, trypsinized cells were stained with propidium iodide by using the Cellular DNA Flow Cytometric Analysis Reagent Set (Boehringer Mannheim, Indianapolis, IN) and analyzed for DNA content by using the FACScan (Becton Dickinson, San Jose, CA). Data were analyzed by Cell Quest software (Becton Dickinson). Dead cells were gated out by using pulse processing.

**Assay for Apoptosis Detection.** To detect and quantify apoptotic cells, FACS analysis was performed. As<sub>2</sub>O<sub>3</sub>-treated cells were trypsinized, washed in cold PBS, fixed in 1% paraformaldehyde and 70% ethanol, and then stored at –20 °C for at least 2 h. TUNEL assay was performed according to the manufacturer's instructions (PharMingen, San Diego, CA). Cells were analyzed by flow cytometry using a FACScan as described previously.

To detect apoptosis in combination with bafilomycin A 1, U373-MG cells were cultured in medium containing 2 μM As<sub>2</sub>O<sub>3</sub> for 24 h, and then 5 nM of bafilomycin A 1 was added in culture medium. After 72 h, tumor cells were collected and stained with propidium iodide. The percentage of sub-G<sub>1</sub> population was determined by flow cytometry.

**Supravital Cell Staining with Acridine Orange for Autophagy Detection.** Cell staining with Acridine orange (Sigma Chemical Co.) was performed according to published procedures (19), adding at a final concentration of 1 mg/ml for a period of 20 min. Bafilomycin A1 (Sigma Chemical Co.) was dissolved in DMSO and added to the cells 30 min before addition of acridine orange. Photographs were obtained with a fluorescence microscope (Axioscop) equipped with a mercury 100-W lamp, 490-nm band-pass blue excitation filters, a 500-nm dichroic mirror, and a 515-nm long-pass barrier filter.

**Quantification of AVOs with Acridine Orange Staining Using Flow Cytometry.** In acridine orange-stained cells, the cytoplasm and nucleolus fluoresce bright green and dim red, whereas acidic compartments fluoresce bright red (19, 20). The intensity of the red fluorescence is proportional to the degree of acidity. Therefore, the volume of the cellular acidic compartment can be quantified

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<sup>2</sup> The abbreviations used are: As<sub>2</sub>O<sub>3</sub>, arsenic trioxide; APL, acute promyelocytic leukemia; AVO, acidic vesicular organelle; FACS, fluorescence-activated cell sorter; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; z-VAD-fmk, z-Val-Ala-Asp-fluoromethyl ketone; AdCMVp53, recombinant adenovirus p53; MM, multiple myeloma; EM, electron microscopy; MOI, multiplicity of infection.

(19, 21). Cells were stained with acridine orange for 17 min, removed from the plate with trypsin-EDTA, and collected in phenol red-free growth medium. Green (510–530 nm) and red (650 nm) fluorescence emission from  $1 \times 10^4$  cells illuminated with blue (488 nm) excitation light was measured with a FACSCalibur from (Becton Dickinson) using CellQuest software.

**EM.** The cells were harvested by trypsinization, washed twice with PBS, and fixed with ice-cold glutaraldehyde [3% in 0.1 M cacodylate buffer (pH 7.4)] for 30 min. After washing in PBS, the cells were postfixed in OsO<sub>4</sub> and embedded in Epon. One- $\mu$ m thin sections were cut, stained with methylene buffer ArumeII, and observed by light microscopy. Representative areas were chosen for ultra-thin sectioning and viewed with a Hitachi 7000 STEM electron microscope.

**Caspase Inhibition Assay.** To determine whether caspase is involved in As<sub>2</sub>O<sub>3</sub>-induced cell death, caspase inhibition assay was performed with pan-caspase inhibitor, z-VAD-fmk (BIOMOL Research Laboratories Inc., Plymouth Meeting, PA) solubilized in DMSO as described previously (22). As a positive control, AdCMVp53 was used. Briefly, U373-MG cells ( $5 \times 10^3$  cells/well) were seeded in 96-well plate and incubated overnight at 37°C. One h before treatment with As<sub>2</sub>O<sub>3</sub> (2  $\mu$ M) or adenoviral infection with AdCMVp53 (50 MOI), 50  $\mu$ M z-VAD-fmk was added. After 2 days, cell viability was determined by trypan blue staining as described above. The cell viability of untreated U373-MG cells in the presence of diluted DMSO was regarded as 100%.

