

Cell cycle arrest and apoptotic cell death in cultured human gastric carcinoma cells mediated by arsenic trioxide

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Abstract

AIM: To investigate the effect of arsenic trioxide on human gastric cancer cell line MKN45 with respect to both cytotoxicity and induction of apoptosis *in vitro*.

METHODS: MKN45 cells were treated with arsenic trioxide (As_2O_3) at the concentration of 1, 5, and 10 $\mu\text{mol/L}$, respectively, for three successive days. Cell growth and proliferation were observed by cell counting and trypan blue exclusion. Cytotoxicity of As_2O_3 was determined by MTT assay. Morphologic changes were studied with light microscopy. Flow cytometry was used to assay cell DNA distribution and apoptotic cells were confirmed with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and DNA electrophoresis.

RESULTS: The growth of MKN45 cells was significantly inhibited by As_2O_3 which was confirmed by colony-forming assay. After 7 d of culture with various concentrations of As_2O_3 , colony-forming capacity of MKN45 cells decreased with As_2O_3 increment in comparison with that of control group. The inhibitory rate of colony-formation was 38.5%, 99.1%, and 99.5% when the concentration of As_2O_3 was 1, 5, and 10 $\mu\text{mol/L}$ in culture medium, respectively. The cell number of a single colony in drug treatment groups was less than that of control group. The cell-killing rate of As_2O_3 to MKN45 cells was both dose- and time-dependent with an IC_{50} of $(11.05 \pm 0.25) \mu\text{mol/L}$. After incubation in 10 $\mu\text{mol/L}$ As_2O_3 for 24 h, the cell-killing rate was 27.1%, and it was close to 50% after 48 h. The results showed that As_2O_3 induced time- and dose-dependent apoptosis in MKN45 cells, blocked at G_2/M phase. The apoptotic peak (sub- G_1 phase) appeared and cell apoptotic rate in MKN45 cells was 18.3-32.5% after treatment by 10 $\mu\text{mol/L}$ As_2O_3 for 48 h. The percentage of G_2/M cell of the experimental groups was 2.0-5.0 times than that of the control group. Gel electrophoresis of DNA from cells treated with each concentration of As_2O_3 for

48 h revealed a "ladder" pattern, indicating preferential DNA degradation at the internucleosomal, linker DNA sections. TUNEL also demonstrated strand breaks in DNA of MKN45 cells treated with As_2O_3 , while control cells showed negative labeling.

CONCLUSION: As_2O_3 can induce apoptosis of human gastric carcinoma cells MKN45, which is the basis of its effectiveness. It shows great potential in the treatment of gastric carcinoma.

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Key words: Arsenic trioxide; Gastric carcinoma; Cell cycle; Apoptosis

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INTRODUCTION

Arsenic trioxide (As_2O_3) is a major ingredient of traditional Chinese medicine (TCM). It is derived from Pi'shi by sublimation. In the practice of TCM, it is used externally to cure hemorrhoids, acute ulcerative gingivitis, and asthma, *etc.* Its anti-tumor activity was discovered by a group of Chinese doctors in 1970s^[1-6]. Since then, the effect of As_2O_3 in treating cancers has been extensively studied. The first use of As_2O_3 in cancer therapy was to treat acute promyelocytic leukemia (APL). Both the results of *in vitro* and clinical trials showed that As_2O_3 was effective in inhibiting the growth of APL. Because of the significant anti-cancer effect of As_2O_3 , studies were carried out on its potential use in cancer treatment of non-APL such as myeloid leukemia, hepatocellular carcinoma, neuroblastoma, esophageal carcinoma as well as head and neck cancers. Reports showed that As_2O_3 was also effective in inhibiting the growth of these cancers^[7-11,13].

Gastric cancer is one of the most common malignant tumors in China. Evidences have demonstrated that gastric cancer is a disease caused not only by excessive cellular proliferation and poor differentiation, but also by decrease in apoptosis of gastric cells^[12,14-19]. Though the disease in its early stage can be treated by surgical resection, in advanced stage its response to conventional chemotherapy or radiotherapy is usually not satisfactory. Moreover, surgical resection and radiotherapy are carried out provided that

the cancer is restricted to a particular region. However, cancer may metastasize to other regions at the later stage of cancer development. For chemotherapy, side effects like toxic hepatitis and heart damage may result. Moreover, prolonged treatment with anticancer drugs may give rise to multidrug-resistant cancer cells, which in turn, poses great obstacle in cancer therapy. Therefore, discovery of new drugs for the treatment of gastric cancer is urgent. Apoptosis is an important mode of cell death that occurs in response to a variety of agents including ionizing radiation or anticancer chemotherapeutic drugs^[20-24].

