Inactivation of Akt by arsenic trioxide induces cell death via mitochondrial-mediated apoptotic signaling in SGC-7901 human gastric cancer cells

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Abstract. Arsenic trioxide (As_2O_3) has been recognized as a potential chemotherapeutic agent, yet the details concerning its mechanism of action in solid cancers remain undetermined. The present study assessed the role of Akt in the cell death induced by As_2O_3. The MTT assay showed that As_2O_3 suppressed the proliferation of SGC-7901 cells in a dose- and time-dependent manner. Characteristic apoptotic changes were observed in the As_2O_3-treated cells by Hoechst 33342 staining, and FACS analysis showed that As_2O_3 caused dose-dependent apoptotic cell death. As_2O_3 activated caspase-3 and -9, and PARP cleavage in a dose-dependent manner. Compromised mitochondrial membrane potential and an increased protein level of Bax indicated involvement of mitochondia. As_2O_3 decreased the levels of p-Akt (Ser473), p-Akt (Thr308) and p-GSK-3β (Ser9), suggesting that As_2O_3 inactivated Akt kinase. In addition, LY294002 (a PI3 kinase inhibitor) augmented the apoptosis induced by As_2O_3. These results demonstrated that inhibition of PI3K/Akt signaling was involved in As_2O_3-induced apoptosis of gastric cancer SGC-7901 cells.

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Introduction

Gastric cancer has declined from the second to the fourth most common cancer in the world, yet it remains the second most common cause of cancer-related mortality among men and the fourth among women (1). It was estimated by the American Cancer Society that 989,600 new cases of gastric cancer and ~738,000 related deaths occurred in 2008, with over 70% of new cases and deaths noted in developing countries (2). Gastric cancer is difficult to cure primarily since the majority of patients are diagnosed with advanced disease. It has been demonstrated that adjuvant therapy improves survival (3,4). Unfortunately, there is no internationally accepted standard chemotherapy for advanced gastric cancer, and uncertainty remains regarding the choice of the optimal regimen (5,6). Thus, the development of more efficacious therapies is critically needed for the treatment of this disease.

Arsenic trioxide (As_2O_3), an inorganic compound of trivalent arsenic, has been used as a drug in traditional Chinese medicine for thousands of years (7). At present, it is recognized as a potent chemotherapeutic agent and has been approved by the Food and Drug Administration for the treatment of certain leukemias (8-10). Previous studies have demonstrated that As_2O_3 may have beneficial effects in the treatment of solid tumors including gastric cancer (11), hepatocellular carcinoma (12), breast cancer (13), lung cancer (14) and neuroblastoma (15); however, there are limitations to its application in the treatment of solid tumors owing to the necessity of high concentrations for antineoplastic efficacy (16,17). Therefore, a better understanding of the underlying mechanisms of action of As_2O_3 may facilitate the development of strategies to induce therapeutic responses using lower concentrations of As_2O_3 (18).

Despite extensive research, the mechanism of action of As_2O_3 is not fully clear. Several studies have indicated that arsenic-induced apoptotic death may be crucial for its anti-leukemic action (19,20). Specifically, the arsenic-induced apoptotic death process involves the reciprocal regulation of Bcl-2/Bax, loss of mitochondrial membrane potential ($\Delta\psi m$), activation of caspases, and fragmentation of DNA,

suggesting that activation of mitochondrial-mediated intrinsic apoptotic signaling may play a major role in arsenic-induced death (21-23). However, with regard to solid tumors, it remains controversial whether apoptosis is involved in the cell death induced by As_2O_3 (11,24-26).

Although the process of apoptosis is mediated primarily by proteolytic activities, there is compelling evidence that signal transduction pathways involving specific protein kinases modulate the apoptotic response (27). The serine/threonine protein kinase (Akt, a member of the PI3K pathway) is involved in widely divergent cellular processes including apoptosis and cell proliferation (28). The aberrant activation of phosphoinositide 3-kinase (PI3K)/Akt has been documented as a frequent occurrence in human types of cancer (29,30), and inhibition of this pathway should provide a therapeutic approach for cancer (31). Several studies indicate that As_2O_3-induced apoptosis is correlated with inactivation of PI3K/Akt in leukemia cells (32,33). Furthermore, abnormal activation of the PI3K/Akt pathway was found to render these cells resistance to As_2O_3 (34,35), and pharmacologic inhibitors of PI3K/Akt enhanced the apoptotic action of As_2O_3 (36). Therefore, inhibition of PI3K/Akt signaling may be critical for As_2O_3 action.

