Tumor Growth Inhibition by Arsenic Trioxide (As$_2$O$_3$) in the Orthotopic Metastasis Model of Androgen-independent Prostate Cancer

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ABSTRACT

Arsenic trioxide (As$_2$O$_3$) induces clinical remission of patients with acute promyelocytic leukemia. As a novel anticancer agent for treatment of solid cancers, As$_2$O$_3$ is promising, but no in vivo experimental investigations of its efficacy on solid cancers have been done at clinically obtained concentrations. In addition, the cell death mechanism of As$_2$O$_3$ has yet to be clarified, especially in solid cancers. In this study, human androgen-independent prostate cancer cell lines, PC-3, DU-145, and TSU-PRI were examined as cellular models for As$_2$O$_3$ treatment, and As$_2$O$_3$-induced cell death and inhibition of cell growth and colony formation were evaluated. The involvement of p38, c-Jun NH$_2$-terminal kinase (JNK), caspase-3, and reactive oxygen species (ROS) were investigated in As$_2$O$_3$-induced cell death. Finally, As$_2$O$_3$ was administered to severe combined immunodeficient mice inoculated orthotopically with PC-3 cells to estimate in vivo efficacy. In all three of the cell lines, at high concentrations, As$_2$O$_3$ induced apoptosis and, at low concentrations, growth inhibition. As$_2$O$_3$ activated p38, JNK, and caspase-3 dose dependently. Treatment with the p38 inhibitor and over-expression of dominant-negative JNK did not guard against As$_2$O$_3$-induced cell death. In contrast with partial protection by the caspase-3 inhibitor, the antioxidant N-acetyl-l-cysteine gave marked protection from As$_2$O$_3$-induced apoptosis and eliminated the activation of p38, JNK, and caspase-3, and the generation of ROS. The orthotopic murine metastasis model showed in vivo tumor growth inhibition in orthotopic and metastatic lesions with no signs of toxicity. This study establishes that As$_2$O$_3$ provides a novel, safe approach for treatment of androgen-independent prostate cancer. Generation of ROS as a therapeutic target for the potentiation of As$_2$O$_3$-induced apoptosis also was shown.

INTRODUCTION

In the United States, prostate cancer is the most common solid cancer in men and the second cause of death. Many forms of prostate cancer initially are androgen dependent, but the response to androgen-ablation therapy is transient, and, after a few years, the majority of prostate cancers relapse to the status of androgen independence, resulting in death. Despite the availability of various therapeutic approaches (1, 2), none has provided a marked survival advantage for patients in the androgen-independent stage of prostate cancer. Novel therapies for androgen-independent prostate cancer, therefore, are urgently needed.

Development of cancer therapies depends on animal models that reproduce the clinical metastases that frequently affect the prognosis and quality of life of patients. Cancer cells have been injected into immunodeficient mice to reproduce cancer metastasis in animal models. Two major routes for this injection have been commonly used: i.v. or s.c. Ectopic models, however, have several disadvantages. For example, the i.v. injection model leaves out invasion, the initial, critical step in cancer metastasis, and in the s.c. injection model, the low incidence of metastasis limits assessment of therapeutic efficacy. Over the past decade, orthotropic inoculation models have been developed to overcome these disadvantages. Nowadays, several orthotopic models have succeeded in reproducing a high incidence of the metastasis similar to that observed in clinical cancers (3). Likewise in prostate cancer, the orthotopic model provides a high incidence of metastasis to the lymph nodes, lungs, and bones, the major targets of metastasis in clinical cancers (4–6). Orthotopic models, therefore, seem very useful for the development of effective therapies and are expected to become the new standard method (3), although the real significance of the drug efficacies of therapies developed from these in vivo models in cancer research will remain to be validated through the clinical studies.

A recent epoch-making advance in cancer treatment is the ATRA treatment, which induces complete remission in most cases of APL (7, 8), but, thereafter, there are many cases of recurrence that result in death. For the treatment of this recurrent APL after ATRA treatment, As$_2$O$_3$ in the pharmacological range below 2 µM is reported to be dramatically effective (9). Since the discovery of As$_2$O$_3$-induced cell death, its molecular mechanisms have been extensively studied, mainly in hematological cancers. The mechanisms of ATRA and As$_2$O$_3$ differ. ATRA induces differentiation in APL cells, whereas As$_2$O$_3$ mainly induces apoptosis (10).