**RESULTS**

**Effect of As<sub>2</sub>O<sub>3</sub> on Proliferation of Malignant Glioma Cells.** To determine whether As<sub>2</sub>O<sub>3</sub> has a therapeutic effect on glioma cells, we performed the cell viability assay using six human glioma cell lines. As<sub>2</sub>O<sub>3</sub> significantly decreased the cell viability of all six of the glioma cell lines tested in a dose dependent manner (Fig. 1A). The IC<sub>50</sub> of

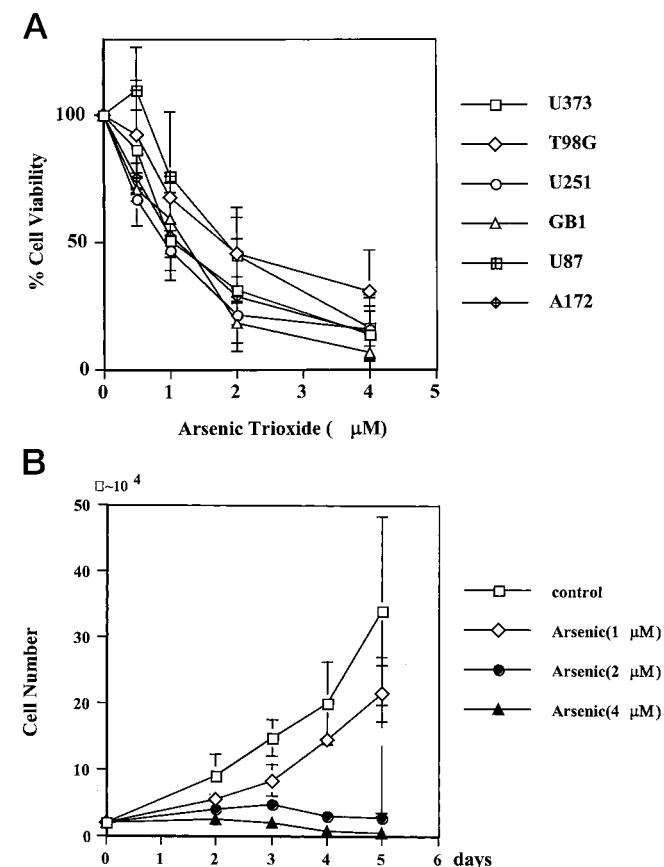


Fig. 1. Dose-dependent effect of As<sub>2</sub>O<sub>3</sub> on cell viability in malignant glioma cell lines. Actual cell numbers were counted with trypan blue dye. A, cell viability of six malignant glioma cell lines 3 days after exposure to increasing doses of As<sub>2</sub>O<sub>3</sub>. B, actual cell number of U373-MG cells nontreated or treated with 1, 2, or 4  $\mu$ M of As<sub>2</sub>O<sub>3</sub>. Data represent cell counts on days 2, 3, 4, and 5 after exposure to As<sub>2</sub>O<sub>3</sub>. Results shown are the means of three independent experiments; bars,  $\pm$ SD.

Table 1 Cell cycle changes 3 days after exposure to 2  $\mu$ M As<sub>2</sub>O<sub>3</sub>

Cell line	G <sub>1</sub> (%)	S (%)	G <sub>2</sub> /M (%)
U373			
Control	60.39	11.23	27.94
Arsenic trioxide	46.31	6.03	45.28
T98G			
Control	66.96	6.87	18.42
Arsenic trioxide	62.27	3.6	27.68
U-251			
Control	53.03	11.03	22.16
Arsenic trioxide	48.04	1.22	37.5
GB-1			
Control	48.6	8.33	27.3
Arsenic trioxide	28	5.51	56.21
U-87			
Control	73.71	12.26	11.73
Arsenic trioxide	56.78	7.21	30.93
A-172			
Control	77.46	4.55	11.3
Arsenic trioxide	61.33	2.76	29.74