In the present study, we present data showing that As_2O_3-induced apoptosis was partly mediated via the activation of mitochondrial-mediated intrinsic apoptotic signaling, and As_2O_3 inactivated Akt kinase via dephosphorylation of Akt. In addition, we showed that LY294002 (a PI3 kinase inhibitor) enhanced the apoptosis induced by As_2O_3.

Materials and methods

Materials. As_2O_3 was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in 1 mmol/l NaOH as a 100-mM stock solution. Antibodies against total Akt, phospho-Akt (Ser473), phospho-Akt (Thr308), phospho-GSK-3β (Ser9), poly(ADP-ribose) polymerase (PARP), cleaved PARP, pro-caspase-3, cleaved caspase-3, pro-caspase-9, cleaved caspase-9, β-actin and LY294002 (a PI3 kinase inhibitor) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibodies against β-actin, Bcl-2 and Bax were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Alkaline phosphatase-linked secondary antibodies were purchased from Promega Corporation (Madison, WI, USA). Annexin V-fluorescein isothiocyanate (FITC)-labeled apoptosis detection kit was obtained from Baosai Biological Technology Co., Ltd. (Beijing, China). Hoechst 33342 and propidium iodide (PI) were purchased from Sigma Chemical Co.

Cell culture. The human gastric cancer SGC-7901 cell line (Heilongjiang Cancer Institute, China) was maintained in RPMI-1640 medium containing 10% fetal bovine serum at 37˚C in a 5% CO_2 atmosphere. All cell samples used were in the logarithmic growth phase.

Cell viability assay. The effect of As_2O_3 on the proliferation of SGC-7901 cells was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion assay. Briefly, SGC-7901 cells were plated at $4x10^3$ cells/well in 96-well plates for 16 to 20 h. Then, the cells were exposed to varying concentrations of As_2O_3 at 37˚C for 24, 48 and 72 h, respectively. Thereafter, 10 μl of MTT (5 mg/ml) was directly added to each well, and the cells were incubated at 37˚C for an additional 4 h. After removal of the culture medium, the cells were lysed in 100 μl of dimethyl sulfoxide (DMSO). The optical density (OD) at 490 nm was measured by a microplate reader (Bio-Rad, Hercules, CA, USA). The following formula was used: Relative percentage of cell viability = (OD of the experimental sample/OD of the control group) x 100%.

Assessment of mitochondrial membrane potential. Mitochondrial membrane potential ($\Delta\psi m$) was quantified using Rhodamine 123, whose accumulation in the mitochondria of living cells depends on $\Delta\psi m$. SGC-7901 cells ($1x10^6$) were collected after various treatments. Cells were washed and resuspended in 500 μl of phosphate-buffered saline (PBS) buffer containing 5 μg/ml of Rhodamine 123 (Molecular Probes, Eugene, OR, USA), and were then incubated at 37˚C for 30 min in the dark, washed and resuspended in PBS buffer. Finally, the intensity of the Rhodamine 123 staining was measured by flow cytometry with an excitation and emission setting of 488 and 530 nm, respectively.

Apoptotic cell staining. Morphological evidence of apoptosis or necrosis was determined by Hoechst 33342 and PI nuclear staining. After the various treatments, the SGC-7901 cells were incubated with 5 μg/ml of Hoechst 33342 and 5 μg/ml PI for 15 min and then observed using fluorescence microscopy (Nikon TE2000-U, Japan) at a magnification of x200. Apoptotic cells were identified by characteristic unequivocal nuclear chromatin condensation and/or fragmentation (37). Digital images were captured from 5 random fields for each sample, and apoptotic cell counts were expressed as a percentage of the total number of nuclei counted.

Analysis of apoptosis by Annexin V-FITC staining. Early apoptosis was determined by staining cells with Annexin V-FITC. Briefly, after the various treatments, both floating and trypsinized adherent SGC-7901 cells were collected. Then the cell pellets were incubated with 5 μl PI and 10 μl Annexin V-FITC. The samples were then analyzed by flow cytometry.

