Arsenic Trioxide Induces Apoptosis in Human Gastric Cancer Cells Through Upregulation of p53 and Activation of Caspase 3

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Abbreviations used: APL, acute promyelocytic leukemia; As_2O_3, arsenic trioxide; PARP, poly-ADP ribose polymerase.

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ABSTRACT

Background and Aims: Arsenic trioxide (As_2O_3) can induce clinical remission in patients

Comment [D1]:

suffering from acute promyelocytic leukemia, through induction of apoptosis and activation

of caspases. We investigated the potential use of As_2O_3 in human gastric cancer and its

possible mechanisms. **_Methods_**: Human gastric cancer cell lines AGS and MKN-28 were

treated with various concentrations (0.1 to 100 μM) of As_2O_3 for 24-72 hours. Apoptosis was

determined by acridine orange staining, flow cytometry and DNA fragmentation. The protein

levels of p53, $p21^{waf1/cip1}$, c-myc, bcl-2 and bax were detected by Western blotting. Effects of

As_2O_3 on caspase 3 protease activity, its protein concentration and cleavage of poly-ADP

ribose polymerase (PARP) were also studied. **_Results_**: Triptolide inhibited cell growth and

induced apoptosis in both cell lines, although AGS cells were more sensitive. As_2O_3 induced

apoptosis in AGS cells following a concentration- and time-dependent manner. Treatment

resulted in a marked increase in p53 protein level as early as 4 hours. Co-incubation with p53

anti-sense oligo-nucleotide suppressed As_2O_3-induced intracellular p53 over-expression and

apoptosis. As_2O_3 increased the activity of caspase 3, with appearance of its p17-kD peptide

fragment, and cleavage of PARP with appearance of the 85-kD cleavage product, both in

parallel with the induction of apoptosis. The tripeptide caspase inhibitor zVAD-fmk or the

specific caspase 3 inhibitor DEVD-fmk both partially suppressed As_2O_3-induced caspase 3

activation and apoptosis. **_Conclusions:_** As_2O_3 inhibits cell growth and induces apoptosis in

gastric cancer cells, involving p53 overexpression and activation of caspase 3. The potential

use of this compound in treatment of gastric cancer is worth further investigation.

Introduction

Recently it has been shown that arsenic compounds, including the inorganic arsenic trioxide (As_2O_3) and arsenic disulfide, are effective for the treatment of patients with acute promyelocytic leukemia (APL) (Sun HD et al, 1992; Huang SL et al, 1995). Arsenic-containing compounds have been used in traditional Chinese medicine for many years. No significant bone marrow suppression was observed in most patients following administration of As_2O_3 (Shen ZX et al, 1997). In a study of fifteen patients with APL, none of those receiving arsenic alone developed significant thrombocytopenia or anemia. Among those who received arsenic with standard chemotherapy, only slight decrease in platelet and hemoglobin level was observed (Shen ZX et al, 1997). Recently the clinical efficacy of As_2O_3 in APL has been confirmed even in patients resistant to conventional chemotherapy (Soignet SL et al, 1998). In vitro studies on the molecular mechanism of As_2O_3 on APL cells showed that the inhibition on cell proliferation was due to a direct induction of apoptosis through down-regulation of bcl-2 expression and modulation of PML/RARα/PML protein (Chen GQ et al, 1996). Furthermore, the activation of caspases was also involved in As_2O_3-induced apoptosis in APL cells (Shen ZX et al, 1997). The apoptotic effect of As_2O_3 can be observed in various cell lines of either myeloid or lymphoid origin (Soignet S et al, 1996; Ishitsuka K et al, 1997). Wang et al reported that As_2O_3 and melarsoprol, the organic arsenical, induced apoptosis in myeloid leukemia cell lines such as HL-60, U937 and KG-1 through down-regulation of Bcl-2 protein (Wang ZG et al, 1998). Akao et al also showed that As_2O_3 induced apoptosis through activation of caspases in B-cell leukemia cell lines (Akao Y et al, 1998). This raised the interest of looking at its apoptotic effect in other tumors, including esophageal cancer, neuroblastoma, head and neck cancers, and cervical cancers (Shen ZY et al,1999; Akao Y et al,1999; Seol JG et al,1999; Zheng J et al,1999). Two studies using As_2O_3 against six and seventeen human cancer cell lines respectively found that the most marked effects were seen in bladder cancer, APL and gastrointestinal cancer cells, including the human gastric cancer

cell line MGC-803 (Zhang TC et al, 1999; Yang CH et al, 1999).

