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

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ABSTRACT

Recent clinical data shows that arsenic trioxide (\(\text{As}_2\text{O}_3\)) causes remission in patients with acute promyelocytic leukemia and multiple myeloma without severe side effects. Laboratory data suggest that \(\text{As}_2\text{O}_3\) induces apoptosis or cell differentiation of hematopoietic or solid tumor cells. To date, there has been no study on the effects of \(\text{As}_2\text{O}_3\) on glioma cells. In this study, we investigated the in vitro effect of \(\text{As}_2\text{O}_3\) on cell growth inhibition and cell death mechanisms in human glioma cells. \(\text{As}_2\text{O}_3\) 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\(_{50}\) of \(\text{As}_2\text{O}_3\) for all of the tumor cell lines was <2 \(\mu\text{M}\). Previous studies have shown that this is a clinically safe concentration. Treatment with 2 \(\mu\text{M} \text{As}_2\text{O}_3\) induced G2/M arrest in all of the glioma cell lines. Apoptophagy (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 \(\mu\text{M} \text{As}_2\text{O}_3\). Caspase inhibitors did not halt \(\text{As}_2\text{O}_3\)-induced cell death. Furthermore, combination of \(\text{As}_2\text{O}_3\) with bafilomycin A1 autophagy inhibitor enhanced the antitumor effect of \(\text{As}_2\text{O}_3\) through induction of apoptosis. These findings suggest that \(\text{As}_2\text{O}_3\) at a clinically safe concentration may be an effective chemotherapeutic agent for malignant gliomas.

INTRODUCTION

Low concentrations (≤2 \(\mu\text{M}\)) of \(\text{As}_2\text{O}_3\)\(^2\) 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 \(\text{As}_2\text{O}_3\) 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 \(\text{As}_2\text{O}_3\)-induced apoptosis in MM cells (13, 14) and solid tumors (15). Although the mechanism of \(\text{As}_2\text{O}_3\)-induced cell death are still under investigation, it has been suggested that \(\text{As}_2\text{O}_3\) 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 \(\text{As}_2\text{O}_3\)-induced apoptosis in esophageal carcinoma cells (17). Moreover, a recent study showed that \(\text{As}_2\text{O}_3\) provides a novel, safe approach for treatment of androgen-independent prostate cancer, activating p38, c-Jun NH\(_2\)-terminal kinase, and caspase-3 (18). These studies suggest that \(\text{As}_2\text{O}_3\) is a safe and promising agent for nonhematopoetic cancer treatment. However, the effects of \(\text{As}_2\text{O}_3\) 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 \(\text{As}_2\text{O}_3\) at low concentration (2 \(\mu\text{M}\)) on malignant glioma cells. Our data show that \(\text{As}_2\text{O}_3\) 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, \(\text{As}_2\text{O}_3\) seems to a promising agent for additional investigations in the treatment of malignant gliomas.

MATERIALS AND METHODS

Reagents. \(\text{As}_2\text{O}_3\) 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 \(\mu\text{g}\)/ml streptomycin.

Growth Inhibition Assay. In vitro growth effect of \(\text{As}_2\text{O}_3\) on glioma cell lines was determined by counting the viable cells with trypan blue staining. Briefly, cells (5 × 10\(^3\) cells/well) were seeded in 96-well plate and preincubated overnight. After exposure of various concentrations of the \(\text{As}_2\text{O}_3\) 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 FACSscan (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. \(\text{As}_2\text{O}_3\)-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 FACSscan as described previously.

To detect apoptosis in combination with bafilomycin A1, U373-MG cells were cultured in medium containing 2 \(\mu\text{M} \text{As}_2\text{O}_3\) for 24 h, and then 5 nm of bafilomycin A1 was added in culture medium. After 72 h, tumor cells were collected and stained with propidium iodide. The percentage of sub-G\(_1\) population was determined by flow cytometry.

Supravitral 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 (Axioskop) 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. The intensity of the red fluorescence is proportional to the degree of acidity. Therefore, the volume of the cellular acidic compartment can be quantified...
(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 × 10^5 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 OsO₄, and embedded in Epon. One-µm thin sections were cut, stained with methylene buffer ArumêII, 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 As₂O₃-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 × 10^5 cells/well) were seeded in 96-well plate and incubated overnight at 37°C. As a positive control for apoptosis induction, induced apoptosis on 68.5% of U373-MG cells treated by z-VAD-fmk (28). As a positive control; induced apoptosis on 68.5% of U373-MG cells treated by z-VAD-fmk (28). As a positive control; induced apoptosis on 68.5% of U373-MG cells treated by z-VAD-fmk (28).

