Anti-apoptotic and apoptotic pathway analysis of arsenic trioxide-induced apoptosis in human gastric cancer SGC-7901 cells

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Abstract. The present study aimed to investigate the effect of arsenic trioxide (As_2O_3) on human gastric cancer SGC-7901 cells. SGC-7901 cells were treated with different concentrations of As_2O_3 in the cell growth media for 24, 48 and 72 h, and the growth rates were determined by WST-1 cell proliferation assays. Analyses of nuclear morphological changes were performed with DAPI fluorescence staining. Cell apoptosis rates as assessed by flow cytometry were determined after cells were grown in media for 48 h containing different As_2O_3 concentrations. The protein expression patterns of the apoptosis factors, Bax, Fas and caspase-8, and anti-apoptosis factors, Akt, p-Akt, mTOR and p-mTOR, were evaluated by western blot analysis following treatment of the cells with different As_2O_3 concentrations in the cell growth media for 48 h. As a result, As_2O_3 inhibited the growth of human gastric cancer SGC-7901 cells in concentrations >5 μmol/l for longer than 24 h. Flow cytometric analysis revealed that the apoptosis of SGC-7901 cells occurred in an As_2O_3 concentration-dependent manner after 48 h ($P<0.001$). Expression levels of Bax, Fas and caspase-8 were increased, whereas expression levels of Akt, p-Akt, mTOR and p-mTOR were decreased in the SGC-7901 cells after a 48-h incubation with different As_2O_3 concentrations. In conclusion, As_2O_3 induced human gastric cancer SGC-7901 cell apoptosis in a time- and concentration dependent manner by inhibiting the activity of anti-apoptosis-related factors.

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Introduction

Gastric carcinoma (GC) remains the third leading cause of cancer-related mortality in men worldwide (1). To date, there are few effective clinical treatments for this highly malignant tumor, and conventional adjuvant treatments have limited effects on the survival of patients with advanced gastric cancer (2). Much research has been conducted to identify efficient chemotherapeutic agents for the cure and prevention of GC, and recently apoptosis has been shown to play a significant role in the treatment of GC cells.

Arsenic trioxide (As_2O_3) is an arsenic compound that has been used as a medicinal agent for more than 2400 years (3). In the 1970s, Chinese researchers were the first to discover its ability to cure acute promyelocytic leukemia (APL) (4). Since then other research groups have demonstrated worldwide that As_2O_3 also inhibits the growth of various solid tumors, including esophageal carcinoma (5,6), breast (3,7,8), bladder (9), lung (10) and liver cancer (11), multiple myeloma (12), neuroblastoma (13), colon (14) and ovarian cancer (15). It has been shown that As_2O_3 regulates proliferation, invasion, differentiation, angiogenesis and apoptosis of cancer cells (16). However, the precise mechanism of As_2O_3-related apoptosis induction of cancer cells is not fully understood. Recent experiments confirm that As_2O_3 affects the activities of protein kinase B (Akt), c-Jun N-terminal kinases (JNK), nuclear factor κB (NF-κB), glutathione and calcium signaling, reactive oxygen species (ROS), caspases, as well as pro- and anti-apoptotic proteins (17-20). It was noted that As_2O_3 can reduce the activation of the Akt/mTOR pathway by reducing Akt, p70S6K and rpS6 phosphorylation in human leukemia cells (21). The PI3K/Akt/mTOR pathway is a crucial regulatory cascade that is central to a variety of physiological functions, including cell cycle regulation, survival, protein synthesis, metabolism, motility, apoptosis, proliferation and angiogenesis (22,23). The phosphoinositide 3-kinase (PI3K) activates Akt, a serine/threonine kinase, which phosphorylates the mammalian target of rapamycin (mTOR) repressor tuberous sclerosis complex 2 (24), which in turn activates mTOR downregulation of autophagy inducing autophagy-related (Atg) proteins (25,26). Recently it has been demonstrated that As_2O_3 suppresses PI3K/Akt activity and induces JNK activa-

tion thereby enhancing chronic B-lymphocytic leukemia cell apoptosis (27).