It has been shown that tumors develop not only from abnormal cell proliferation, but also from reduced cell death due to inhibition of apoptosis. (Bergamaschi G et al, 1994) The possibility of modulating apoptosis of tumor cells suggests new strategies for improving chemotherapy. (Steller H et al, 1996) Apoptosis is regulated by gene products, which are conserved from nematodes to mammals (Penninger JM et al, 1998). Among all apoptosis-related genes, *p53* is of particular importance. (Lowe SW et al, 1993) *P53* is well known for suppression of cellular proliferation through two mechanisms, each operating in a distinct manner. In normal fibroblasts, *p53* induces G_1 arrest in response to DNA-damaging agents, presumably allowing the cells to perform critical repair functions before progressing through the cell cycle (Linke SP et al, 1997). On the other hand, in abnormally proliferating cells or irradiated thymocytes, induction of *p53* leads to apoptosis (Midgley CA et al, 1995). Furthermore, wild-type p53 protein level was increased during apoptosis induced by DNA-damaging agents (Zhan Q et al, 1996). The increased expression of wild type *p53* can induce apoptosis in myeloid leukemia and colon cancers (Yonish-rouach E et al, 1991; Shaw P et al, 1992). Recently, changes of DNA methylation in the *p53* gene promoter induced by arsenic treatment have been shown in cultured human lung cells, which indicated *p53* could be a target of arsenic (Mass MJ et al, 1997). On the contrary, arsenic treatment in JB6 cell lines did not induce *p53*-dependent transactivation (Huang CS et al, 1999). This may indicate that the response or involvement of *p53* in apoptosis is cell type specific. Our previous studies showed that indomethacin induced apoptosis in AGS gastric cancer cells without induction of *p53* mRNA and protein level while protein kinase C inhibitors induced apoptosis in the same cells with increase in p53 protein level (Zhu GH et al, 1999a, 1999b, 1999c).

Members of the caspase family play key roles in the execution phase of apoptosis (Cohen GM et al, 1997). These cysteine proteases are synthesized as inactive proenzymes

which are activated by different apoptotic stimuli, leading to specific cleavage of a range of cellular protein substrates (Alnemri ES et al, 1997). These substrates include enzymes that are involved in genome surveillance and DNA repair such as poly ADP-ribose polymerase (PARP). Among the family of ten or more different caspases already described, caspase 3 is a major effector of apoptosis. Caspase 3 is activated in a variety of cell types during apoptosis including As_2O_3-treated leukemia cell lines (Soignet SL et al, 1998; Schlegel J et al, 1996; Datta R et al, 1996). The importance of caspase 3 has been further confirmed by the study that caspase 3 knockout mice suffered from severe developmental abnormalities attributed to the disturbed regulation of apoptosis (Kueda K et al, 1996).

In this study, we showed that As_2O_3 is a potent inducer of apoptosis in AGS cells. The apoptosis is associated with upregulation of p53 and a rapid activation of caspase 3 protease, with the proteolytic degradation of PARP. Our results suggest that both p53 and caspase 3 are involved in apoptotic cell death induced by arsenic trioxide in gastric cancer.

Materials and Methods

Drugs

0.1% As_2O_3 solution for intravenous administration was kindly provided by the Pharmacy of Chinese Traditional Medicine in the First Hospital affiliated to Harbin Medical University (Harbin, People's Republic of China). Stock solutions were made at the concentration of 1 mmol/L with phosphate-buffered saline (PBS) and diluted to working concentrations before use.