Western blot analysis. After the various treatments, the SGC-7901 cells were collected and lysed in lysis buffer. The supernatants were then collected by centrifugation at 12,000 x g for 5 min and analyzed for total protein content by the Bradford method. Equal amounts of lysate were loaded on a 10% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane, and the blots were probed by corresponding primary antibodies, followed by incubation with alkaline phosphatase (AP)-conjugated secondary antibodies (Promega Corporation). The positive bands representing protein were developed using the Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega Corporation).

Statistical analyses. Data are expressed as means ± SD of three repeated experiments. The one-way analysis of variance (ANOVA) was used for statistical analyses. $p<0.05$ was considered to indicate a statistically significant result. All

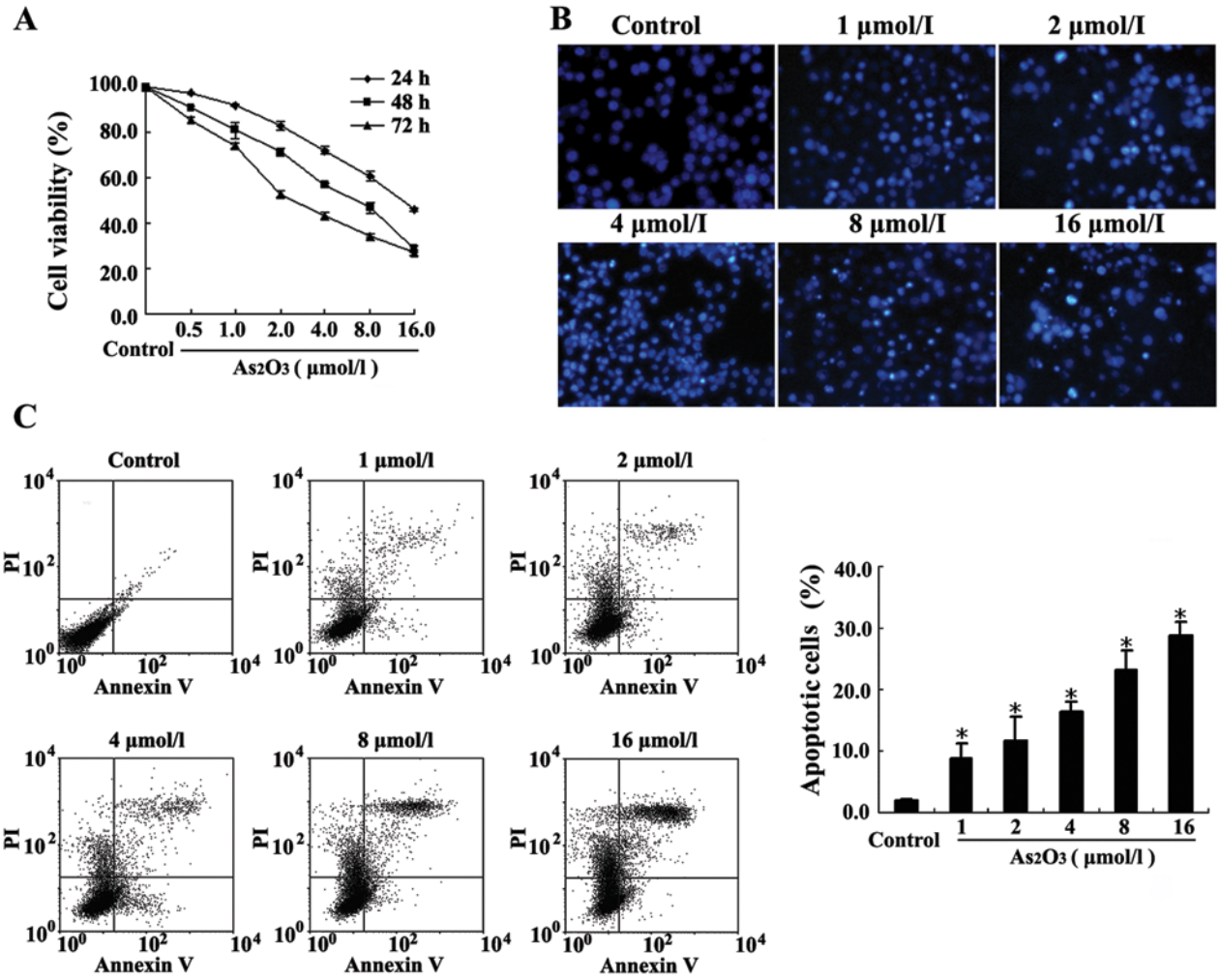

Figure 1. Effect of As_2O_3 treatment on cell growth and apoptosis. (A) Cell viability analysis. SGC-7901 cells were treated with As_2O_3 (0-16 μmol/l) for 24, 48 and 72 h, and the cell growth was determined by MTT assay. Data shown are the mean ± SD of 3 separate experiments for which each treatment was repeated in 96-well plates. (B and C) SGC-7901 cells were treated with As_2O_3 (0-16 μmol/l) for 24 h, and apoptosis was determined by fluorescence microscopy and flow cytometry as detailed in Materials and methods. (B) Morphological evidence of apoptosis or necrosis was determined by Hoechst 33342 and PI nuclear staining. Cells that stained blue with condensed chromatin and/or fragmentation were recorded as apoptotic cells. (C) Quantification of apoptosis by flow cytometry. The cells were labeled with Annexin-FITC and PI. Annexin V-positive cells were considered to be apoptotic cells, and their percentage is indicated. Data from 3 independent experiments are shown. *$p<0.01$ vs. control. As_2O_3, arsenic trioxide; PI, propidium iodide.