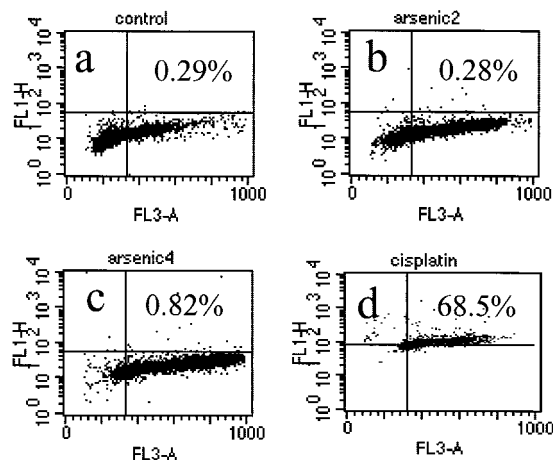


Fig. 2. FACS analysis of TUNEL staining in As<sub>2</sub>O<sub>3</sub>-treated U373-MG malignant glioma cells. Tumor cells were cultured in medium containing As<sub>2</sub>O<sub>3</sub> for 3 days, and then analyzed. a, control; b, 2  $\mu$ M-As<sub>2</sub>O<sub>3</sub> treatment; c, 4  $\mu$ M-As<sub>2</sub>O<sub>3</sub> treatment; d, cisplatin treatment used as positive control. Note that the percentage of TUNEL-positive cells after exposure to As<sub>2</sub>O<sub>3</sub> treatment is <1%.

As<sub>2</sub>O<sub>3</sub> was <2  $\mu$ M (approximately 0.90–1.85  $\mu$ M). Recent clinical studies have shown that at this concentration, bone marrow suppression and other severe side effects were not observed (3). Additionally, the cell number of U373-MG treated by As<sub>2</sub>O<sub>3</sub> (2 or 4  $\mu$ M) decreased below the initial cell number on day 4 or day 3, respectively (Fig. 1B). These data indicate that As<sub>2</sub>O<sub>3</sub> not only inhibits cell proliferation but also induces cell death.

**As<sub>2</sub>O<sub>3</sub> Induced Cell Cycle Arrest of G<sub>2</sub>/M Phase on All of the Glioma Cells.** To investigate the effect of As<sub>2</sub>O<sub>3</sub> on cell cycle, we performed DNA cell cycle analysis using FACScan. Three days after exposure to As<sub>2</sub>O<sub>3</sub> (2  $\mu$ M), the cell population in the G<sub>1</sub> and S phases of cell cycle decreased, and the G<sub>2</sub>/M population increased in all of the cell lines tested (Table 1). Additionally, This cell cycle arrest induced by As<sub>2</sub>O<sub>3</sub> was in a dose-dependent manner (data not shown).

**Detection of Apoptosis on As<sub>2</sub>O<sub>3</sub>-treated Glioma Cells.** These results show that As<sub>2</sub>O<sub>3</sub> can induce cell death associated with G<sub>2</sub>/M cell cycle arrest in malignant glioma cells. To investigate the mechanism of As<sub>2</sub>O<sub>3</sub>-induced cell death, TUNEL staining was performed on U373-MG cells. As<sub>2</sub>O<sub>3</sub>-treated cells showed only a few TUNEL-positive cells. Data quantified by FACS analysis showed a TUNEL positive rate of <1% on days 3 and 4 (data not shown) after exposure to 2 or 4  $\mu$ M of As<sub>2</sub>O<sub>3</sub> (Fig. 2, b and c). Cisplatin, used as positive control for apoptosis induction, induced apoptosis on 68.5% of