Cell Culture and As_2O_3 Treatment

AGS, a gastric adenocarcinoma cell line bearing wild-type p53, was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). MKN-28, a gastric adenocarcinoma cell line, does not contain wild-type *p53*, but has missense mutation (codon 251, isoleucine to leucine (Matozaki T et al, 1992). This was kindly donated by Professor SD Xiao (Shanghai Second Medical University). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Life Technologies, NY, USA) in a humidified atmosphere of 95% air with 5% CO_2 at 37^0C as a monolayer. The cells were seeded 24h before drug treatment and were 60-70% confluent at the time of treatment. Different concentrations of As_2O_3 (0.1μM to 100μM) were added to the medium for different time period. After treatment, adherent cells were removed by trypsinization and combined with floating cells in the medium for further analysis.

MTT Assay

Cell growth was measured by a modified MTT assay (Boehringer Mannheim, BMGH, Germany) (Carmichael J et al, 1985). About 8,000 cells per well were grown in 96-well plates

and incubated overnight in 100 μl of culture medium. Then cells were treated with different concentrations of As_2O_3 for fixed time intervals. Ten μl MTT labeling reagent (final concentration 0.5mg/ml) was added to each well, and the cells were further incubated at 37^0C for 4 hours. The supernatant was removed and 100μl of 0.04 M hydrochloric acid in isopropanol was added to each well. A micro ELISA reader (BioRad, CA, USA) was used to measure the absorbency at a wavelength of 595 nm. The blank control contained the cell culture medium only. Each assay was performed three times in triplicates.

Acridine Orange Staining

Single cell suspensions were fixed in 1% formalin/ PBS and stained with acridine orange (AO, 10 μg/ml, Sigma, St. Louis, USA). Cells were spotted on glass slides and visualized under fluorescence microscope. Apoptotic cells were defined as cells showing cytoplasmic and nuclear shrinkage and chromatin condensation or fragmentation morphologically. At least 300-cells/ field were counted to determine the apoptotic index.

Flow cytometry

Cells were collected and fixed in ice-cold 70% ethanol in PBS and stored at -20^0C before use. After resuspension, cells were washed and incubated with 100 μl of RNAase I (1mg ml^{-1}) and 100 μl of propidium iodide (PI, 400 μg/ml, Sigma) at 37^0C for 30 min. The analysis of samples was performed by a flow cytometry (Coulter Epics XL, UK). The cell cycle phase distribution was calculated from the resultant DNA histogram using Multicycle AV software (Phoenix Flow System, San Diego, CA, USA). The apoptotic cells can be observed as a subdiploid or 'pre-G_1' peak on the DNA histogram.

DNA Fragmentation Analysis

DNA fragmentation was analyzed as described previously (Grant S et al, 1992) with some

modification. Briefly, cells were harvested and rinsed twice in ice-cold PBS. The final pellets were lysed in 0.3 ml 10 mM Tris-HCl (pH7.4) buffer containing 25 mM EDTA, 0.5% SDS and 0.1mg/ml proteinase K (Sigma, St. Louis, USA) and incubated at 50^0C for 12-18 hours. DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with two volumes of ice-cold absolute ethanol and 1/10 volume 3M sodium acetate. DNA was collected, washed once with 70% ethanol, and dissolved in TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0). The samples were incubated with 10 μg/ml RNase I for 1 hour at 37^0C. Equal amounts (10μg per well) of DNA was electrophoresed in 1.8% agarose gels impregnated with ethidium bromide (0.1μg/ml) for 2 hours at 80V. DNA fragments in the form of laddering pattern were visualized by ultraviolet transillumination.