Until now, As$_2$O$_3$-induced apoptosis has been approached in three major apoptotic mechanisms by using pharmacological inhibitors: MAPKs, caspase, and ROS. MAPKs include JNK, p38, and ERK (11–13). Of these, JNK and p38 belong to the SAPKs and have been investigated in As$_2$O$_3$-induced apoptosis (11, 12). An APL cell line, NB4, also undergoes As$_2$O$_3$-induced apoptosis through activation of the ASK1-SEK1-JNK kinase cascade in PML bodies (4). The second mechanism in As$_2$O$_3$-induced apoptosis has been postulated to be mediated through the cascade reaction of caspases, a family of aspartate-specific cysteine proteases (14, 15). Functionally, these caspases are divided into two subgroups: initiator (caspase-8, -9, and -10) and effector (caspase-3, -6, and -7) caspases (16). Of the effector caspases, caspase-3 is divided into two main molecules, and the other effectors, caspase-6 and -7, are called caspase-3-like caspases generically (16). The last mechanism involves ROS such as hydrogen peroxide, superoxide, hydroxyl radicals, all-trans-retinoic acid; APL, acute promyelocytic leukemia; MAPK, Mitogen-activated protein kinase; ROS, reactive oxygen species; JNK, c-Jun NH$_2$-terminal protein kinase; ERK, extracellular signal-regulated kinase; SAPK, stress-activated protein kinases; ASK1, apoptosis-signaling regulating kinase; SEK1, SAPK-ERK kinase; PML, promyelocytic leukemia; z-DEVD-fmk, carbobenzoxyl-Asp-Glu-Val-Asp-fluoromethane; NAC, N-acetyl-l-cysteine; WST-8, 2-(2-methoxy-4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt; TUNEL, terminal deoxynucleotidyl transferase-mediated nucleotide nick-end labeling; DAPI, 4′,6-diamidino-2-phenylindole; MKK, MAPK kinase; EGFP, enhanced GFP; 7-ADD, 7-aminoactinomycin D; Ac-DEVD- AFC, acetyl-Asp-Glu-Val-Asp-amido-4-trifluoromethyl coumarin; CM-H$_2$DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; SCID, severe combined immunodeficient; GFP, green fluorescent protein.

radicals, and nitric oxide (17). In healthy organisms, ROS are inevitably generated through a respiratory chain of mitochondria but are scavenged by antioxidant defense systems. When this system is compromised, oxidative stress is considered to produce senescence and various diseases including cancer, inflammation, and neurodegenerative disorder. Recently, the generation of ROS has been reported to regulate As2O3-induced apoptosis (15, 18, 19). Among these three mechanisms suggested in As2O3-induced apoptosis, the most critical mechanism in As2O3-induced apoptosis and the interactions between the mechanisms are still not clear, especially in solid cancers.

We used in vitro assays of androgen-independent prostate cancer cell lines and found that As2O3 induces apoptosis at high concentrations, and it inhibits growth at low ones. Analysis of intracellular signaling showed that the inhibition of ROS generation protected androgen-independent prostate cancer cells from As2O3-induced cell death, whereas the inhibition of SAPKs and caspase did not. In in vivo experiments that used the murine orthotopic metastasis model of human androgen-independent prostate cancer, treatment with As2O3 inhibited tumor growth in both orthotopic and lymph nodal metastatic lesions. This treatment gave no signs of toxicity to major organs. These findings establish that As2O3 is a safe, promising treatment for androgen-independent prostate cancer and show that the ROS-scavenging system is a therapeutic target for the potentiation of As2O3-induced apoptosis.

**MATERIALS AND METHODS**

**Reagents.** As2O3 and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions of As2O3 were diluted in PBS at 6.4 mg/ml for the in vitro and 50 mg/ml for the in vivo experiments. SB203580 and z-DEVD-fmk were purchased from Calbiochem (La Jolla, CA). NAC was obtained from Nacalai tesque (Kyoto, Japan).

**Cell Culture and Cell Viability.** Cell lines PC-3, DU-145, and TSU-PR1 were cultured in RPMI 1640 plus 10% FCS. A growth inhibition assay was carried out to determine the growth inhibition effect of As2O3 using a cell-counting kit (Nacalai tesque). A cell proliferation assay modified from the MTT assay, in which WST-8, a tetrazolium salt, is used as the substrate (20). Briefly, a CellTiter 96 Aqueous Cell Proliferation Assay kit (Promega, Madison, WI) was added to the culture medium, and the absorbance of each well was determined in a microplate reader by the absorbance spectrophotometry at the wavelength of 450 nm. The assay gave an absorbance that correlated linearly with the number of cells and was not affected by As2O3 itself (data not shown). Cell growth was expressed as a percentage of the absorbance in the vehicle-treated control wells. Dose-response curves were drawn, and IC50, the concentration that inhibits 50% of the growth of control cells, were both calculated by the computer software, PRISM (GraphPad, San Diego, CA). These inhibitors were added 2 h before the addition of As2O3 in experiments with the enzyme inhibitors or NAC.

**Colonies Formation Assay.** Exponentially growing PC-3 cells (3 x 103 cells/well) in RPMI 1640–0.3% agar containing various concentrations of As2O3 (0, 0.5, 1.25, 5, and 10 μM) were layered in 6-well culture dishes on top of a basal 0.6% agar layer containing the same concentrations of As2O3. Triplicate tests were made for each concentration. Three weeks later, cell colonies were stained with 1 ml of 1% Nile red in methanol. Colonies larger than 60 μm2 were counted under a phase-contrast microscope, and the results expressed as percentages of the control.