The major apoptosis pathways are the extrinsic pathways (death receptor) and intrinsic pathways (mitochondrial) (28). The common effector for extrinsic apoptotic pathway initiation is FASL, which regulates apoptosis via binding to FASR, a member of the tumor necrosis factor (TNF) receptor family of proteins. Deregulation of the FAS pathway has been implicated in various malignancies and diseases (29). After stimulation of the death receptor pathway by the FAS ligand, conformational changes to the FAS receptor lead to cleavage of pro-caspase 8 into its activated form, which then cleaves other effector caspases eventually leading to apoptosis. During the apoptotic process, Bcl-2-associated X (Bax) inhibits the anti-apoptotic B-cell lymphoma 2 (Bcl-2) protein (30) and permeabilizes the mitochondrial outer membrane, leading to cytochrome *c* release (31).

In the present study, we explored the effects of As_2O_3 on expression levels of FAS, caspase-8 and Bax proteins in human gastric cancer SGC-7901 cells. Then we further analyzed the role of As_2O_3 in the Akt/mTOR pathway in As_2O_3-exposed cells.

Materials and methods

Materials. As_2O_3 solution was purchased from YiDa Pharmacy. The stock solution of arsenic trioxide was 8 mM and was stored at 4˚C. RPMI-1640 medium and FBS were purchased from Hyclone. Anti-Akt polyclonal antibodies (9272) were purchased from Cell Signaling Technology. Anti-phospho-Akt (s473), anti-mTOR, anti-phospho-mTOR (s2448), anti-Bax, anti-caspase-8 and anti-FAS polyclonal antibodies were purchased from ImmunoWay. Anti-β-actin monoclonal, goat anti-mouse and goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase were purchased from Santa Cruz Biotechnology.

Cell culture and treatment. Human SGC-7901 gastric cancer cells were cultured in RPMI-1640 medium containing 10% FBS in a 5% CO_2 humidified atmosphere chamber at 37˚C. For the experiments, FBS was reduced to 2%, and exponentially growing cells were incubated for the indicated time periods with different concentrations of As_2O_3 (0, 2.5, 5, 7.5, 10, 12.5 and 15 μmol/l).

WST-1 proliferation assay. The effect of As_2O_3 on *in vitro* growth inhibition of SGC-7901 cells was measured using the WST-1 Cell Proliferation and Cytotoxicity Assay Kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. SGC-7901 cells were seeded in 96-well plates at a density of $1.0x10^4$ cells per well in 200 μl RPMI-1640 medium containing 10% FBS for 24 h. Then the cells were exposed to different concentrations of As_2O_3 (0, 2.5, 5, 7.5, 10, 12.5 and 15 μmol/l) in RPMI-1640 medium containing 2% FBS for 24, 48 and 72 h. Finally 20 μl of WST-1 solution was added to each well, and the cells were incubated for another 1 h. The absorbance at 450 nm was measured using a microplate reader (Finnpipette MK3 Multiskan). The amount of the formazan dye, which is generated by activities of dehydrogenases in the cells, is proportional to the number of living cells. Inhibitory rates of cellular growth were calculated with the following formula: Inhibitory rate (%) = (1 - A value of experimental group/A value in the control group) x 100%. The 0 μmol/l group was used as the control group. A graph with inhibitory cell growth rates (y-axis) against the concentrations of As_2O_3 (x-axis) was plotted.

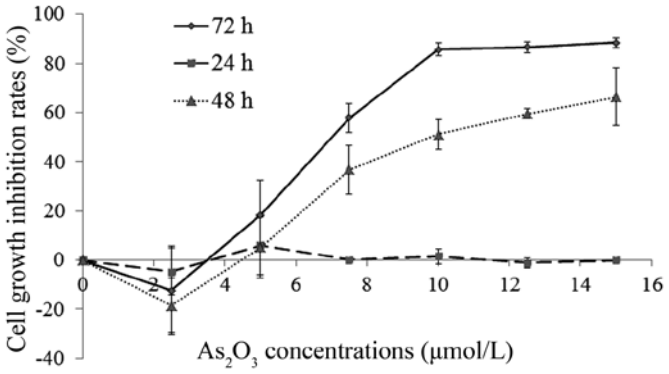

Figure 1. Cell growth inhibitory effects of arsenic trioxide (0, 2.5, 5, 7.5, 10, 12.5 and 15 μmol/l) on SGC-7901 cells after a 24-, 48- and 72-h incubation.