experiments were performed at least three times independently.

Results

As_2O_3 reduces the proliferation of SGC-7901 cells. To investigate the inhibitory effect of As_2O_3 on the proliferation of gastric cancer cells, SGC-7901 cells were treated with various concentrations of As_2O_3 (0 to 16 μmol/l) for 24, 48 and 72 h. The results of the MTT assay (Fig. 1A) demonstrated that As_2O_3 significantly inhibited cell viability in a dose- and time-dependent manner. The 50% inhibitory concentration (IC_{50}) of As_2O_3 was 3.32 μmol/l at 72 h.

As_2O_3 induces the apoptosis of SGC-7901 cells through the mitochondrial pathway. To determined whether As_2O_3 inhibits the growth of SGC-7901 cells via induction of apoptosis, SGC-7901 cells were treated with As_2O_3 (0 to 16 μmol/l) for 24 h, and apoptosis was determined using Hoechst 33342 staining. Characteristic apoptotic changes such as condensed, fragmented, and intensely fluorescent nuclei were observed in the SGC-7901 cells following treatment with As_2O_3 for 24 h (Fig. 1B). The extent of apoptosis was next quantified using flow cytometry with Annexin V and PI staining. As_2O_3 treatment for 24 h resulted in dose-dependent induction of apoptosis in the SGC-7901 cells (Fig. 1C).

To determine whether the mitochondrial pathway is involved in the induction of apoptosis induced by As_2O_3 treatment, changes in $\Delta\psi m$ were measured by Rhodamine 123 staining and subsequent flow cytometry. With the increasing concentrations of As_2O_3, the numbers of SGC-7901 cells increased in the hypofluorescent portion, which indicated that As_2O_3 decreased the $\Delta\psi m$ in SGC-7901 cells (Fig. 2A).

Since pro-apoptotic Bax and anti-apoptotic Bcl-2 play crucial roles in apoptosis, changes in the protein levels in the SGC-7901 cells treated with As_2O_3 were determined using western blotting. As_2O_3 did not significantly modify the protein level of Bcl-2, but increased the protein level of Bax