**Assays for Apoptosis Detection.** Apoptosis was determined three ways. For nuclear morphology and mitochondrial transmembrane potential, cells that had been cultured on glass-bottom dishes were incubated with 1 μg/ml HOECHST 33342 and 10 μg/ml rhodamine 123 (Sigma Chemical Co.) at 37°C for 30 min and then was observed under a fluorescent microscope. For DNA flow cytometry, cells were trypsinized, washed with ice-cold PBS, fixed in 70% ethanol, and stored at 4°C for 60 min. After another wash with PBS, cells were incubated with 100 μg/ml DNA-free RNase at 37°C for 30 min and then stained at 4°C for 10 min with 50 μg/ml of propidium iodide. DNA-propidium iodide fluorescence was measured with FACSscan (Becton Dickinson, San Jose, CA) with respective excitation and emission wavelengths of 488 and 620 nm. TUNEL assays were performed with an In Situ Cell Death Detection kit, Fluorescein (Roche Molecular Biochemicals, Mannheim, Germany) according to the vendor’s protocol with minor modifications.

**Western Blotting.** One x 106 cells were incubated for the period indicated, harvested, and lysed in 150 mM NaCl, 1.0% NP40, 0.1% SDS, 0.5% deoxycholate 12 mM β-glycerophosphate disodium salt hydrate, 1 mM sodium dihydrogenphosphate dihydrate, 5 mM sodium fluoride, 15 μg/ml aprotinin, 2 mM diithiothereitol, 1 mM phenylmethylsulfonl fluoride, and 1 mM sodium vanadate. Protein sample (10 μg each) were boiled through a standard method. Each blot was treated with primary antibodies purchased from New England Biolabs, Inc. (Beverly, MA) as follows: anti-phospho-specific p38 (Thr180/Tyr182), anti-p38, anti-phospho-specific SAPK/JNK (Thr183/Tyr185), anti-SAPK/JNK, anti-phospho-specific ERK1/2 (Ser217/221), anti-ERK1/2, anti-phospho-specific ATF2 (Thr71), anti-ATF2, anti-phospho-specific c-Jun (Ser73), anti-c-Jun, anti-phospho-specific MKK3/6 (Ser189/207), anti-MKK3, and anti-phospho-specific SEK1/MKK4 (Thr223). Anti-SEK1/MKK4 and anti-ASK1 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Primary antibody was detected by horseradish peroxidase-conjugated antibody (1:2000). Signals were detected by the enhanced chemiluminescence detection system (ECL; Amersharm Pharmacia Biotech).

**Immunocytochemistry.** After incubation with 50 μM As2O3 for 4 h in a slide chamber, cells were fixed for 10 min in 4% formaldehyde, treated for three min with 0.2% Triton X-100 in PBS for permeability, blocked for 30 min with 1% normal goat serum in PBS, then incubated at 4°C overnight with the first antibodies (1:1000 dilution): a mouse monoclonal anti-PML (Santa Cruz Biotechnology), a rabbit polyclonal anti-ASK1(Santa Cruz Biotechnology), and a rabbit polyclonal anti-phospho-specific SEK1/MKK4 (Thr223). New England Biolabs, Inc). Subsequently, the cells were incubated at room temperature for 3 h with Texas Red- or FITC-conjugated secondary antibody (1:500 dilution; Vector Laboratories) then were mounted in antifade solution with DAPI (Vector Laboratories). Labeled cells were analyzed by laser confocal microscopy.

**Plasmids.** Rat dominant-negative SAPK/JNK was made by substitution of the phosphorylation sites Thr-183 and Tyr-185, respectively, with Val and Phe. For pGFP-DN-SAPK/JNK, an entire DNA fragment encoding BirBII was excised from pCDL-DR-DN-SAPK/JNK and subcloned into the BirBII site located 3’ to the GFP coding region of pEGFPc1 (Clontech, Palo Alto, CA). The resulting plasmid was named pGFP-DN-SAPK/JNK. The plasmid that encodes the dominant-negative SEK, pGFP-DNSEK1 has been described previously (21).

**Transfection and Cell Death Assays.** DNA transfection was done with Lipofectamine Plus (Life Technology), 3.5 x 103 cells in a six-well dish being transfected with 1.0 μg of pEGFP vector containing each CDNA. Twenty-four h later, the medium with or without As2O3 was changed. Forty-eight h after transfection, cell death was determined by flow cytometry analysis with 7-AAD (Molecular Probe, Eugene, OR). 7-AAD (10 μg/ml) was used instead of propidium iodide because of better discrimination from the fluorescence of EGFP. Fluorescences of DNA-7-AAD and EGFP were measured with FACScan, using excitation and emission wavelengths of 488 and 620 nm, and 488 and 530 nm, respectively. The cell death rate was determined by dividing the number of 7-AAD- and EGFP-positive cells by the number of EGFP-positive cells.