Western Blotting

After treatment, the whole cell lysates were extracted with lysis buffer containing 1% Triton-100, 50 mM sodium chloride, 50 mM sodium fluoride, 20 mM Tris (pH7.4), 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 0.2 mM phenymethylsulfonyl floride and 0.5% Nonidet P-40. The protein concentration was determined by using Bicinchoninic acid assay (BCA)(Smith PK et al, 1985) with bovine serum albumin (Sigma, St. Louis, USA) as standard. Western blotting was carried out as described previously (Li Y et al, 1996). In brief, equal amount of cell lysates (60μg) were solublized in 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Sigma, St. Louis, USA). After blocking, the membranes were incubated with the appropriate diluted primary antibody. Then membranes were incubated with a horseradish peroxidase-conjugated secondary antibody. Proteins were detected by the enhanced chemiluminescence (ECL) detection system (Amersham, Buckinghamshire, England). Mouse monoclonal antibodies against p53 (Ab-6), p21$^{waf1/cip1}$ (Ab-1), c-myc (Ab-1) and peroxidase-conjugated anti-mouse IgG were purchased from Oncogene Research Products (CalBiochem, La Jolla, CA, USA).

Monoclonal antibodies against bcl-2 (100) and bax (B-9) were purchased from Santa-Cruz Biotechnology (CA, USA). Rabbit polyclonal antibody against cpp-32 and mouse monoclonal antibody against PARP (c-20) were purchased from Pharmingen (San Diego, CA, USA).

Caspase 3 Activity Assay

Caspase 3 activity was determined using the Clontech ApoAlert CPP32 Assay Kit according to the manufacturer's instructions (Clontech, Palo Alto, CA). These assays depended upon the cleavage of a specific colorimetric caspase substrate, DEVD-ρNA (Asp-glu-val-asp-ρ-nitroanilide). Cells were plated into 60mm dishes at 2×10^6cells/dish and cultured for 24 hours before treatment. After treatment, cells were collected by scraping in cold PBS, centrifuged (2000rpm, 8min) and lysed in the Cell Lysis Buffer provided in the kit on ice for 10 min. The extracts were frozen and maintained at -70^0C until the time of assay. At that time, the extracts were thawed and reacted with an equal volume of 2×Reaction Buffer containing DTT (10mM) and the colorimetric caspase-3 substrate (DEVD-ρNA). The mixtures were maintained in a water bath at 37^0C for 45 min and then analyzed in a spectrophotometer at 405nm.

Statistical Analysis

The data shown were mean values of at least three different experiments and expressed as means±S.D. Student's t-test was used to compare the result. A p-value of less than 0.05 is considered statistically significant.

Results

Effect of As$_2$O$_3$ on Cell Growth

We examined the antiproliferative effect of As$_2$O$_3$ on human gastric cancer cell lines AGS and MKN-28 by exposing them to different concentrations of As$_2$O$_3$ for 24 to 72hrs. Cell viability was determined by MTT assay. When AGS and MKN-28 cells were exposed to As$_2$O$_3$ ranging from 0.1μM to 100μM, the growth of both cell lines was inhibited in a dose dependent manner. However, AGS cells were much more sensitive to growth inhibition by As$_2$O$_3$ than MKN-28 cells (data not shown).

Apoptosis of Gastric Cancer Cells Induced by As$_2$O$_3$

Morphological changes typical of apoptosis, including chromatin condensation, fragmentation of the nuclei, and formation of apoptotic bodies were observed in AGS cells after 12 hours with the concentration of more than 1 μM (Figure 1). The percentage of apoptotic cells increased in a time- and dose-dependent manner. Approximately 25% of the AGS cells underwent apoptosis with treatment of 10 μM As$_2$O$_3$ for 24 hours. However, only about 4.8% MKN-28 cells died after 24 hours treatment with the same concentration (data not shown). Apoptosis was further confirmed by agarose gel electrophoresis of the genomic DNA. A characteristic pattern of nucleosomal-sized ladders of DNA fragments, which characterized programmed cell death, was detected after 24 hours exposure to As$_2$O$_3$ (1μM, 10μM) in AGS cells (Figure 2). FACS analysis showed that As$_2$O$_3$ could induce apoptosis in AGS cells, with a typical sub-diploid peak on the DNA histogram (Figure 3). Taken together, these results demonstrated that AGS cells, which contained wild type *p53*, were more sensitive to As$_2$O$_3$-induced apoptosis than MKN-28 cells.