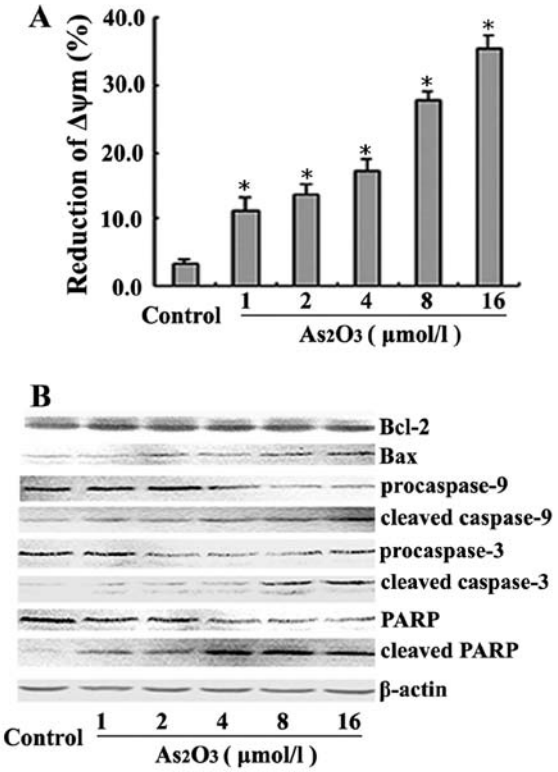

Figure 2. As_2O_3 induces the apoptosis of SGC-7901 cells through the mitochondrial pathway. (A) Assessment of mitochondrial membrane potential (Δψm). SGC-7901 cells were treated with As_2O_3 (0-16 μmol/l) for 24 h, and Rhodamine 123 fluorescence was analyzed by flow cytometry. Data represent the percentage of cells with decreased fluorescence, which reflects the number of cells with low Δψm. *p<0.01 vs. control. (B) Analysis of the protein expression of Bax and Bcl-2, activation of caspase-3 and -9, and cleavage of PARP protein. SGC-7901 cells were incubated with As_2O_3 (0-16 μmol/l) for 24 h, and the levels of the proteins were analyzed by western blotting with antibodies against Bcl-2, Bax, pro-caspase-3, cleaved caspase-3, pro-caspase-9, cleaved caspase-9, PARP and cleaved PARP. As_2O_3, arsenic trioxide; PARP, poly(ADP-ribose) polymerase.

in a dose-dependent fashion (Fig. 2B), which resulted in an increase in the ratio of Bax to Bcl-2, therefore favoring apoptosis.

To determine whether the activation of caspase-3 and -9 is involved in the apoptosis induced by As_2O_3, the proteolytic maturation of pro-caspase-3 and -9 was detected using western blotting. As_2O_3 treatment resulted in a decrease in the protein levels of pro-caspase-3 and -9 and concomitant increase in the corresponding active cleavage products in the SGC-7901 cells (Fig. 2B), which indicated that As_2O_3 may increase the cleavage maturation of caspase-3 and -9. Meanwhile the cleavage of PARP, an executioner caspase substrate, was also detected using western blotting. Likewise, As_2O_3 treatment resulted in cleavage of PARP from 116 to 89 kDa (Fig. 2B). Therefore, these results suggested that As_2O_3-induced apoptosis was partly mediated through the mitochondrial pathway.

As_2O_3 inhibits the phosphorylation of Akt in SGC-7901 cells. Akt has been reported to be involved in the signaling pathway mediated by As_2O_3, and its activation was found to be correxlated with phosphorylation at Thr308 and Ser473 residues. Therefore, the effects of As_2O_3 (0 to 16 μmol/l) treatment for 24 h on the amount and phosphorylation of Akt were evaluated using western blotting. As_2O_3 did not significantly change the protein level of Akt, but decreased the levels of p-Akt (Ser473) and p-Akt (Thr308) (Fig. 3), which indicated that inactivation of Akt kinase following As_2O_3 treatment was due to dephosphorylation of Akt, rather than reduction in total Akt protein. It was found that activated Akt phosphorylates and inactivates glycogen synthase kinase 3 (GSK-3), which plays an important role in the apoptotic pathway. Thus, changes in the phosphorylation of serine 9 of GSK3β were detected using an antibody that specifically recognizes phosphoserine-9-GSK3β. Likewise, a reduction in GSK3β phosphorylation was found in the SGC-7901 cells treated with As_2O_3 (Fig. 3), suggesting that As_2O_3 treatment decreases the activity of Akt.

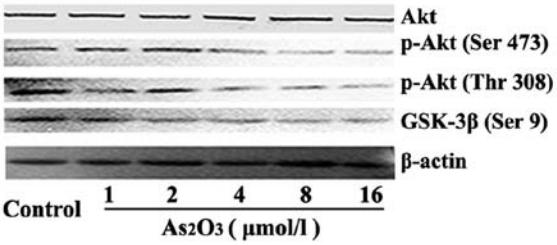

Figure 3. Effects of As_2O_3 on the activation and expression of Akt proteins. Exponentially growing cells were treated with As_2O_3 (0-16 μmol/l) for 24 h, and the Akt, phospho-Akts and phosphoserine-9-GSK3β levels were evaluated using western blotting. As_2O_3, arsenic trioxide.