**Caspase-3-like Cleavage Activity Assay.** PC-3 cells were incubated with As2O3 for 8 h at 37°C after a 2-h pretreatment with 10 mM NAC, 20 μM z-DEVD-fmk and 20 μM SB203580. The caspase-3-like cleavage activity assay was done with ApoAlert caspase assay kits (Clontech) according to the manufacturer’s protocol. For the positive control, cells were treated for 8 h with anti-APO-1/Fas mouse monoclonal antibody (Bender MedSystems) after a 2-h pretreatment with 2.5 μg/ml cycloheximide with or without 20 μM z-DEVD-fmk.

**Assays for ROS Detection.** Intracellular ROS accumulation was monitored with CM-H2DCFDA (Molecular Probes), which passively diffuses into cells and then is decetylated by intracellular esterases. Hydrolyzed, oxidized CM-H2DCFDA emits green fluorescence at 520 nm. Briefly, after treatment with various concentrations of As2O3 and NAC, cells were incubated at 37°C.
for 30 min with serum-free medium containing 10 μM CM-H_{2}DCFDA and then were observed under a fluorescent microscope. At the same time, fluorescence was monitored by FACScan with excitation at 488 nm and emission at 530 nm.

**In Vivo Murine Studies.** The in vivo therapeutic effect of As_{2}O_{3} was evaluated in the orthotopic inoculation mouse model as reported previously (6). Twenty-four 10-week-old male SCID mice (BALB/c; Charles River Japan Inc., Tokyo, Japan) were maintained in a specific, pathogen-free environment and handled in accordance with the Guidelines for Animal Experiments of Kyoto University. For the orthotopic inoculation, a transverse incision was made in the lower abdomen under i.p. anesthesia with pentobarbital sodium salt. After the abdominal wall muscles were split, the bladder and seminal vesicles were exposed then retracted anteriorly to reveal the dorsal prostate. Five × 10^{5} PC-3 cells suspended in 20 μl of medium were carefully inoculated under the prostate capsule. Mice were divided into three subgroups according to the daily dose: Group 1 (n = 8), saline alone; Group 2 (n = 8), 2 mg/kg As_{2}O_{3}; Group 3 (n = 8), 5 mg/kg As_{2}O_{3}. The stock solution of As_{2}O_{3} was diluted with physiological saline, i.p. administration was carried out from 3 days after surgery and continued every day for 32 days. Five weeks after inoculation, the mice were killed by deep anesthesia. Body weight measurement and blood sampling by cardiac aspiration were done immediately after inoculation, the samples were made in the lower abdomen under i.p. anesthesia with pentobarbital sodium salt.

**RESULTS**

**In Vitro Inhibition of Cell Growth and Colony Formation in Androgen-independent Prostate Cancer Cells Treated with As_{2}O_{3}.** At the clinically obtainable concentration of 2 μM As_{2}O_{3}, in vitro growth inhibition (58–70% of the control) was induced in all three of the androgen-independent prostate cancer cell lines (Fig. 1A). Sensitivity to As_{2}O_{3} varied with the cell line, the respective IC_{50}s of the TSU-PR1, PC-3, and DU-145 cell lines being 2.4, 2.5, and 4.8 μM. In the PC-3 cell colony formation assay, anchorage-independent growth was inhibited by As_{2}O_{3} dose dependently (Fig. 1B). Remarkable inhibition (60% that of the control) was induced at 2 μM As_{2}O_{3}, and the IC_{50} was 3.3 μM (Fig. 1B).

**Fig. 2. In vitro induction of apoptosis in PC-3 cells by As_{2}O_{3}.** In A, cells were incubated with [HoList 33342 (1 μg/ml) and the potential-sensitive probe rhodamine 123 (10 μg/ml) to make the nuclear morphology and mitochondrial membrane potential visible; a and b, untreated; c and d, treated with As_{2}O_{3} (50 μM, 8 h). In B, floating cells stained by the TUNEL method were collected on the slide glass by the use of a cytopin, and mounted in antifade solution with DAPI; a and b, untreated; c and d, treated with As_{2}O_{3} (20 μM, 36 h).
Fig. 3. As$_2$O$_3$-induced apoptosis and growth inhibition in PC-3 cells shown by flow cytometry analysis. A, dose-response of As$_2$O$_3$-induced apoptosis detected by DNA flow cytometry analysis. Sub-G$_1$ fractions increased with the increase in the As$_2$O$_3$. B, dose-response of As$_2$O$_3$-induced apoptosis detected by flow cytometry analysis in the TUNEL assay. C, quantitation of the kinetics of As$_2$O$_3$-induced apoptosis by DNA flow cytometry analysis. Sub-G$_1$ fractions increased with time during treatment with 20 μM As$_2$O$_3$. D, As$_2$O$_3$-induced growth inhibition shown by DNA flow cytometry analysis. PC-3 cells were treated with As$_2$O$_3$ at low concentrations for 24 h. G$_2$-M fractions increased with the increase in the As$_2$O$_3$ dose, but the increase was interrupted at 8 μM As$_2$O$_3$.