Effects of As$_2$O$_3$ on the cell cycle phase distribution

To investigate the antiproliferative mechanisms of As_2O_3, the cell cycle phase distribution of AGS cells treated with different concentrations of As_2O_3 were analyzed by flow cytometry. The results showed that after the indicated treatments for 24 hours, there was no obvious alteration of each cell cycle phase when compared with the control (Figure 3).

Effects of As_2O_3 on p53, p21$^{waf1/cip1}$, c-myc, bcl-2 and bax Protein Levels

To further evaluate the mechanism of As_2O_3-induced apoptosis in gastric cancer, we examined the changes in protein expression of apoptotic-related genes including p53, p21$^{waf1/cip1}$, c-myc, bcl-2 and bax. Treatment with 1 µM As_2O_3 resulted in a marked increase in p53 protein level noted as early as 4 hours and reached a maximum at 24 hours which was approximately 3 times that of the control (Figure 4). However, there were no apparent changes of protein levels in other apoptosis-related genes, i.e. p21$^{waf1/cip1}$, c-myc, bcl-2 and bax during the course of treatment (Figure 4). In contrast, in MKN-28 cells there was no detectable change of these apoptotic-related genes during the whole period of treatment (data not shown).

Down-Regulation of p53 with Its Antisense Oligonucleotides on AGS-Induced Apoptosis

The role of p53 in AGS-induced apoptosis was confirmed by using various concentrations of antisense oligonucleotides. The phosphorothioate antisense oligonucleotide for p53 and the control CG-matched randomized-sequence phosphorothioate oligonucleotides were purchased from Biognostik (GÖttingen, FRG). Cells growing in log phase were seeded in 24-well plates at a density of 1×10^5 cells per well. In order to enhance the uptake of oligonucleotide, we pre-incubated 0.8µl oligos with lipofectin (5 µg/ml, Life technologies, Gaithersburg, MD, USA) for 30 minutes, then add to cultured cells and incubate for another 30 minutes. After washing, supplemented medium containing 5µM oligos were added for 18 hours. Then As_2O_3 (1 µM) was added to the culture medium for a fixed time period for further analysis. Figure 5 showed

that 5 μM p53 antisense oligonucleotide could effectively inhibit the As_2O_3-induced increase in p53 protein level after 12 hours, whereas the control oligonucleotide showed no effect on p53 expression. The p53 antisense oligonucleotide also inhibited the As_2O_3-induced apoptosis. At 1 μM of As_2O_3, the percentage of apoptotic cells decreased from 29.5% ± 2.3% to 8.9% ± 1.4% by the addition of 5 μM of p53 antisense oligonucleotide ($p<0.05$). The same concentration of control oligonucleotide showed no effect on apoptosis induced by As_2O_3 (Figure 6).

Effect of As_2O_3 in activation of caspase 3 and cleavage of poly (ADP-ribose) polymerase (PARP)

Caspases, especially caspase 3 (CPP-32), have been shown to participate in apoptosis. To see if apoptosis induced by As_2O_3 was regulated by caspase 3, we examined several aspects of the caspase 3 activation. In the first experiment, different concentrations of As_2O_3 were added to AGS cells for 0, 3, 6, 12, 24 and 48 hours. After treatment, caspase 3 protease activity was assessed in cell lysates by measuring hydrolysis of colorimetric caspase 3 substrate DEVD-ρNA. Figure 7 illustrated the time course of caspase 3 protease activity in AGS cells after treatment with 1 μM As_2O_3. Caspase 3 activity began to increase after 3 hours and reached a maximal level after 24 hours in a linear fashion. In the next experiment, the expression of caspase 3 during apoptosis was studied by Western blot analysis. The 32 kD procaspase 3, when activated, was cleaved into two subunits of 17 and 12 kD assembled into enzymatically active heterotetramers as shown in Figure 8*A*. The amount of proenzyme decreased with the appearance of the 17 kD active form with increasing concentration of As_2O_3, in parallel with the degree of apoptosis. To further confirm the involvement of caspase 3 in As_2O_3-induced apoptosis, we took advantage of the general property of the caspase family of leading to the specific cleavage of certain substrates during apoptosis, such as poly (ADP-ribose)