PI3K/Akt inhibitor enhances the apoptosis induced by As_2O_3. To investigate whether the inhibition of PI3K/Akt signaling alters the extent of apoptosis induced by As_2O_3, LY294002 (a specific inhibitor of PI3K) was selected to inhibit PI3K/Akt signaling. Pretreatment with 25 μmol/l LY294002 for 1 h before exposure to 16 μmol/l As_2O_3 for 24 h completely blocked Akt activation (Fig. 4A). LY294002 significantly decreased the viability of the SGC-7901 cells from 60.0±1.0 to 24.9±1.7% following treatment with As_2O_3 (Fig. 4B). LY294002 alone induced typical morphological changes and increased the percentages of the apoptotic population, which were greatly potentiated in the presence of As_2O_3 (Fig. 4C). Moreover, LY294002 significantly decreased Δψm, which was further decreased in the presence of As_2O_3 (Fig. 4D). When As_2O_3 was used in combination with LY294002, the protein level of Bcl-2 was decreased, the protein level of Bax was significantly increased, the proteolytic activation of pro-caspase-3 and -9 was greatly potentiated, and the cleavage of PARP was enhanced. Therefore, LY294002 enhanced the extent of apoptosis induced by As_2O_3.

Discussion

As_2O_3 has attracted much attention due to its beneficial effects in the treatment of patients with acute promyelocytic leukemia (APL) without severe side-effects (38). Moreover, its antitumor activity has been confirmed in non-APL leukemia (9) and solid tumor cell lines (16,25,39). As_2O_3 induces apoptosis and inhibits the growth of various types of tumor cells *in vitro*, but relatively higher concentrations are required for solid tumor cells than for hematologic cancer cells (40,41). In the present study, we