Fig. 4. Activation of p38 and JNK and the PML protein in the nuclear PML bodies in PC-3 cells treated with As$_2$O$_3$. In A, cell extracts of PC-3 cells, prepared after 12 h of treatment at the indicated As$_2$O$_3$ concentrations, were examined for three major MAP kinases by Western blotting. POS., positive control. 0.7 M NaCl treatment of HeLa cells for p38 and JNK. Purified ERK protein (New England Biolabs, Inc.). In B, cell extracts of PC-3 cells, treated with 50 μM As$_2$O$_3$ at the times indicated, were examined by Western blotting using p38 and JNK1/2. In C, cell extracts of PC-3 cells prepared after 3 h (phospho ATF2 and ATF2) and 12 h (phospho c-Jun and c-Jun) treatment at the indicated concentrations of As$_2$O$_3$ were examined by Western blotting. In D, cell extracts of PC-3 cells prepared after 12 h treatment at the indicated As$_2$O$_3$ concentrations were examined in phosphorylated MEK3/6, MEK3, phosphorylated SEK1, and SEK1 by Western blotting. In E, after incubation with 50 μM As$_2$O$_3$ for 4 h, immunocytochemistry for PML was performed as described in “Materials and Methods” a–c, untreated; d–f, As$_2$O$_3$-treated.
Apoptosis and Growth Inhibition Determined by Concentrations of As$_2$O$_3$. Treatment of PC-3 cells with 50 µM As$_2$O$_3$-induced morphological change characteristic of apoptosis. HOECHST 33342 staining showed nuclear shrinkage with chromatin condensation and fragmentation, indicative that these cells were on the way to death showing an apoptotic morphology (Fig. 2A). Simultaneous rhodamine 123 staining detected a decrease in mitochondrial transmembrane potential (Fig. 2A), whereas nontreated PC-3 cells had healthy nuclei and bright rhodamine 123 staining, indicative of intact mitochondrial transmembrane potential (Fig. 2A). Apoptosis was made visible by TUNEL-positive nuclear staining which detected nuclear DNA fragmentation in cells treated with As$_2$O$_3$ (Fig. 2B). No signal was present in the nontreated cells. The relation between apoptosis and As$_2$O$_3$ dose was clearly shown by flow cytometry analysis. Dose-dependent induction of apoptosis was detected by DNA flow cytometry (Fig. 3A) and by the flow-cytometric TUNEL assay (Fig. 3B) in PC-3 cells treated for 36 h with As$_2$O$_3$. Time-dependent induction of apoptosis was also shown by DNA flow cytometry in PC-3 cells treated with 20 µM As$_2$O$_3$ (Fig. 3C). Treatment of PC-3 cells with low concentrations (≤4 µM) of As$_2$O$_3$-induced cell cycle arrest at the G$_2$-M phase, whereas fewer cells underwent apoptosis at these concentrations (Fig. 3D). The growth inhibition seen in Fig. 1A, therefore, was mostly attributable to G$_2$-M phase arrest. The respective IC$_{50}$ of PC-3 cells was 2.5 µM at 48 h and 2.4 µM at 96 h. Time-dependent induction of apoptosis was not shown by DNA flow cytometry in PC-3 cells treated with 2 µM As$_2$O$_3$ until 96-h incubation (data not shown). These results indicate that As$_2$O$_3$ at high concentrations induces apoptosis in PC-3 cells but only causes growth inhibition at low concentrations.

As$_2$O$_3$-induced Activation of p38 and JNK Pathway. Western blots of PC-3 cell extracts treated with As$_2$O$_3$ were done with antibodies that recognize activated phosphorylated forms to determine the involvement of MAPKs in As$_2$O$_3$-induced cell death. The blots showed dose- and time-dependent activations of p38 and JNK1/2, but ERK1/2 was not activated (Fig. 4A and B). Activation of p38 and JNK1/2 were also observed in the DU-145 cell line (data not shown). Other molecules in the signal transduction pathways of p38 and JNK were investigated. Of the transcription factors regulated by these two kinases, ATF2 and c-Jun were activated (Fig. 4C). Of the MKKs regulating p38 and JNK, MEKK3/6 was markedly activated (Fig. 4D), but there was no activation of SEK1 (Fig. 4D). ASK1, a member of the MKK kinase family regulating both MEKK3/6 and SEK1 (23), was not detected in the Western blots of the PC-3 cells (data not shown). p38, JNK1/2, and their downstream molecules, therefore, were activated, whereas, of the upstream regulators, the activation only of MEKK3/6 could be identified. The involvement of the PML body in As$_2$O$_3$-induced apoptosis of PC-3 cells, therefore, was investigated. Immunocytochemistry clearly showed that As$_2$O$_3$ induced an increase in the PML protein expression of PML bodies (Fig. 4E), but there was no colocalization of PML bodies with ASK1 or phosphorylated-SEK1 (data not shown).