Analysis of nuclear morphology by DAPI staining. Apoptosis was assessed based on changes in the nuclear morphology by staining the cells with the fluorescent DNA dye 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI) (Roche). Briefly, cells were treated with As_2O_3 (0 and 10 μmol/l) in RPMI-1640 medium containing 2% FBS for 48 h. Then the cells were washed with PBS and incubated with 1 μg/ml DAPI in methanol for 30 min at 37˚C in darkness. Slides were viewed using a fluorescence microscope with ultraviolet (UV) excitation at 300-500 nm. Cells were evaluated as normal or apoptotic depending on morphological characteristics. Normal nuclei (smooth nuclei) and apoptotic nuclei (condensed or fragmented chromatin) were observed.

Analysis of apoptosis. Cells were treated with different concentrations of As_2O_3 (0, 7.5, 10, 12.5 and 15 μmol/l) in 2% FBS and RPMI-1640 for 48 h, collected and then stained using the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences) for flow cytometric analyses. The 0 μmol/l group served as the control.

Protein extraction and western blot analysis. Cells were treated with different concentrations of As_2O_3 (0, 5, 7.5, 10, 12.5 and 15 μmol/l) in 2% FBS and RPMI-1640 medium for 48 h. Both adherent and floating cells were harvested and lysed with RIPA lysis buffer and phenylmethanesulfonyl fluoride (Beyotime Institute of Biotechnology), incubated at 4˚C for 40 min and centrifuged for 10 min at 12,000 rpm. Total protein in the cell lysate was measured with an enhanced BCA protein assay kit (Beyotime Institute of Biotechnology). For western blot analysis, equal amounts of protein were separated by SDS-PAGE and then transferred onto PVDF membranes (Millipore). The membranes were blocked for 1.5 h in a non-fat dried milk solution containing 1% Tween-20. The membranes were then incubated with primary antibodies for β-actin (1:800), Akt (1:800), p-Akt (1:800), mTOR (1:800),