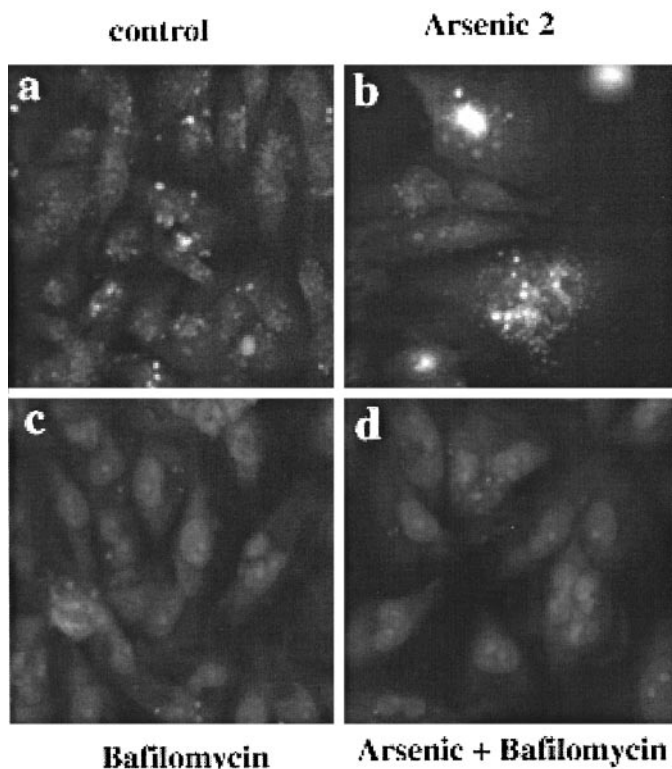


Fig. 3. Microphotograph using the supravital cell-stain acridine orange in  $2 \mu M$   $As_2O_3$ -treated U373-MG malignant glioma cells. *a*, control; *b*,  $2 \mu M$   $As_2O_3$ -treated cells; *c*, bafilomycin A1 (200 nM)-treated cells; *d*,  $2 \mu M$   $As_2O_3$  and bafilomycin A1 (200 nM)-treated cells. Note the large amount of AVOs after treatment with  $As_2O_3$  (*b*). This is consistent with autophagic changes. Acidification of AVO was inhibited by bafilomycin A1 (*d*). In control cells, the cytoplasm and nucleus basically revealed the green fluorescence, but a small accumulation of acidic component was occasionally observed (*a*). Bar (*a-d*),  $14 \mu m$ .

U373-MG treated cells. Additionally, apoptotic percentage (sub- $G_1$  population) was measured by flow cytometry in all of the cell lines. However, there was no significant increase in apoptotic percentage before and after  $As_2O_3$  treatment (Table 1). These results indicate that  $As_2O_3$  caused  $G_2/M$  arrest and cell death, but not apoptosis, in all six of the malignant glioma cell lines tested. Thus, cell death induced by  $2 \mu M$   $As_2O_3$  was not caused by apoptosis in malignant glioma cells.

**Induction of Autophagy in Malignant Glioma Cells.** Cell death by autophagy (programmed type II cell death) has been proposed recently (23, 24). Autophagy is characterized by AVO formation, which is detected and measured by vital staining of acridine orange. Acridine orange moves freely to cross biological membranes and accumulates in acidic compartment, where it is seen as fluorescence bright red (19–21). As shown in Fig. 3*b*, vital staining of U373-MG cells with acridine orange showed the accumulation of AVO in the cytoplasm of cells exposed to  $2 \mu M$   $As_2O_3$ . This was inhibited by addition of bafilomycin A1 (200 nM), an  $H^+$ ATPase inhibitor (Fig. 3, *a* and *d*).

To gain ultrastructural knowledge on the morphology of the  $As_2O_3$ -induced cell death in malignant glioma cells, we performed EM. Four days after  $As_2O_3$  treatment ( $2 \mu M$ ), U373-MG cells were collected and analyzed. Numerous AVOs were observed in the cytoplasm of U373-MG glioma cells treated with  $As_2O_3$  (Fig. 4*b*). Lamellar structures were frequently observed in many AVOs (Fig. 4*c*). AVOs were induced in  $\sim 10\%$  of cells treated with  $As_2O_3$  ( $2 \mu M$ ), which exhibited numerous vacuoles with ultrastructurally different contents, and the cell surface of the cells was characterized by loss of microvilli (Fig. 4*b*). In contrast, there were relatively few AVOs induced in the cytoplasm of control cells (Fig. 4*a*). The cytoplasmic membrane

showed numerous microvilli (Fig. 4*a*). These ultrastructural features seen in  $As_2O_3$ -treated cells indicated that the cytotoxic effect of  $As_2O_3$  was characterized by progressive AVO formation and degradation of the cytoplasmic organelles. These findings are consistent with autophagy as described previously (22, 24).