Involvement of p38 and JNK in As$_2$O$_3$-induced Cell Death. To determine the effect of p38 on intracellular signal transduction, SB203580, a selective inhibitor of p38 kinase, was added to PC-3 cells treated with As$_2$O$_3$. This addition suppressed the As$_2$O$_3$-induced activation of ATF2 dose dependently (Fig. 5A). Slight enhancement of p38 activation was detected (Fig. 5B), whereas there was remarkable enhancement of the activation of JNK1/2 and its downstream c-Jun (Fig. 5B). Next, DNA flow cytometry assay was used to evaluate whether p38 kinase is involved in As$_2$O$_3$-induced apoptosis. Few PC-3 cells, treated with SB203580 alone, underwent apoptosis (Fig. 5C), whereas addition of SB203580 to PC-3 cells treated with As$_2$O$_3$ produced an increase in the apoptotic fraction, which was not statistically significant (Fig. 5C). Addition of SB203580 to the DU-145 cells treated with As$_2$O$_3$ did not decrease the apoptotic fraction (data not shown). These findings indicate that the inhibition of p38 kinase...
enhances activation in the JNK-c-Jun signal cascade, which suggests that p38 may function as a survival signal mediator for protection against As$_2$O$_3$-induced cell death.

To clarify the involvement of the SEK1-JNK cascade in As$_2$O$_3$-induced cell death, dominant-negative SAPK/JNK and SEK1 tagged with GFP were introduced into PC-3 cells, and the cells analyzed by flow cytometry. After transfection and the addition of As$_2$O$_3$, flow cytometry analysis using 7-AAD and EGFP showed that As$_2$O$_3$-induced cell death was not inhibited by the over-expression of pGFP-DN SAPK/JNK1 and pGFP-DN-SEK1 (data not shown), evidence that the inhibition of the SEK1-JNK cascade did not guard against As$_2$O$_3$-induced cell death. This result suggests the contribution of pathways other than SEK1-JNK to As$_2$O$_3$-induced cell death.

**Involvement of Caspase in As$_2$O$_3$-induced Apoptosis.** The role of caspase in As$_2$O$_3$-induced cell apoptosis was investigated. In the caspase-3-like cleavage activity assay, combined treatment with cycloheximide and anti-APO-1/Fas antibody induced marked activation, which was eliminated by the caspase-3-like protease specific inhibitor, z-DEVD-fmk (Fig. 6A). In contrast, treatment with As$_2$O$_3$ caused only a slight increase in caspase-3-like cleavage activity (Fig. 6A). The addition of z-DEVD-fmk returned the activity to the basal level, but few PC-3 cells were rescued by this addition from As$_2$O$_3$-induced apoptosis (Fig. 6B). These findings suggest that, in PC-3 cells, caspase-3-like proteases do not have major roles in the signaling pathway of As$_2$O$_3$-induced cell death. Next, signal cross-talk between caspase and SAPKs was investigated. Combined treatment of PC-3 cells with As$_2$O$_3$ and SB203580 had a limited effect on caspase-3-like cleavage activity (Fig. 6A). Similarly, in the Western blots, the addition of z-DEVD-fmk did not produce a remarkable change in the phosphorylated levels of p38 and JNK1/2 (data not shown). No signal cross-talk between caspase and these two SAPKs, therefore, was found.

**Relation between ROS and As$_2$O$_3$-induced Apoptosis.** The ROS-sensitive fluorogenic dye CM-H$_2$DCFDA was used in the flow cytometry and fluorescent microscopy to investigate the relation between ROS and As$_2$O$_3$-induced apoptosis in PC-3 cells treated with As$_2$O$_3$. The generation of ROS was clearly shown by fluorescent microscopy (Fig. 7A) and flow cytometric analysis (Fig. 7A). This generation of ROS was completely suppressed by the addition of the antioxidant NAC (Fig. 7A). In addition, NAC, remarkably, protected PC-3 cells from As$_2$O$_3$-induced growth inhibition. Addition of 10 mM NAC increased the IC$_{50}$ of As$_2$O$_3$ from 2.1 to 6.1 $\mu$m. Similarly, NAC dramatically inhibited As$_2$O$_3$-induced apoptosis in the flow cytometric TUNEL assay (Fig. 7B) and DNA flow cytometry (Fig. 7C). Likewise, the generation of ROS and inhibition of As$_2$O$_3$-induced apoptosis by NAC were also observed in DU-145 cells (data not shown). Furthermore, this addition attenuated the As$_2$O$_3$-induced activation of caspase-3-like cleavage activity (Fig. 6A), of p38 (Fig. 7C) and JNK (Fig. 7C), evidence that the suppression of ROS inhibits As$_2$O$_3$-induced apoptosis, and that ROS regulates the intracellular signal transduction of caspases and SAPKs.