In the present study, the effects of As₂O₃ on human gastric cancer cell line, MKN45, was investigated by *in vitro* study.

MATERIALS AND METHODS

Cell culture and drug treatment

Poorly differentiated human gastric adenocarcinoma cell line, MKN45, was grown in RPMI 1640 (Gibco BRL, Life Technologies, Inc., Rockville, MD, USA) supplemented with 2 mol/L L-glutamine, 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL, Life Technologies, Inc.) and 5% mixture of 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B (Antibiotic-Antimycotic, Gibco BRL, Life Technologies, Inc.). After being subcultured for 24 h, exponentially growing cell suspensions were distributed into 25-cm² cell culture flasks at a density of 5×10⁶ cells/mL (5 mL medium). Cells were passaged twice weekly, routinely examined for mycoplasma contamination, maintained in 37 °C incubator in a humidified atmosphere consisting of 50 mL/L CO₂ in air.

As₂O₃ (Sigma Chemical, St. Louis, MO, USA) was dissolved in PBS at 1 mol/L as a stock solution, stored at 4 °C. For *in vitro* use, the stock solution was diluted to the appropriate concentration in growth medium without FBS. Exponentially growing cells were treated with As₂O₃ at a final concentration of 1, 5, 10 µmol/L respectively. Control cultures were treated with distilled PBS at a final concentration of 0.1% in culture medium. All experiments were performed in triplicate.

Cell growth and proliferation assay

The proliferation of MKN45 cells during the period of experiments was monitored by counting cell number, IC₅₀ and mitotic indices. Cell suspension was mixed with equal volume of 0.08% trypan blue solution (Sigma Chemical Co., Ltd, St. Louis, MO, USA). The mixture was then transferred to the hemacytometer. Only viable cells (unstained cells) were counted.

Inhibition rate of cell growth (%) = [viable control cells - viable treated cells] / viable control cells × 100%.

To avoid possible influence of cell density on cell growth and survival, cells were maintained at less than 1×10⁵/mL with daily adjustment by addition of fresh culture medium containing the corresponding concentration of As₂O₃.

Colony-forming assays

To assess effects of As₂O₃ on MKN45 cell lines, exponentially growing cells at a density of 5×10⁶ cells/mL (5 mL

medium) were mixed with RPMI 1640 supplemented with 2 mol/L L-glutamine, 20% heat-inactivated fetal bovine serum and 5% mixture of 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B. One microliter of liquid culture was plated in a 35-mm plastic culture plate in the presence of various concentrations of As₂O₃, and maintained in 37 °C incubator in a humidified atmosphere containing 50 mL/L CO₂ in air. After 7 d, more than 40 colony formation in methylcellulose was assessed by inverted phase-contrast microscopy. All experiments were performed in triplicate.

MTT cytotoxicity assay

In vitro growth inhibition effect of As₂O₃ on MKN45 cells was determined by measuring MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) dye absorbance of living cells. Briefly, cells (1×10⁵ cells per well) were seeded in 96-well microtiter plates (Nunc, Roskilde, Denmark). After exposure to various As₂O₃ for 48 and 72 h respectively, 100 µL (1 g/L) MTT (Sigma) solution was added to each well and the plates were incubated for an additional 4 h at 37 °C. MTT solution in medium was aspirated. To achieve solubilization of the formazan crystal formed in viable cells, 100 µL DMSO was added to each well before absorbance at 570 nm was measured. Each concentration treatment was done in triplicate wells. The cytotoxicity rates were measured by the formula:

$$\text{the cytotoxicity rate} = [1 - A_{570 \text{ test}} / A_{570 \text{ control}}] \times 100\%$$

Morphologic assessment of apoptosis by light microscopy and DAPI staining

Following exposure to As₂O₃, cells were obtained, washed with PBS, cytospun onto slides, and stained with Wright-Giemsa for morphologic assessment of apoptosis by light microscopy. Evidence of apoptosis was indicated by the presence of cell shrinkage, membrane blebbing, fragmentation of nuclei, and formation of apoptotic bodies. Apoptosis was also detected by DAPI (Sigma) as previously described, and the percentages of cells in interphase, mitosis, and apoptosis were quantified.