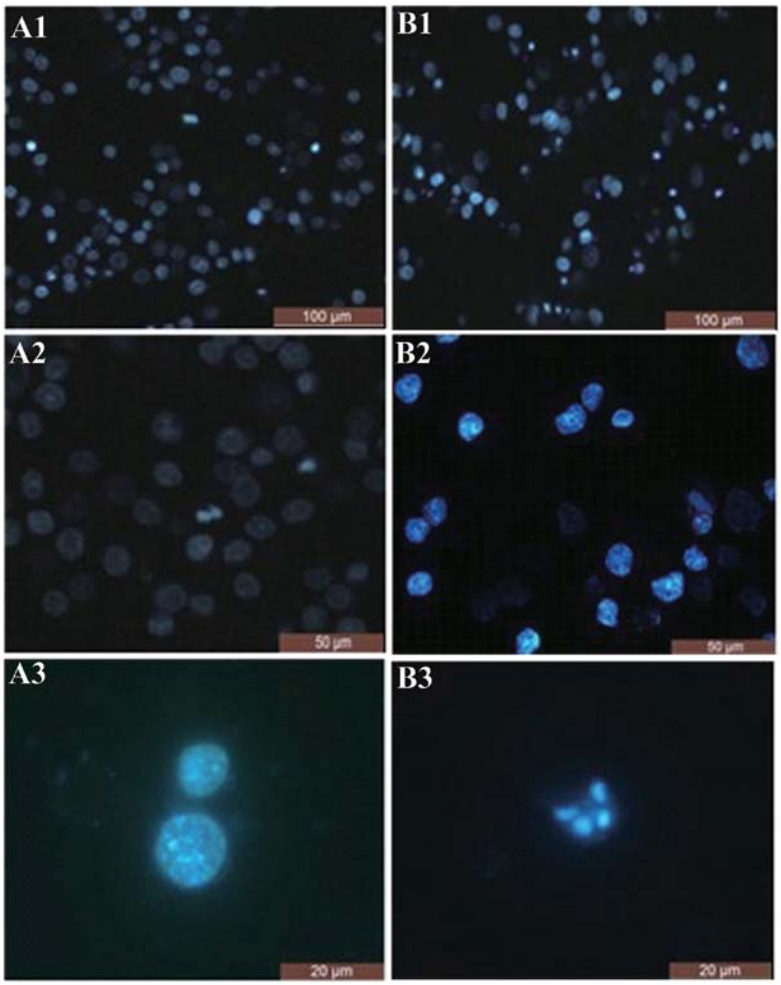

Figure 2. Changes in SGC-7901 cell nuclear morphology visualized with DAPI under a fluorescence microscope. (A1-A3) Control group after 48 h. (B1-B3) Cells after exposure to 10 μmol/l As_2O_3 for 48 h.

p-mTOR (1:800), Bax (1:1000), caspase-8 (1:1000) and FAS (1:1000) overnight at 4˚C, followed by incubation with anti-mouse or anti-rabbit (1:5000) secondary antibodies for 1 h. Finally, protein bands were detected using a chemiluminescent substrate (HRP) kit (Beyotime Institute of Biotechnology). The β-actin level was used as an internal standard.

Statistical analyses. All experiments were performed at least three times. Data for each series of experiments (performed in triplicates) are expressed as the mean values ± standard deviation of the mean (SD). Statistical significance of differences between groups was analyzed using ANOVA analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Extended incubation with As_2O_3 leads to cell growth inhibition. Human SGC-7901 gastric cancer cells were incubated with different concentrations of As_2O_3 (0, 2.5, 5, 7.5, 10, 12.5 and 15 μmol/l) for 24, 48 and 72 h and the cell growth inhibition was recorded using the WST-1 assay. When the As_2O_3 solution concentrations were >5 μmol/l, the cell growth was significantly reduced after 48 and 72-h incubation periods, whereas after a 24-h incubation none of the As_2O_3 concentrations had an effect on cell growth. Concentrations <5 μmol/l led to reduced growth inhibition (Fig. 1).

As_2O_3 leads to apoptosis of SGC-7901 cells

Analysis of nuclear morphology as assessed by DAPI staining. The SGC-7901 cells were treated with 10 μmol/l As_2O_3 for 48 h, and apoptosis was visualized by DAPI staining using fluorescence microscopy. Compared with the control, a large number of cells displayed morphological changes exhibiting the typical characteristics of apoptotic cell death, including cell shrinkage, chromatin condensation, chromatin crescent formation/margination, DNA fragmentation and apoptotic body formation (Fig. 2).

Analysis of apoptosis by flow cytometry. With increasing concentrations (0, 7.5, 10, 12.5 and 15 μmol/l) of As_2O_3 in the growth media, the apoptosis rates increased after 48 h from 2.83±0.88, 9.85±2.18, 25.81±2.17 and 29.92±3.30 to 35.40±0.58%, which indicated that As_2O_3 induced the apoptosis of human gastric cancer SGC-7901 cells in a dose-dependent manner (Fig. 3).

Protein extraction and western blot analysis

As_2O_3 induces Bax, Fas and caspase-8 activation. SGC-7901 cells were incubated with different As_2O_3 concentrations