To quantify the accumulation of the acidic component, we performed FACS analysis of acridine orange-stained cells using fl3 mode ( $>650$  nm) to value the bright red fluorescence and fl1 mode (500–550 nm) to value the green fluorescence. As shown in Fig. 5*b*,  $As_2O_3$  treatment increased the strength of red fluorescence from 3.4% to 30.9%. Bafilomycin A1 decreased the strength of red fluorescence from 3.4% to 1.5% in control (Fig. 5, *a* and *c*) or from 30.5% to 12.9% (Fig. 5, *b* and *d*) in  $As_2O_3$ -treated cells. These results corroborate the observation that  $As_2O_3$  treatment induced AVOs in U373-MG cells.

**Caspase Inhibitors Do Not Prevent  $As_2O_3$ -induced Cell Death.** Whereas apoptosis is mediated by caspases, the role of caspases in autophagy is debatable (25–29). Therefore, caspase inhibition assay was performed to determine the involvement of caspase in  $As_2O_3$ -induced cell death. As shown in Fig. 6,  $50 \mu M$  z-VAD-fmk significantly increased the cell viability of U373-MG cells infected with AdCMVp53 (50 MOI) from 16.3% to 29.7% ( $P = 0.027$ ). This finding indicated that the concentration of  $50 \mu M$  was sufficient for caspase inhibitor z-VAD-fmk to suppress the activation of caspases. On the other hand,  $50 \mu M$  z-VAD-fmk did not significantly affect the cell viability of U373-MG cells treated with  $As_2O_3$  ( $2 \mu M$ ; Fig. 6). These results indicate that  $As_2O_3$ -induced cell death is independent of caspase activation. These data taken, together with EM and acridine orange results, corroborate the concept that  $As_2O_3$ -induced cell death occurs by autophagy.

**Inhibition of Autophagy and Induction of Apoptosis by Bafilomycin A1 in  $As_2O_3$ -treated Malignant Glioma Cells.** Recent data showed that bafilomycin A1, an autophagy inhibitor, suppresses autophagy in irradiated cancer cells and consequently induces apoptosis (21). Therefore, we investigated whether bafilomycin A1 has a similar effect in  $As_2O_3$ -treated glioma cells. As shown in Fig. 7, in the presence of bafilomycin A1, the sub- $G_1$  population increased from 3.8% to 22%, and the  $G_2/M$  population decreased (Fig. 7, *b* and *d*). There was no significant difference in the sub- $G_1$  population between untreated and bafilomycin A1-treated U373-MG cells (Fig. 7, *a* and *c*). These results indicated that apoptosis was induced in  $As_2O_3$ -treated tumor cells when autophagy was inhibited by bafilomycin A1.

## DISCUSSION

In this study, we have shown that  $As_2O_3$  successfully inhibited the cell proliferation of all six of the malignant glioma cell lines tested. These findings support the results of other previous studies showing similar effects on human MM cell lines (30), relapse case in APL (3), myeloma (30), human T-cell leukemia virus type 1 cells (5), and gynecological cancers (31, 32).  $As_2O_3$  showed a dose-dependent inhibition of cellular proliferation of malignant glioma cell lines. We found that the  $IC_{50}$  of  $As_2O_3$  was  $<2 \mu M$  in all of the malignant glioma cell lines tested. This is similar to what was shown in MM (14) and APL (33) studies. Clinical studies showed that at this concentration severe side effects are not seen (34). The results of our study suggest that  $As_2O_3$  may be clinically useful in patients with malignant gliomas as an adjuvant chemotherapeutic agent.