**In Vivo Tumor Growth Inhibition in the Orthotopic Metastasis Model of Prostate Cancer.** Lastly, the in vivo therapeutic efficacy of As$_2$O$_3$ was assessed by the orthotopic mouse metastasis model. Five weeks after inoculation of PC-3 cells to mouse prostate, orthotopic tumor and lymph node metastasis were identified macroscopically and were confirmed by histochemical analysis with H&E staining. Neither gross nor microscopic metastases were detected in the liver, lungs, or kidneys (data not shown). As shown in Fig. 8A, orthotopic tumor growth and lymph node metastasis were inhibited markedly by As$_2$O$_3$ treatment. Dose-dependent inhibition of orthotopic tumor growth was statistically significant (Fig. 8B). Lymph node metastasis showed a tendency for dose-dependent inhibition as well, but the statistical difference was marginal ($P = 0.06$; Fig. 8C). Neither apparent severe toxicity nor body weight loss occurred during the treatment period. No significant difference between groups was found for peripheral blood cell counts of RBCs and WBCs and platelets (data not shown). The histological examination showed there was no severe damage to the liver, lungs, or kidneys (data not shown). H&E staining could not identify any therapeutic effects between groups (Fig. 8D), but in situ TUNEL analysis of the tumor tissue showed a marked difference between the control and the As$_2$O$_3$-treated groups. More positive cells for in situ TUNEL analysis were identified in orthotopic tumors of As$_2$O$_3$-treated mice than in those of the control mice (Fig. 8D). These findings indicate that As$_2$O$_3$ induces both in vitro and in vivo tumor growth inhibition in the orthotopic murine metastasis model with no severe signs of toxicity.

**DISCUSSION**

The first objective of our study was to show the feasibility of As$_2$O$_3$ treatment in advanced prostate cancer for future clinical trial. In the in vitro assays, treatment with As$_2$O$_3$ at high concentrations induced apoptosis, but at low concentrations it only inhibited growth (Figs. 2 and 3). In the in vivo study, carried out with our orthotopic murine model of prostate cancer, in the orthotopic murine metastasis model with no severe toxicity.
The role of SAPKs and JNK in As2O3-induced apoptosis has been investigated. A previous analysis of NB4 cells showed that As2O3-induced apoptosis occurs through the ASK1-SEK1-JNK kinase pathway regulated by the generation of ROS (manuscript in preparation4 and Ref. 19). These reports suggest that the three pathways are related rather than independent. Analysis of the relationship of the three pathways in androgen-independent prostate cancer cell lines showed that ROS is the regulator of the caspases and SAPKs, but no relationship between the caspases and SAPKs was found.

Whether SAPK activation is involved in apoptosis generally depends on the type of cells and stimuli (27). In our study, p38 as well as JNK was activated in As2O3-induced apoptosis, but the inhibition of p38 did not protect androgen-independent prostate cancer cell lines from As2O3-induced apoptosis, and rather it activated the JNK-c-Jun pathway. This indicates that in androgen-independent prostate cancer cell lines, p38 is not involved in As2O3-induced apoptosis, and some signaling cross-talk exists between p38 and JNK. When we assumed that the activation of JNK is critical to As2O3-induced apoptosis of PC-3 cells, but its roles could not be clearly determined from the overexpression of its dominant-negative forms. Unlike in NB4 cells, neither ASK1, SEK1 activation, nor their colocalization with PML was detected in PC-3 cells (data not shown). These differences between NB4 and PC-3 cells may complicate the interpretation of the role of JNK. Other studies have reported that JNK has a critical role in As2O3-induced apoptosis of both rat primary cortical neuron and the JB6 CI41 mouse epidermal cell line. Currently, selective inhibitors of JNK are commercially unavailable. Further investigation with JNK-specific inhibitors is required to clarify what that involvement is.

As stated, our in vitro analysis showed that low concentrations of As2O3 could induce growth inhibition alone but not apoptosis. To reproduce this in vitro growth inhibition in in vivo experiments, we administered As2O3 to an SCID mouse whose prostate had been inoculated with PC-3 cells. In a unique study of the in vivo therapeutic effect of As2O3 on solid cancer, As2O3 was very effective both for the metastasis model, As2O3 induced a marked inhibition of tumor growth in orthotopic tumors and a marginal inhibition in retroperitoneal lymph node metastases (Fig. 8). Results of this in vivo study showed there was no significant toxicity to major organs. Our findings indicate that As2O3 treatment may prove a novel, promising approach to the favorable treatment for androgen-independent prostate cancer. Another goal was to clarify the intracellular signaling in As2O3-induced apoptosis. Analysis showed that the suppression of ROS inhibited As2O3-induced apoptosis, whereas SAPKs and caspase did not. This suggests that drugs that produce intracellular ROS may be potent enhancers of As2O3-induced apoptosis.