Cell cycle analysis

Cell cycle and apoptotic cells were detected by flow cytometry which was performed as described previously. After drug treatment, about 1×10⁶ cells at each time point were collected by trypsin digestion and centrifugation, then fixed in 70% ethanol/PBS for at least 12 h at 4 °C. After 100 µL (1 g/L) RNase treatment, cells were stained with 50 mg/L propidium iodide. Cells were examined by flow cytometry using an FACScan (FACS-420, USA). Also, in cell cycle analysis, cells were considered to be in apoptosis if they exhibited sub-G₁ DNA fluorescence and a forward angle light scatter, the same as or slightly lower than that of cells in G₁. The results were analyzed with Lysis II software (FACS-420, USA).

DNA gel electrophoresis

A total of 10⁶ cells with or without As₂O₃ treatment were gently scraped from the dishes and washed twice in cold PBS. The pellets were collected by centrifugation and

resuspended in 1 mL of buffer containing 150 mol/L NaCl, 10 mol/L Tris-HCl pH 8.0, 20 mol/L EDTA pH 8.0, and 0.5% SDS. After thorough mixing, 20 μ L of proteinase K (10 mg/mL) was added and incubated at 50 °C for 2 h and cooled to 0 °C, 2 mL of ethanol (at -20 °C) was added to precipitate DNA, which was collected by centrifugation (1 200 r/min, 20 min), dried in air and dissolved in 50 μ L of 10 mol/L Tris, 1 mol/L EDTA at pH 8.0 (TE buffer). Each DNA preparation was mixed with 2 mL RNase (10 mg/mL) and 6 μ L of loading buffer (30% glycerol, 0.1% bromophenol blue) and electrophoresed for approximately 3.5 h (2 V/cm gradient). A marker was obtained from Boehringer Mannheim, Penzberg, Germany. The gels were stained with ethidium bromide, and the DNA bands were visualized under ultraviolet light and photographed.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

TUNEL assay was used to monitor the extent of DNA fragmentation due to apoptosis. The assay was performed according to the recommendations of the manufacturer (Boehringer, Mannheim, Germany). In brief, following exposure to As₂O₃, cells were obtained, washed with PBS, cytospun onto slides, and dried in air. The cells were fixed with a freshly prepared paraformaldehyde solution (4% in PBS, pH 7.4) for 30 min at room temperature. The slides were rinsed with PBS and incubated in permeabilization solution (0.1% Triton[®] X-100, 0.1%_[s1] sodium citrate) for 2 min on ice. They were then rinsed twice with PBS and the area around the samples was dried. Fifty microliters of TUNEL reaction mixture were placed on the sample, and the slides incubated in a humidified chamber for 60 min at 37 °C in the dark. After rinsing of the slides thrice with PBS, samples were analyzed under a fluorescence microscope (Zeiss, Jena, Germany). The percentage of fluorescence-positive apoptotic cells was calculated out of more than 500 cells on each slide.

Statistical analysis

Most experiments were performed in triplicate, and the results from separate experiments are expressed as mean \pm standard deviation (mean \pm SD). The $P < 0.05$ was taken as a significant difference between groups as determined by χ^2 -test.

RESULTS

Inhibitory effect of As₂O₃ on growth of MKN45 cell line

After exposure of exponentially growing MKN45 cells to As₂O₃ for 72 h, the inhibitory effect of As₂O₃ on cell growth

was significant as revealed by cell counting, and was both dose- and time-dependent (Table 1). There were significant differences between each concentration and control group ($P < 0.01$) respectively. The IC₅₀ of As₂O₃ on MKN45 was 11.05 \pm 0.25 μ mol/L.

Effect of As₂O₃ on colony formation of MKN45 cells

The growth of MKN45 cells was significantly inhibited by As₂O₃ which was confirmed by colony-forming assay. After 7 d of culture with various concentrations of As₂O₃, colony-forming capacity of MKN45 cells decreased with As₂O₃ increment in comparison with that of control group. The inhibitory rate of colony forming was 38.5%, 99.1%, and 99.5% when the concentration of As₂O₃ was 1, 5, and 10 μ mol/L in culture medium, respectively. The cell number of a single colony in drug treatment groups was less than that of control group.

Effect of As₂O₃ on MKN45 cells

The cell-killing rate of As₂O₃ on MKN45 cells was significant as revealed by MTT, and was both dose- and time-dependent. After incubation in 1 μ mol/L As₂O₃ for 24 h, the cell-killing rate was 11.5%, and after treatment with 10 μ mol/L As₂O₃ for 48 h, the cell-killing rate was close to 50% (Table 2). These results indicated that the cytotoxic effect of As₂O₃ was strong.