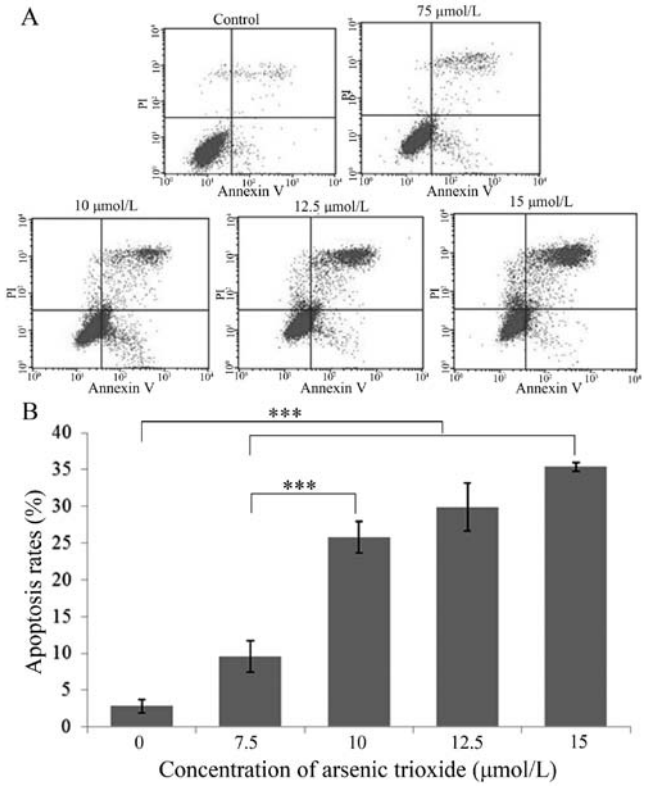

Figure 3. Apoptosis rates of the SGC-7901 cells following addition of As_2O_3 (0, 7.5, 10, 12.5 and 15 μmol/l) to the medium for 48 h. (A) Representative flow cytometric images. (B) Histogram of the data derived from the flow cytometric analyses (n=3). *** P<0.001.

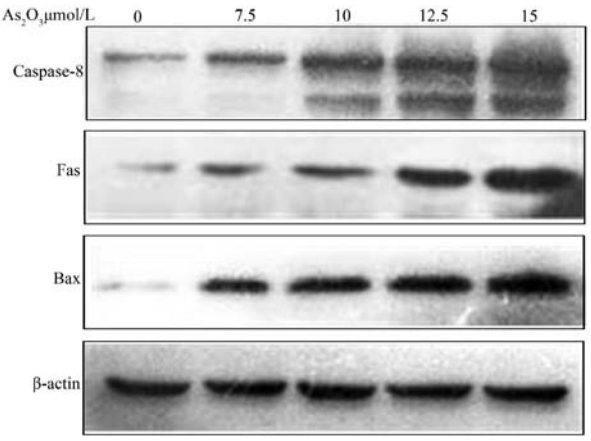

Figure 4. Effect of As_2O_3 concentrations (0, 7.5, 10, 12.5 and 15 μmol/l) on protein expression levels of Bax, Fas and caspase-8 in SGC-7901 cells after a 48-h incubation.

(0, 7.5, 10, 12.5 and 15 μmol/l) for 48 h, and then Bax, Fas and caspase-8 protein expression levels were analyzed via western blotting. As shown in Fig. 4, expresssion of Bax, Fas and caspase-8 protein was increased with increasing As_2O_3 concentrations.

As_2O_3 suppresses Akt, p-Akt, mTOR and p-mTOR activation. SGC-7901 cells were incubated with different As_2O_3 concentrations (0, 7.5, 10, 12.5 and 15 μmol/l) for 48 h and Akt, p-Akt, mTOR and p-mTOR protein expression levels were analyzed via western blotting. As shown in Fig. 5, Akt, p-Akt, mTOR and p-mTOR protein expression levels decreased with increasing As_2O_3 concentrations.

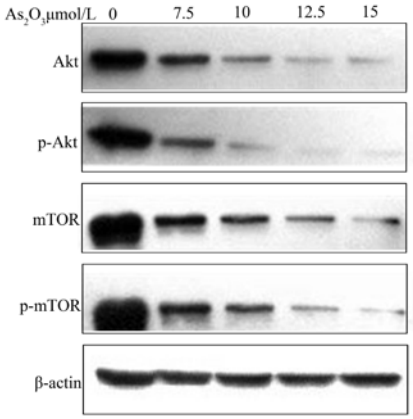

Figure 5. Effect of As_2O_3concentrations (0, 7.5, 10, 12.5 and 15 μmol/l) on protein expression levels of Akt, p-Akt, mTOR, p-mTOR in SGC-7901 cells after a 48-h incubation.