polymerase (PARP). An anti-PARP antibody capable of detecting both the uncleaved 116 kD proform and the active cleaved 85 kD fragments of PARP was used in Western blotting. The 85 kD fragment cleaved from the 116 kD PARP was detected in extracts from AGS cells treated with As_2O_3 at concentrations of 1 μM and 10 μM after 24 hours (Figure 8*B*). Both caspase 3 activation and PARP cleavage followed a concentration-dependent manner, which were in parallel with the induction of apoptosis.

Caspase inhibitors suppressed As_2O_3-induced caspase 3 activation and apoptosis

Although caspase activation and proteolysis are hallmarks of apoptosis, inhibition of caspases does not always prevent cells from undergoing apoptosis, suggesting the existence of caspase-independent pathway. To confirm the essential role of caspase 3 in As_2O_3-induced apoptosis, we next analyzed the effect of a pan-inhibitor of the caspase family, zVAD-fmk, and a specific inhibitor for caspase 3, DEVD-fmk, on apoptosis. We first determined the concentration of zVAD-fmk and DEVD-fmk required to inhibit caspase 3 activity. For this, we studied the inhibition by zVAD-fmk and DEVD-fmk of the proteolytic cleavage of caspase 3 induced by As_2O_3 in AGS cells. As shown in Figure 9, the cleavage of caspase 3 was completely blocked by 100 μM zVAD-fmk and 100 μM DEVD-fmk. At this concentration, both zVAD-fmk and DEVD-fmk suppressed As_2O_3–induced apoptosis (Figure 10).

Discussion

Since the first observation of the relationship between arsenic and skin cancer in the 1820's, arsenic compounds have been generally accepted as a potent environmental carcinogen, more likely as a comutagen and cocarcinogen for human skin and lung cancers (Bishop C et al, 1978). However, at a low concentration, some arsenic compounds have beneficial effects, such as the stimulation of human hematopoiesis. The use of arsenic compounds as therapeutic drug has a long history in traditional Chinese medicine. Recently arsenic trioxide (As_2O_3) induced clinical remission in all-trans-retinoic acid-resistant APL patients. (Shen ZX et al, 1997) The plasma concentration of As_2O_3 was sustained at 1-2 μM, regarded as a therapeutic and cancer-selective range in treating APL patients with minimal toxicity. The mechanism of action of arsenic trioxide was shown to be the induction of apoptosis (Shen ZX et al, 1997). Subsequently a lot of tumors have been tested and the apoptosis-inducing effects were more profound in bladder cancer, APL and gastrointestinal cancer cells, including the human gastric cancer cell line MGC-803 (Zheng J et al, 1999; Penninger JM et al, 1998).

In the present study, we have shown that As_2O_3 inhibited the growth and induced apoptosis in gastric cancer cells in a time- and concentration-dependent manner. AGS cells bearing wild-type p53 gene were more sensitive to the growth inhibitory effect of As_2O_3 than MKN-28 cells with mutant p53. Similarly, As_2O_3-induced apoptosis occurred more readily in AGS than in MKN-28 cells. We have also shown that As_2O_3 upregulated p53 expression at the protein level only in AGS cells, which contributed to the induction of apoptosis. This finding agrees