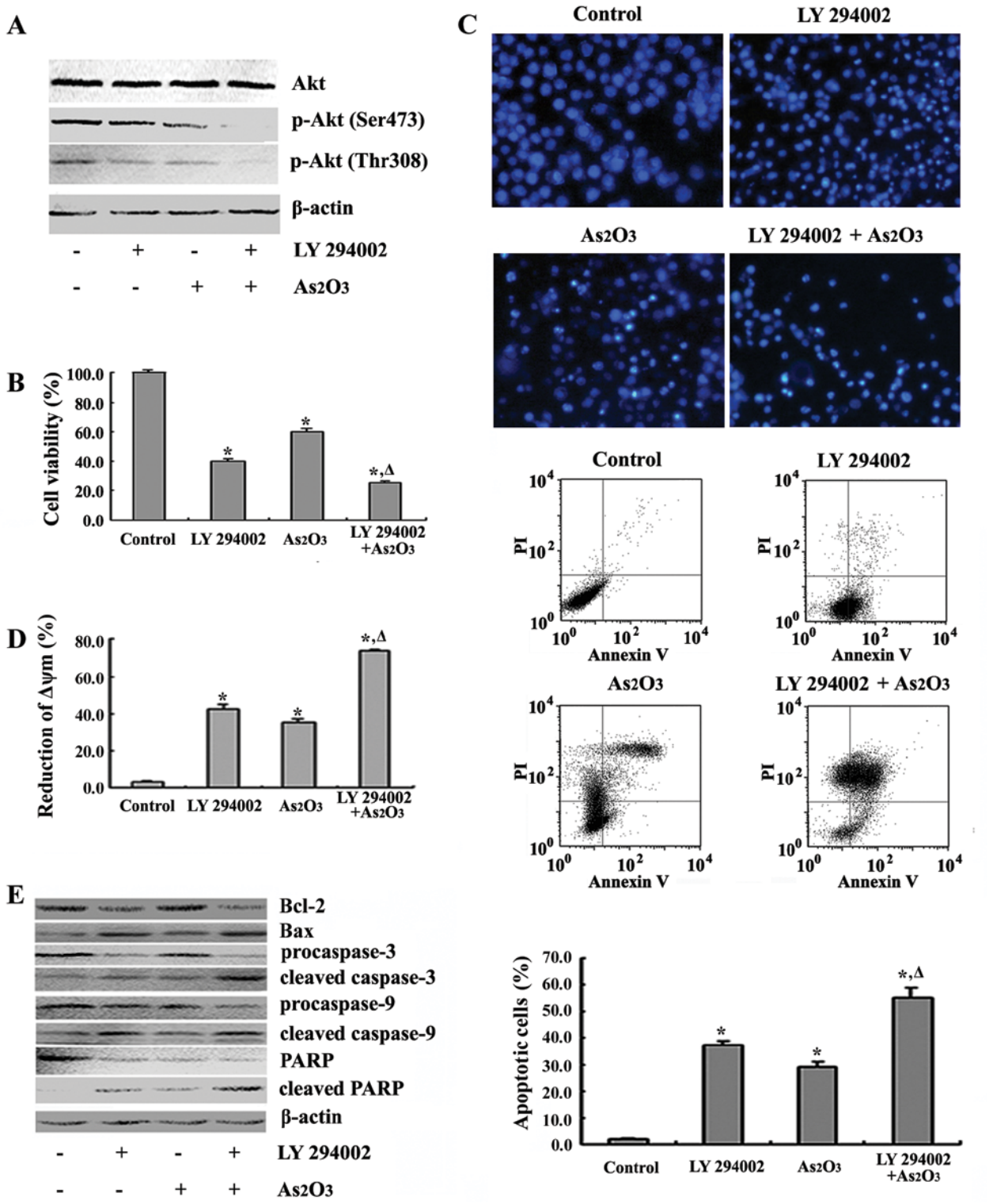

Figure 4. Effects of LY294002 on the action of As_2O_3 in SGC-7901 cells. Exponentially growing cells were pretreated with 25 μmol/l LY294002 for 1 h, and were then exposured to 16 μmol/l As_2O_3 for 24 h. (A) The levels of Akt or phospho-Akts were evaluated using western blotting. (B) Cell viability was analyzed by MTT assay. (C) Morphological evidence of apoptosis was determined by Hoechst 33342 and PI nuclear staining and quantified using flow cytometry with Annexin-FITC and PI staining. (D) Mitochondrial membrane potential ($\Delta\psi$m) was assessed by Rhodamine 123 staining. (E) The protein expression of Bax and Bcl-2, activation of caspase-3 and -9, cleavage of PARP protein were analyzed by western blotting. *p<0.01 vs. control; $^{\Delta}$p<0.01 vs. LY294002. As_2O_3, arsenic trioxide; PARP, poly(ADP-ribose) polymerase.

demonstrated that As_2O_3 inhibited proliferation and viability of SGC-7901 cells in a dose- and time-dependent manner, which supports the results of previous studies (11,42,43). The IC_{50} of As_2O_3 in SGC-7901 cells was 3.32 μmol/l at 72 h, and was greater than the optimum plasma level (2 μmol/l) used for the treatment of hematological malignancies (44,45), indicating that SGC-7901 cells are less susceptible to As_2O_3 than hematological cancer cell lines. However, pharmacokinetic

studies, which were performed in 8 APL patients successfully treated with As_2O_3, revealed that peak plasma concentrations rangeg from 5.54 to 7.30 μmol/l (38). Therefore, these results suggest that As_2O_3 may be clinically useful in patients with gastric cancer as an adjuvant chemotherapeutic agent. It has been indicated that the effective dose range of As_2O_3 being used to treat solid tumors in mice is from 2 to 6.5 mg/kg (40,46). These dosages are ~12- to 40-fold higher than the standard dosage of 0.16 mg/kg used to treat APL patients. Such high dosages carry the risk of severe side-effects due to toxicity (40,47). Therefore, it is essential to determine the mechanisms of action of As_2O_3 and to develop strategies to enhance its efficacy.

The mechanisms responsible for the antitumor action of As_2O_3 have been extensively investigated. Apoptosis appears to be one of the main mechanisms by which As_2O_3 induces cell death and inhibits cell growth (19,20,48). However, several studies indicate that As_2O_3 primarily induces arrest at the G1 or G2/M phases in solid cancer cells (24,26,49). In support of these findings, our previous research found that As_2O_3 effectively inhibited the growth of hepatocellular carcinoma cells and induced G2/M phase arrest (50). In the present study, apoptotic morphological changes were observed in the nuclei of the SGC-7901 cells following treatment with As_2O_3, and FACS analysis showed that As_2O_3 caused dose-dependent apoptotic cell death. This result is similar to previous reports (11,51). Consistent with a previous report (52), As_2O_3 increased active cleaved caspase-3 (17 kDa) and cleaved fragment of PARP (89 kDa). These results revealed that As_2O_3 has the capability to induce apoptosis in SGC-7901 cells.