The cell cycle analysis of our study showed that  $As_2O_3$  induced a prominent cell cycle arrest in the  $G_2/M$  phase of glioma cells after their exposure to  $As_2O_3$  ( $2 \mu M$ ). These results are consistent with those of other investigators who showed that the antiproliferative effects of arsenical compounds were linked to a  $G_2/M$  phase arrest in lymphoid neoplasms (32), NB4 cells (33), and myeloma cells (14).

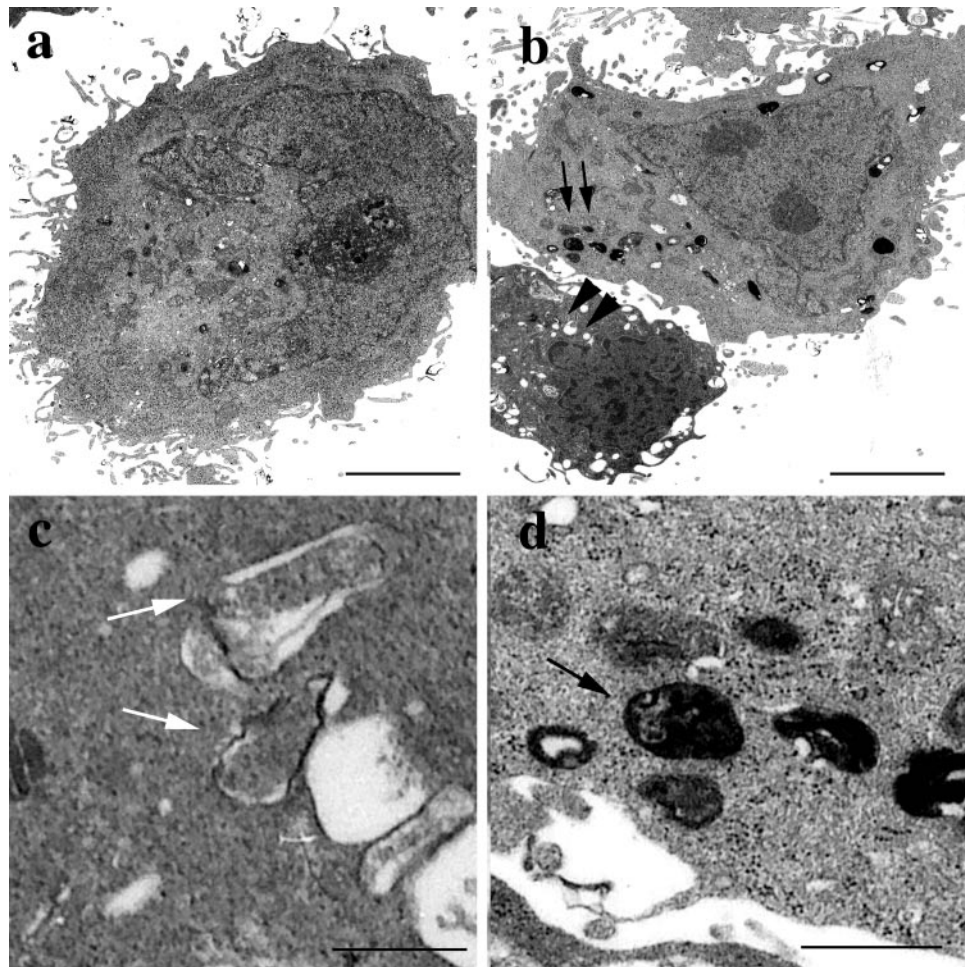


Fig. 4. EM microphotographs showing the ultrastructure of  $2 \mu M$   $As_2O_3$ -treated U373-MG malignant glioma cells. *a*, control cell; very few autophagosomes were observed in nontreated U373-MG cells. Microvilli are preserved around the cytoplasm. *b*,  $2 \mu M$   $As_2O_3$ -treated U373-MG malignant glioma cells. Note the loss of microvilli and the presence of numerous autophagosomes (arrows and arrowheads). These were characterized by different content. *c*, high magnification of typical autophagosomes (white arrows). *d*, high magnification of an autolysosome. Bars (*a* and *b*)  $5.5 \mu m$ , (*c* and *d*)  $1 \mu m$ .