Discussion

In the present study, we demonstrated that As_2O_3 induced the apoptosis of human gastric cancer SGC-7901 cells in a dose- and time-dependent manner, which is in agreement with previous findings of the As_2O_3-triggered apoptosis of lung cancer cells (32).

Further analyses revealed that the apoptotic proteins Bax, Fas and caspase-8 were upregulated and the anti-apoptotic proteins Akt, p-Akt, mTOR as well as phosphorylated mTOR (p-mTOR) were downregulated. The PI3K (phosphatidylinositol 3 kinase) pathway is a signal transduction cascade, which is at the center of many physiological functions including cell cycle regulation, cell survival, protein synthesis, metabolism as well as blood vessel formation. There are two key elements (Akt and mTOR) in the PI3K transduction pathway. Akt (serine/threonine kinase) is the regulator of the PI3K transduction pathways by regulating a variety of downstream effectors. A variety of growth factors, cytokines and hormones lead to the phosphorylation of Akt, which in turn activates downstream effectors including mTOR directly or indirectly by preventing the combination of mTORC1 and mTORC2 thereby promoting protein synthesis and cell growth (33,34). Akt also inactivates cell cycle inhibitors (p21 and p27) and promotes cell cycle proteins (c-Myc and cyclinD1) to maintain cell survival (35,36). Another study found that Akt suppressed the apoptosis inhibition genes (BIM and BAD) and reduced the expression of the tumor-suppressor protein (p53) restricting programmed cell death and promoting cell survival (37). Our results showed that As_2O_3 concentrations <5 μmol/l promoted cell growth and during the 24-h incubation cell growth was not inhibited by any As_2O_3 concentration (Fig. 1). In previous studies, the apoptotic effect of As_2O_3 was attributed to reactive oxygen species development (38,39), and As_2O_3 has also been shown to inhibit mitochondrial respiration, thereby enhancing ROS occurrence (40), which has been used to sensitize tumor cells for radiation therapy (41). Autophagy constitutes a stress

adaptation that avoids cell death, and cells can compensate oxidative stress damages to a certain extent through autophagy, which was demonstrated by different cell reactions upon low and high dosage exposures to safingol, which is a ROS inducer (42). Autophagy following As_2O_3 exposure has also been reported (43). We suggest that at low doses of As_2O_3 up to 5 μmol/l, autophagy is the main mechanism triggered in SGC-7901 cells and apoptosis is blocked (44) leading to somewhat reduced growth inhibition. Moreover, in short periods (24 h), the ROS development is under the threshold for inducing apoptosis, probably also due to oxygen radical squelching mechanisms (45). This is supported by the finding that apoptotic effects of As_2O_3 are most pronounced in tumor cells with low GSH levels, and ascorbic acid could further enhance its capacity for apoptosis induction (45).

A drawback of our study was that apoptotic mechanisms are complex and this study is a preliminary study of the Akt/mTOR anti-apoptotic pathway, while other anti-apoptosis pathways need further investigation. In addition, the effective dose of As_2O_3 was >5 μmol/l, which is higher than the allowed clinical therapeutic dose of 1-2 μmol/l, thus further long-term and sensitizing agent evaluations are warranted (45).

In conclusion, our *in vitro* results showed that As_2O_3 can induce apoptosis in human gastric cancer SGC-7901 cells. As_2O_3 treatment led to enhanced expression of the apoptotic proteins Bax, Fas and caspase-8, and reduced the expression of the anti-apoptotic proteins Akt and mTOR as well as their phosphorylated forms p-Akt and p-mTOR in a time- and dose- dependent manner. Since the effective dose of As_2O_3 was higher than the therapeutic limit and growth inhibition rate reductions were incubation time-dependent, further research is necessary to establish As_2O_3 for the treatment of gastric cancers.

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