Since the discovery of the dramatic effects that As2O3 has on APL, several studies have investigated the use of As2O3 in the treatment for solid cancers that include neuroblastoma, gastric cancer, and head and neck cancer (11, 14, 24–26). Except for one (26), all of the studies were in vitro ones and showed antitumor effects that could not be compared with those of APL. As2O3 was reported to induce apoptosis or growth inhibition in each cell line, but concentrations used in those studies were higher than used in studies done on hematological cancers. At clinically obtainable As2O3 concentrations (<2 μM), all of the studies failed to induce total cell death. Likewise, in our study on prostate cancer cell lines, As2O3 induced apoptosis with reduced mitochondrial transmembrane potential at high concentrations but only growth inhibition at low ones. These facts suggest that to potentiate cytotoxicity of As2O3 at low concentrations, it is essential to develop the enhancer of As2O3 toxicity.

We, therefore, investigated the intracellular signaling mechanism of As2O3-induced apoptosis. Three major pathways have been proposed as the critical mechanism for As2O3-induced apoptosis: SAPKs, caspase, and ROS, but which mechanism actually is critical for As2O3-induced apoptosis, especially in solid cancers, has yet to be determined. In our study of PC-3 cells from prostate cancer, ROS proved to be the most important of the three signaling pathways. Similar results have been reported in another study done with CHO cells from ovarian cancer (15). It also showed the association of ROS with caspase-dependent apoptosis (15).
tumor itself and the tumor-feeding vessels (26). Although these findings may be very important experimentally, there must be two important points to be overcome to get clinical relevance. The first point is that a high dose (10 mg/kg) used for the single injection of As$_2$O$_3$ (26) was lethal in two of the three SCID mice used in our study (data not shown). Multiple administration at this dosage, therefore, must be lethal to all of the SCID mice. Less toxic agents and less invasive administration protocols are required in a clinical setting, especially for older patients with prostate cancer. In our murine study, the daily As$_2$O$_3$ dose was reduced by one-half, to 5 mg/kg, and continuous administration was shown to be safe over a 5-week period with no significant toxicity to the peripheral blood cells, liver, kidneys, or lungs. The other point was the therapeutic effects of As$_2$O$_3$ on metastases. Our orthotopic model overcomes this point by establishing sizable metastases in the retroperitoneal lymph nodes, and enables simultaneous evaluation of the compound’s therapeutic effects on primary and metastatic lesions. Although additional studies are required to determine correlations given the As$_2$O$_3$ dose, and serum and tissue concentrations of As$_2$O$_3$, we believe that our study is more clinically relevant and shows the feasibility of the clinical use of As$_2$O$_3$ for the treatment of androgen-independent prostate cancer.

We succeeded in reproducing in vitro growth inhibition in an in vivo orthotopic metastasis model, but monotherapy with As$_2$O$_3$ did not induce complete tumor disappearance. We think that this in vivo tumor growth inhibition indicates the limit of monotherapy with As$_2$O$_3$ because it could not induce total cell death because of its low intracellular concentrations. To obtain in vivo tumor disappearance without increasing the dose of As$_2$O$_3$, it would be necessary to develop potentiators of As$_2$O$_3$-induced apoptosis. Our study has shown that the generation of ROS has a central role in As$_2$O$_3$-induced apoptosis. Drugs that produce intracellular ROS or interfere ROS scavengers, therefore, are potential candidates for the enhancement of As$_2$O$_3$-induced apoptosis. In fact, l-buthionine sulfoximine, an intracellular glutathione-depleting agent, produces a large amount of ROS, and has been shown to greatly potentiate As$_2$O$_3$-induced apoptosis in other solid cancer cell lines (28). In the future, combined

Fig. 8. In vivo tumor growth inhibition by As$_2$O$_3$ in the orthotopic mouse model of PC-3 cells. A, representative cases 5 weeks after orthotopic inoculation of PC-3 cells. Seminal vesicles (SV) and the bladder (B) were exposed in the control mouse (left panel), and mouse treated with 5 mg/kg As$_2$O$_3$ (right panel). Growth inhibition is clear both in the orthotopic tumor (black arrowheads) and retroperitoneal lymph node metastases (white arrows) in the mouse treated with 5 mg/kg As$_2$O$_3$. B, dose response of As$_2$O$_3$-induced growth inhibition on orthotopic tumor weight in the mouse treated with As$_2$O$_3$. The difference between the control and 5-mg/kg-As$_2$O$_3$-treatment groups was significant. C, dose response of As$_2$O$_3$-induced growth inhibition on the number of retroperitoneal lymph node metastases. Retroperitoneal lymph nodes larger than 0.5 mm in diameter were counted under a microscope, and the pathology was confirmed. The difference between the control and the 5-mg/kg-As$_2$O$_3$-treatment group was marginal and not significant (P = 0.06). D, representative histology of an orthotopic tumor formed by PC-3 cells, after treatment. H&E staining (a, b) and the in situ TUNEL assay (c, d) were performed in the control (a, c) and 5-mg/kg-As$_2$O$_3$-treated (b, d) mice.
therapy that uses $\text{As}_2\text{O}_3$ together with these drugs may prove useful for treating androgen-independent prostate cancer.

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