**RESULTS**

**Effect of As₂O₃ on Proliferation of Malignant Glioma Cells.** To determine whether As₂O₃ has a therapeutic effect on glioma cells, we performed the cell viability assay using six human glioma cell lines. As₂O₃ significantly decreased the cell viability of all six of the glioma cell lines tested in a dose dependent manner (Fig. 1A). The IC₅₀ of As₂O₃ was <2 µM (approximately 0.90–1.85 µM). 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 As₂O₃ (2 or 4 µM) decreased below the initial cell number on day 4 or day 3, respectively (Fig. 1B). These data indicate that As₂O₃ not only inhibits cell proliferation but also induces cell death.

**As₂O₃ Induced Cell Cycle Arrest of G₂/M Phase on All of the Glioma Cells.** To investigate the effect of As₂O₃ on cell cycle, we performed DNA cell cycle analysis using FACSscan. Three days after exposure to As₂O₃ (2 µM), the cell population in the G₁ and S phases of cell cycle decreased, and the G₂/M population increased in all of the cell lines tested (Table 1). Additionally, this cell cycle arrest induced by As₂O₃ was in a dose-dependent manner (data not shown).

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**Table 1: Cell cycle changes 3 days after exposure to 2 µM As₂O₃**

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<thead>
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<th>Cell line</th>
<th>G₁ (%)</th>
<th>S (%)</th>
<th>G₂/M (%)</th>
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<td>1.22</td>
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<tr>
<td>Arsenic trioxide</td>
<td>61.33</td>
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<td>29.74</td>
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Fig. 1. Dose-dependent effect of As₂O₃ on cell viability in malignant glioma cell lines. Actual cell numbers were counted with trypan blue dye. A: viability of six malignant glioma cell lines 3 days after exposure to increasing doses of As₂O₃. B: actual cell number of U373-MG cells nontreated or treated with 1, 2, or 4 µM of As₂O₃. Data represent cell counts on days 2, 3, 4, and 5 after exposure to As₂O₃. Results shown are the means of three independent experiments; bars, ±SD.

Fig. 2. FACS analysis of TUNEL staining in As₂O₃-treated U373-MG malignant glioma cells. Tumor cells were cultured in medium containing As₂O₃ for 3 days, and then analyzed. a: control; b: 2 µM-As₂O₃ treatment; c: 4 µM-As₂O₃ treatment; d: cisplatin treatment used as positive control. Note that the percentage of TUNEL-positive cells after exposure to As₂O₃ treatment is <1%.

As₂O₃ is a potential therapeutic agent for glioma treatment, as it selectively induces cell death in malignant glioma cells with minimal effect on normal brain cells. The cellular and molecular mechanisms underlying As₂O₃-induced cell death are being actively investigated to optimize its clinical use.
The addition of bafilomycin A1 (200 nM), an H+/ATPase inhibitor, caused G2/M arrest and cell death, but not apoptosis, in all six malignant glioma cell lines tested. Thus, cell death induced by arsenic trioxide was characterized by progressive AVO formation and degradation of the cytoplasm 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. 5b, As2O3 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 As2O3-treated cells. These results corroborate the observation that As2O3 treatment induced AVOs in U373-MG cells.

Caspase Inhibitors Do Not Prevent As2O3-induced Cell Death.

Whereas apoptosis is mediated by caspases, the role of caspases in autophagy is debatable (25–29). Therefore, caspase inhibition assays were performed to determine the involvement of caspase in As2O3-induced cell death. As shown in Fig. 6, 50 μM 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 μM was sufficient for caspase inhibitor z-VAD-fmk to suppress the activation of caspases. On the other hand, 50 μM z-VAD-fmk did not significantly affect the cell viability of U373-MG cells treated with As2O3 (2 μM; Fig. 6). These results indicate that As2O3-induced cell death is independent of caspase activation. These data, taken together with EM and acridine orange results, corroborate the concept that As2O3-induced cell death occurs by autophagy.

Inhibition of Autophagy and Induction of Apoptosis by Bafilomycin A1 in As2O3-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 on As2O3-treated glioma cells. As shown in Fig. 7, in the presence of bafilomycin A1, the sub-G1 population increased from 3.8% to 22%, and the G2/M population decreased (Fig. 7, b and d). There was no significant difference in the sub-G1 population between untreated and bafilomycin A1-treated U373-MG cells (Fig. 7, a and c). These results indicated that apoptosis was induced in As2O3-treated tumor cells when autophagy was inhibited by bafilomycin A1.