Our study showed that cell death after malignant glioma cell exposure to a low concentration of  $As_2O_3$  ( $2 \mu M$ ) was not because of apoptosis. These results are consistent with those reported for prostate cancer (18). On the other hand, at higher concentration of  $As_2O_3$  (approximately  $8-16 \mu M$ ) apoptosis was induced (data not shown), similar to

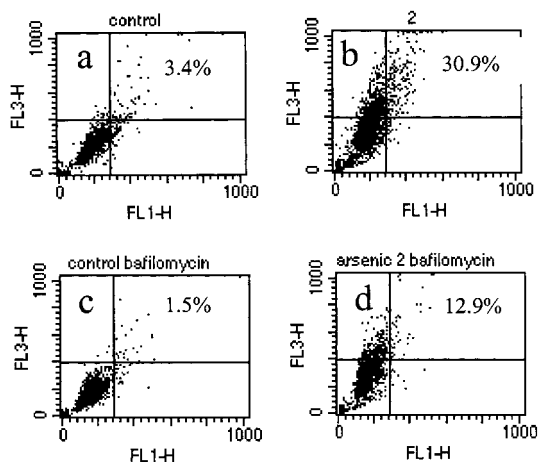


Fig. 5. Quantification of AVOs with acridine orange using FACS scan. Cells were exposed to the supravital stain acridine orange 3 days after treatment with  $As_2O_3$ . Bafilomycin was added before FACS analysis (see "Materials and Methods"). *a*, control; *b*,  $2 \mu M$ - $As_2O_3$  treatment; *c*, bafilomycin A ( $200 \text{ nm}$ ) treatment; *d*,  $2 \mu M$ - $As_2O_3$  and bafilomycin A ( $200 \text{ nm}$ ) treatment. Note that after the exposure to  $As_2O_3$ , there is an increased number of AVO in  $As_2O_3$ -treated cells (31%). AVO formation is inhibited by bafilomycin.

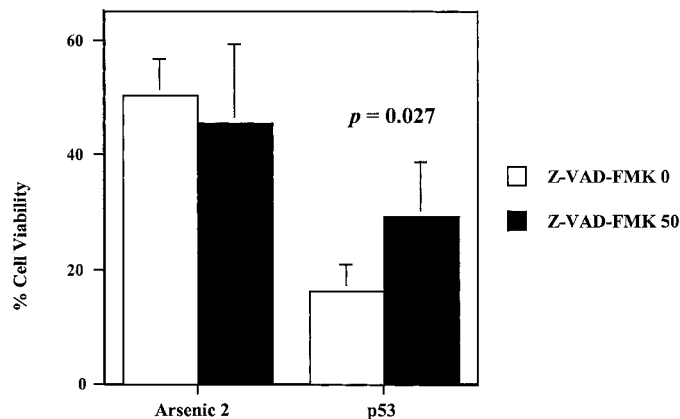


Fig. 6. Effect of pan-caspase inhibitor, z-VAD-fmk, on the cell viability of U373-MG cells treated with  $As_2O_3$ . Fifty  $\mu M$  z-VAD-fmk were added to cells 1 h before the treatment with A ( $2 \mu M$ ). After 2 days, the cell viability was determined. As a positive control of caspase-dependent apoptosis, the AdCMVp53 was used at MOI of 50. The cell viability of untreated U373-MG cells in the presence of diluted DMSO was regarded as 100%. Results shown are the means of three independent experiments; bars,  $\pm$  SD. Note that pan-caspase inhibitor z-VAD-fmk ( $50 \mu M$ ) did not prevent the induction of autophagic cell death by  $As_2O_3$ .

the results shown in myeloma (14) and prostate cancer (18). In our study, we used the supravital stain acridine orange and EM to elucidate the molecular mechanisms involved in cell death of human brain tumor after exposure to low doses of  $As_2O_3$ . We found that glioma cells exposed to low doses of  $As_2O_3$  showed AVO development in the