with previous study showing that sodium arsenate induced an increase in p53 protein level which might be activated in response to DNA damage (Salazar AM et al, 1997). The tumor suppressor gene p53 is an essential mediator of the cellular response to DNA damage in mammalian cells. Both G_1 cell cycle arrest (Kastan,MB et al,1991) and apoptosis (Clarke,AR et al,1993; Lowe,SW et al,1993) after DNA damage were shown to depend on normal p53 function (Bates, S et al,1996). The exact mediators of apoptosis after p53 activation are not known. Müller M et al showed that wt p53 bound to and transactivated the CD95 gene, whereas mt p53 failed to induce apoptosis via activation of the CD95 gene (Müller, M et al, 1998). In our experiment, the role of p53 was further confirmed by using p53 antisense oligonucleotide, which significantly suppressed the As_2O_3- induced apoptosis in AGS cells. Thus, the reduced sensitivity to growth inhibition and apoptosis induction in MKN-28 cells compared to AGS cells may be in part explained by the effect of p53 mutation. Some clinical studies indicated a trend for association between wtp53 status and sensitivity to chemotherapy (Tetu, B et al, 1998). Our results showed that p53 status in human gastric cancer cells might contribute to the sensitivity to cancer treatment, mainly in the readiness to undergo apoptosis. Furthermore, we suggest a p53-independent pathway might exist in As_2O_3- induced apoptosis in MKN-28 cells.

We showed no changes in the expression of p21$^{waf1/cip1}$, as well as cell cycle phase distribution. Similar results have been reported for myeloid leukemia S6 cells and hepatoma HepG2 cells, where the induction of wild type *p53* failed to arrest cell growth during apoptosis (Youish-Rouach E et al, 1993; Jiang MC et al, 1996). The role of *p21$^{waf1/cip1}$* in arsenic-induced apoptosis was only reported in PCI-1 cell line in which the increased protein level of p21$^{waf1/cip1}$ was accompanied by G_2/M arrest and reduction of cdc2 kinase activity (Seol JG et al, 1999). Increased p21$^{waf1/cip1}$ expression has been correlated with both apoptosis

and cell survival, indicating that its role is cell type specific (Sheikh MS et al, 1995; Kobayashi T et al, 1995). Our previous studies on AGS cells suggested that overexpression of p21[waf1/cip1] enhanced cell survival and protected against indomethacin-induced apoptosis (Zhu GH et al, 2000). This suggests that the effect of arsenic on p21[waf1/cip1] expression and involvement in apoptosis is cell type specific.

We have shown that As_2O_3-induced apoptosis was controlled through caspase activation, especially caspase 3. Caspase 3 or CPP32 is one of the important candidates for cell death-inducing proteases that cleave PARP and other vital proteins (Patel T et al, 1996). Recently, it was reported that caspase 3 was involved in As_2O_3-induced apoptosis in APL, B-cell leukemia cell lines and neuroblastoma cell lines (Soignet SL et al, 1998; Akao Y et al, 1998; Akao Y et al, 1999). In this study, As_2O_3 induced caspase 3 activation and PARP cleavage in parallel with the induction of apoptosis. Furthermore, caspase inhibitors could effectively block the cleavage of caspase 3. However, both zVAD-fmk and DEVD-fmk could only partially inhibit As_2O_3-induced apoptosis in AGS cells. These results indicated that the activation of caspase 3 was involved in arsenic-induced apoptosis. But it is likely that As_2O_3 could also induce apoptosis partly via a caspase-independent pathway.

As_2O_3 has been intensively investigated as a new treatment for cancer. It has become apparent that the mechanism of action of As_2O_3 is closely related to its ability to induce apoptosis. Recent studies showed that As_2O_3 produced a preferential vascular shutdown in a fibrosarcoma animal model, leading to massive necrosis of the tumor, while normal skin, muscle and kidney were relatively unaffected (Lew YS et al, 1999). Our study and others have shown that gastric cancer cells were more sensitive to As_2O_3 than other tumors. The potential use of As_2O_3 in treatment of gastric cancer is worth further exploration.