DISCUSSION

In this study, we have shown that As2O3 successively 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). As2O3 showed a dose-dependent inhibition of cellular proliferation of malignant glioma cell lines. We found that the IC50 of As2O3 was <2 μM 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 As2O3 may be clinically useful in patients with malignant gliomas as an adjuvant chemotherapeutic agent.

The cell cycle analysis of our study showed that As2O3 induced a prominent cell cycle arrest in the G2/M phase of glioma cells after their exposure to As2O3 (2 μM). These results are consistent with those of other investigators who showed that the antiproliferative effects of arsenical compounds were linked to a G2/M phase arrest in lymphoid neoplasms (32), NB4 cells (33), and myeloma cells (14).
Our study showed that cell death after malignant glioma cell exposure to a low concentration of As$_2$O$_3$ (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$_2$O$_3$ (approximately 8–16 $\mu$M) apoptosis was induced (data not shown), similar to 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$_2$O$_3$. We found that glioma cells exposed to low doses of As$_2$O$_3$ showed AVO development in the

![Fig. 4. EM microphotographs showing the ultrastructure of 2 $\mu$M As$_2$O$_3$-treated U373-MG malignant glioma cells. a, control cell; very few autophagical vacuoles were observed in nontreated U373-MG cells. Microvilli are preserved around the cytoplasm. b, 2 $\mu$M As$_2$O$_3$-treated U373-MG malignant glioma cells. Note the loss of microvilli and the presence of numerous autophagical vacuoles (arrows and arrowheads). These were characterized by different content. c, high magnification of typical autophagical vacuoles (white arrows). d, high magnification of a autolysosome. Bars (a and b) 5.5 $\mu$m, (c and d) 1 $\mu$m.](image)

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![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$_2$O$_3$. Bafilomycin was added before FACS analysis (see “Materials and Methods”). a, control; b, 2 $\mu$M-As$_2$O$_3$ treatment; c, bafilomycin A (200 nM) treatment; d, 2 $\mu$M-As$_2$O$_3$ and bafilomycin A (200 nM) treatment. Note that after the exposure to As$_2$O$_3$, there is an increased number of AVO in As$_2$O$_3$-treated cells (31%). AVO formation is inhibited by bafilomycin.](image)

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![Fig. 6. Effect of pan-caspase inhibitor, z-VAD-fmk, on the cell viability of U373-MG cells treated with As$_2$O$_3$. 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 AdCMV-p53 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, ± SD. Note that pan-caspase inhibitor z-VAD-fmk (50 $\mu$M) did not prevent the induction of autophagic cell death by As$_2$O$_3$.](image)

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In our study, we demonstrated that after treatment with low doses of As$_2$O$_3$, bafilomycin A1 was added before the FACS analysis as described in “Materials and Methods.” a, control; b, 2 μM-As$_2$O$_3$ treatment; c, bafilomycin A (200 nM) treatment; d, 2 μM-As$_2$O$_3$ and bafilomycin (200 nM) treatment. Note that after exposure to As$_2$O$_3$, there is an increased number of AVO in As$_2$O$_3$-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 μM-As$_2$O$_3$ treatment; c, bafilomycin A1 (5 μM) treatment; d, 2 μM-As$_2$O$_3$ and bafilomycin A1 (5 μM) treatment. Note that there is a significant increase in the sub-G$_1$ population (22%) after exposure to bafilomycin A1 and As$_2$O$_3$.

cytoplasm before undergoing nuclear changes. Taken together, these findings support the evidence that human glioma cell death after exposure to low doses of As$_2$O$_3$ occurs by autophagy. Several investigators have recently proposed two types of programmed cell death (24). Type I, apoptosis, is mediated by a cascade of proteins from the caspase family and factors released by mitochonddria. 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$_2$O$_3$, 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$_2$O$_3$ was found when we demonstrated that inhibition of caspase did not alter As$_2$O$_3$-induced cell death. Finally, we corroborated the mechanism of As$_2$O$_3$-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$_2$O$_3$ and bafilomycin A1, autophagy was inhibited and apoptosis occurred alternatively.

In conclusion, we showed that As$_2$O$_3$ produces in vitro growth inhibition, G$_2$/M cell cycle arrest, and cell death in six glioma cell lines at a concentration of 2 μM. We have demonstrated that the cytotoxic effects after exposure to low-concentration As$_2$O$_3$ is caused by autophagy. However, when autophagy is inhibited, apoptosis occurs. These findings suggest that As$_2$O$_3$ should be additionally investigated as a potential novel chemotherapeutic agent for the adjuvant treatment of malignant human gliomas.

**Acknowledgments**

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**References**


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