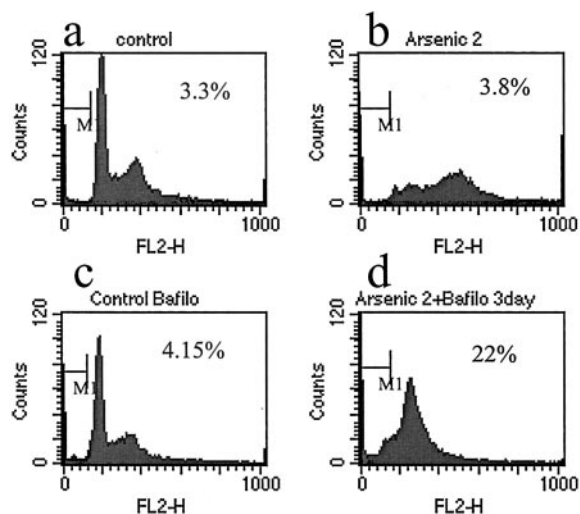


Fig. 7. Effect of  $As_2O_3$  and bafilomycin A1 on AVO and apoptosis. *a*, cells were exposed to acridine orange 3 days after treatment with  $As_2O_3$ . Bafilomycin A1 was added before the FACS analysis as described in "Materials and Methods." *a*, control; *b*, 2  $\mu M$ - $As_2O_3$  treatment; *c*, bafilomycin A (200 nM) treatment; *d*, 2  $\mu M$ - $As_2O_3$  and bafilomycin (200 nM) treatment. Note that after exposure to  $As_2O_3$ , there is an increased number of AVO in  $As_2O_3$ -treated cells (30.9%). Acidification of AVO is inhibited by bafilomycin A1 (12.9%). *b*, the sub- $G_1$  population (M1) was calculated using Cell Quest Software. *a*, control; *b*, 2  $\mu M$ - $As_2O_3$  treatment; *c*, bafilomycin A1 (5 nM) treatment; *d*, 2  $\mu M$ - $As_2O_3$  and bafilomycin A1 (5 nM) treatment. Note that there is a significant increase in the sub- $G_1$  population (22%) after exposure to bafilomycin A1 and  $As_2O_3$ .

cytoplasm before undergoing nuclear changes. Taken together, these findings support the evidence that human glioma cell death after exposure to low doses of  $As_2O_3$  occurs by autophagy. Several investigators have recently proposed two types of programmed cell death (23, 24). Type I, apoptosis, is mediated by a cascade of proteins from the caspase family and factors released by mitochondria. In contrast, type II programmed cell death, autophagy, is characterized by the presence of AVO formation in the cell cytoplasm (35). This leads to disruption of cytoplasm organelles before nuclear collapse (24). Autophagy has been documented in human breast carcinoma cells after treatment with ionizing radiation (21) or chemotherapeutic drugs (30). In our study, we demonstrated that after treatment with low doses of  $As_2O_3$  the AVO formation occurred as documented by EM and occupied the cytoplasm before nuclear changes. Additional support to the fact that autophagy caused cell damage after exposure to  $As_2O_3$  was found when we demonstrated that inhibition of caspase did not alter  $As_2O_3$ -induced cell death. Finally, we corroborated the mechanism of  $As_2O_3$ -induced cell death by using bafilomycin A1, an autophagy inhibitor. It has been suggested that AVO formation and digestion of material in the AVO are dependent on acidification of cellular organelles. Bafilomycin A1 specifically inhibits AVO formation and digestion of material in the AVO in the process of autophagy (36). In our study, we showed that after exposure to bafilomycin A1 apoptosis occurred. Thus, after exposure to low doses of  $As_2O_3$  and bafilomycin A1, autophagy was inhibited and apoptosis occurred alternatively.

In conclusion, we showed that  $As_2O_3$  produces *in vitro* growth inhibition,  $G_2/M$  cell cycle arrest, and cell death in six glioma cell lines at a concentration of 2  $\mu M$ . We have demonstrated that the cytotoxic effects after exposure to low-concentration  $As_2O_3$  is caused by autophagy. However, when autophagy is inhibited, apoptosis occurs. These findings suggest that  $As_2O_3$  should be additionally investigated as a potential novel chemotherapeutic agent for the adjuvant treatment of malignant human gliomas.

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