Morphologic changes of MKN45 cells

By inverted phase-contrast microscopy, we found that the attaching ability of MKN45 cells to the flask treated with 1, 5, and 10 μ mol/L As₂O₃ was weaker as compared with that of controls, and cell growth markedly inhibited.

MKN45 cells treated with As₂O₃ underwent significant changes as seen under microscope, the nucleocytoplasmic ratio enlarged, with indentation of nuclei. The nucleocytoplasmic ratio in MKN45 cells treated with 1 μ mol/L As₂O₃ was much smaller than that in controls, and the nuclei appeared round, with loss of nuclear indentation, but with well-differentiated organelles in the cytoplasm. When treated with As₂O₃ at 5 and 10 μ mol/L for three successive days, one could find intact cell membrane, nuclear condensation and apoptotic body formation, but much less than in 1 μ mol/L As₂O₃ group.

Effect of As₂O₃ on cell cycle of MKN45 cells

The effect of As₂O₃ on MKN45 cells showed remarkable cell cycle specificity. There was no significant change in cell cycle after 1-10 μ mol/L treatment for 24 h, being similar to control group. The fraction of G₀/G₁ was decreased from 55.6%, 57.1%, 55.2% to 37.2%, 19.9%, 13.1% after 1, 5, 10 μ mol/L treatment for 48 h, respectively, while the

Table 1 The effects of As₂O₃ on the growth of MKN45 cells at different times and concentrations

Groups	Cell number (1 \times 10 ⁵ /mL)		
	24 h	48 h	72 h
PBS control	7.15 \pm 1.08	9.18 \pm 0.99	13.34 \pm 0.54
As ₂ O ₃ (μ mol/L) 1	2.47 \pm 0.99	3.34 \pm 1.02	2.73 \pm 1.38
5	2.51 \pm 0.63	2.84 \pm 1.12	1.75 \pm 1.12
10.0	2.09 \pm 0.67	1.34 \pm 0.51	0.25 \pm 0.24

Table 2 Cytotoxic effect of As₂O₃ on MKN45 cells (%)

	As ₂ O ₃ (μ mol/L)	Treatment time (t/h)		
		24 h	48 h	72 h
	1	11.5 \pm 5.2	18.9 \pm 5.6	34.8 \pm 6.8
	5	19.1 \pm 3.8	34.3 \pm 3.7	46.9 \pm 7.6
	10.0	23.9 \pm 7.3	48.8 \pm 8.2	69.5 \pm 10.6

fraction of G₂/M phase was increased from 16.3%, 16.7%, 17.5% to 22.6%, 41.5%, 69.0%, respectively.

The results showed that As₂O₃ induced time- and dose-dependent apoptosis in MKN45 cells, blocked at G₂/M phase. The apoptotic peak (sub-G₁ phase) appeared and cell apoptotic rate was 18.3-32.5% after being treated by 10 μmol/L As₂O₃ for 48 h (Tables 3 and 4). The percentage of G₂/M cells of the experimental groups was 2.0-5.0 times that of the control group. It demonstrated that As₂O₃ arrested cell cycle at G₂/M phase, inhibited cell proliferation and induced apoptosis at G₁ phase.

Table 3 Effect of As₂O₃ on apoptosis of MKN45 cells

Groups	Apoptosis (%)		
	24 h	48 h	72 h
Control	0.5±0.1	0.4±0.3	0.5±0.2
As ₂ O ₃ (μmol/L) 1	2.7±2.1 ^a	7.2±2.5 ^a	12.3±3.6 ^a
5	7.3±5.7 ^a	17.3±5.7 ^a	21.1±5.6 ^a
10.0	11.6±3.4 ^a	25.4±7.1 ^a	35.8±8.1 ^a

^aP<0.05 vs control.

Gel electrophoresis

Gel electrophoresis of DNA from cells treated with each concentration of As₂O₃ for 48 h revealed a "ladder" pattern, indicating preferential DNA degradation at the internucleosomal, linker DNA sections.

TUNEL assay

TUNEL assay also demonstrated strand breaks in DNA of MKN45 cells treated with As₂O₃, while control cells showed negative labeling.

DISCUSSION

The medicinal effect of As₂O₃ is of great significance, though it is also well known for its toxicity. As₂O₃ has been used as medicine for thousands of years in both the Chinese and Western societies. However, its use in cancer treatment was not discovered until 1970s^[1-5,8]. Since then, As₂O₃ has been found to be an effective anticancer drug in APL as well as non-APL leukemia^[9-11,16-18]. Recently, studies were carried out to expand the use of As₂O₃ in the treatment of solid tumors. The present study explored its effect on human gastric cancer cell line.