Mitochondria play a key role in apoptotic signal transduction in mammalian cells (53). It has been suggested that As_2O_3 induces apoptosis in tumor cells by affecting the mitochondria, due to the loss of $\Delta\Psi$m (54-58) and cytochrome *c* release from mitochondria (59,60). In the present study, the loss of $\Delta\Psi$m was demonstrated by an increase in the proportions of cells with reduced Rhodamine 123 staining. Bcl-2 and Bax are members of the Bcl-2 family that regulate apoptosis by controlling mitochondrial integrity. Although they have highly similar amino acid sequences, their functions are opposed; Bcl-2 acts to inhibit apoptosis, whereas Bax counteracts this effect by heterodimerization with Bcl-2. The ratio of Bcl/Bax dictates the sensitivity of cells to apoptosis through destabilization of the mitochondrial membrane and activation of the caspase cascade (61). Zheng *et al* found that As_2O_3 triggered apoptosis through induction of Bcl-2 conformational change, Bax activation and upregulation of total Bax expression in human gastric cancer SGC7901 cells (11). In the present study, As_2O_3 had no effect on the protein level of Bcl-2, but increased the protein level of Bax in a dose-dependent fashion, thus increasing the ratio of Bax to Bcl-2. In mitochondrial-dependent apoptosis, the disruption of the mitochondrion leads to the release of cytochrome *c* into the cytosol. Apoptosomes containing cytochrome *c*, Apaf-1 and pro-caspase-9 are then assembled, resulting in proteolytic processing and activation of pro-caspase-9. Active caspase-9 in turn activates pro-caspase-3 initiating a caspase signaling cascade to induce apoptosis (62). In the present study, we also demonstrated that As_2O_3 induced apoptosis in parallel with the activation of caspase 9. Therefore, As_2O_3 has the ability to induce the mitochondrial-intrinsic apoptosis signaling pathway in gastric cancer.

Akt, a key mediator of the PI3K signaling pathway, promotes cell survival partially by phosphorylation and inactivation of several pro-apoptotic proteins, including GSK-3 (63), BAD (64) and caspase-9 (65). The role of Akt in As_2O_3-induced death has been investigated, yet the results are conflicting and confusing (18,66,67). It has been found that the activity of the PI3K/Akt signaling pathway determines the sensitivity of leukemia cells to As_2O_3-induced apoptosis (32,34,35). Moreover, pharmacologic inhibitors of PI3K/Akt were found to enhance the apoptotic action of As_2O_3 (36), while another report showed that pretreatment with inhibitors of PI3K had no effect on As_2O_3-induced apoptosis in the leukemic cell line NB4 (34). Our results showed that As_2O_3 decreased not only phosphorylated Akt protein levels but also Akt activity, which is coincident with a previous report (68). Meanwhile, we found that pretreatment with the PI3K/Akt inhibitor LY294002 strongly increased As_2O_3-induced apoptosis in SGC-7901 cells. Moreover, when As_2O_3 was used in combination with LY294002, $\Delta\psi$m was further decreased, the protein level of Bcl-2 was decreased, the protein level of Bax was significantly increased, the proteolytic activation of pro-caspase-3 and -9 was greatly potentiated, and the cleavage of PARP was enhanced, indicating that LY294002 enhanced the apoptosis induced by As_2O_3 via the mitochondrial-intrinsic apoptosis signaling pathway.

Taken together, our results suggest that As_2O_3 inactivated Akt kinase via dephosphorylation, which then induced apoptosis via activation of mitochondrial-mediated intrinsic apoptotic signaling. In addition, the PI3K inhibitor LY294002 enhanced the apoptosis induced by As_2O_3. Therefore, the PI3K/Akt pathway plays a role in As_2O_3-induced death of SGC-7901 cells and the addition of PI3K inhibitors may be valuable for improving the efficacy of As_2O_3 treatment for human gastric cancer.

Acknowledgements

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