As₂O₃ has been used in clinical trials of APL for years and 10 mg/d of As₂O₃ was shown to be effective in inducing

complete remission of both the newly diagnosed and relapsed APL patients. The action mechanism of As₂O₃ is complicated. In APL, it has been reported that As₂O₃ exerted its effect by degradation of the fusion protein, PML/RARα, in turn, inducing differentiation and triggering apoptosis. This was supported by clinical trials. However, some studies showed that As₂O₃ mediated its effect in a PML/RARα independent manner. This suggested that the effect of PML/RARα is not restricted to cancers which express PML/RARα^[24-27,33]. In fact, apoptosis is one of the key pathways of As₂O₃, regardless of cell types. Studies showed that caspase-3 is activated upon As₂O₃ treatment. Caspases can be considered as the central trigger of apoptosis because they bring about most of the visible changes that characterize apoptotic cell death. Among various caspases, caspase-3 is one of the most crucial caspases^[28-32,34].

Mitochondria play an important role in apoptosis. As₂O₃ causes the collapse of mitochondrial membrane potential, indicating that mitochondria participate in As₂O₃-induced apoptosis. Disruption of the mitochondrial membrane potential and release of cytochrome *c* to the cytosol can be considered as the major event through which mitochondria participate in the induction of apoptosis. The change in the mitochondrial membrane potential upon As₂O₃ treatment was examined by flow cytometry, while the release of cytochrome *c* to the cytosol was detected by Western analysis. As₂O₃ recruits a number of pathways but the whole picture of its action mechanism is not fully understood^[35-39].

The present study showed that As₂O₃ exhibited strong anticancer activity on MKN45 cells via inhibition of proliferation and induction of apoptosis, which was both dose- and time-dependent in a certain range of dose with an IC₅₀ of (11.05±0.25) μmol/L. The apoptosis rate was increased 3.5 times when the concentration of As₂O₃ increased from 1 to 10 μmol/L. Gel electrophoresis of DNA and TUNEL demonstrated existence of apoptotic cells. Flow cytometry analysis showed that the apoptotic peak (sub-G₁ phase) changed considerably with the increase of concentration of As₂O₃ and, cell were blocked at G₂/M phase. The effect of As₂O₃ on cell cycle was obvious, while no distinct changes occurred in cell cycle treatment by different concentrations of As₂O₃ for 24 h being similar to control group. However, the cell cycle changed markedly after drug treatment for 48 h, G₀/G₁ phase decreased from 55.6%, 57.1%, 55.2% to 37.2%, 19.9%, 13.1% and the G₂/M phase increased from 16.3%, 16.7%, 17.5% to 22.6%, 41.5%, 69.0% in 1, 5, 10 μmol/L As₂O₃ treatment groups, respectively. The arrest of G₂/M phase become more

Table 4 Effect of As₂O₃ on cell cycle of MKN45 cells

Group	Cell cycle (%)								
	24 h			48 h			72 h		
	G ₁ /G ₀	S	G ₂ /M	G ₁ /G ₀	S	G ₂ /M	G ₁ /G ₀	S	G ₂ /M
Control	54.5	25.8	19.7	56.7	30.1	13.2	55.5	28.1	16.4
As ₂ O ₃ (μmol/L) 1	55.6	28.1	16.3	37.2	40.2	22.6 ^a	35.1	36.8	28.1 ^a
5	57.1	26.2	16.7	19.9	38.6	41.5 ^a	18.4	35.2	46.4 ^a
10.0	55.2	27.3	17.5	13.1	17.9	69.0 ^a	10.9	15.2	73.9 ^a

^aP<0.05 vs control.

apparent after treatment with As₂O₃ for 72 h, its proportion increased with the increase of concentration of As₂O₃ in a dose- and time-dependent manner. The proportion of cells in G₂/M phase was 2.0-5.0 times that of untreated cells after treatment with As₂O₃ for 48 h, demonstrating that the anticancer effect of As₂O₃ on MKN45 cells was attributed to the inhibition of cell proliferation, arrest of cell cycle and induction of apoptosis.

In conclusion, our study demonstrates the proliferation inhibition and apoptosis induction effects of As₂O₃ at the concentrations of 1, 5, and 10 μmol/L on human gastric cancer cell line MKN45. These results shed light on the use of As₂O₃ in treating human gastric cancer. To fully utilize As₂O₃ in cancer treatment, however, much more efforts should be made on the study of its action mechanism, pharmacokinetic characteristics, dosing schedules as well as potential adverse effects.

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