Acknowledgement

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Legend

Figure 1. Effect of As_2O_3 treatment on the morphology of AGS cells. Cells were treated with $1\mu M$ As_2O_3 for 24 hours, stained with acridine orange, and analyzed under fluorescence microscope. (A) Control, (B) treated with As_2O_3.

Figure 2. Induction of DNA fragmentation by As_2O_3 treatment. AGS cells were exposed to As_2O_3 and the formation of DNA fragmentation was determined by agarose gel electrophoresis. M, DNA marker; Lane 1, $10\mu M$ As_2O_3; Lane 2, $1\mu M$ As_2O_3; Lane 3, 0.1 μM As_2O_3; Lane 4, control.

Figure 3. The results of FACS analysis of AGS cells treated with As_2O_3. Cells were treated with As_2O_3 for 24 hours and their DNA content was determined by FACS, as described in Materials and Methods. (A) Control (B) $10\mu M$ As_2O_3

Figure 4. The expression of p53, $p21^{waf1/cip1}$, c-myc, bcl-2 and bax protein in AGS cells treated by As_2O_3. (A) AGS cells were exposed to $1\mu M$ As_2O_3 for different intervals (0, 4, 8, 12, 24, 48 hours). The protein levels of p53, $p21^{waf1/cip1}$, c-myc, bcl-2 and bax were determined by Western blotting. Each blot is the representative of three similar experiments.

Figure 5. Effect of oligonucleotides on p53 protein expression. AGS cells were cultured with antisense or control oligonucleotide (5 μM) for 18 hours before incubation with $1\mu M$ As_2O_3 for another 24 hours, after which cell lysates were collected and p53 protein was determined by Western blotting. Lane 1, control; Lane 2, 1 μM As_2O_3 for 12 hours; Lane 3, 1 μM As_2O_3 + 5 μM control oligos for 12 hours; Lane 4, 1 μM As_2O_3 + 5 μM antisense oligos for 12

hours; Lane 5, 1 μM As_2O_3 for 24 hours; Lane 6, 1 μM As_2O_3 + 5 μM control oligos for 24 hours; Lane 7, 1μM As_2O_3 + 5 μM antisense oligos for 24 hours. This experiment was repeated three times and one representative result is shown in this figure.

Figure 6. Suppression of As_2O_3-induced apoptosis by p53 antisense oligonucleotides. AGS cells were pretreated with 5 μM control oligos or anti-sense oligos for 18 hours followed by 1 μM As_2O_3 for 48 hours. The percentage of apoptotic cells was measured by acridine orange staining. The results are expressed as mean $\pm$ SE from three different experiments.   *, $P < 0.05$ Vs untreated control; **, $p < 0.05$ Vs As_2O_3 alone.

Figure 7. Effect of As_2O_3 on caspase 3 activity in AGS cells. AGS cells were treated with 1 μM As_2O_3 for 0, 3, 6, 12, 24 and 48 hours. Protease activity at each time point was assessed against the substrate DEVD-ρNA. The data are expressed as the mean $\pm$ SD of three different experiments.

Figure 8. Effect of As_2O_3 on caspase 3 and PARP expression in AGS cells. Cells were treated with 0.1, 1, and 10 μM As_2O_3 for 24 hours. Caspase 3 expression and PARP cleavage were analyzed by Western blotting with specific antibodies. (A) Caspase 3; (B) PARP. This experiment was repeated three times and one representative result is shown in this figure.

Figure 9. Inhibition of caspase 3 expression and cleavage in As_2O_3-treated AGS cells by specific caspase inhibitors. Cells were cultured for 24 hours in the absence (-) or presence of 1μM As_2O_3 and 100 μM zVAD-fmk or 100 μM DEVD-fmk. The expression and cleavage of caspase 3 was determined by immunoblotting. This experiment was repeated three times and one representative result is shown in this figure.

Figure 10. Effect of caspase inhibitors on As_2O_3-induced apoptosis in AGS cells. Cells were pretreated with 100 μM zVAD-fmk or 100 μM DEVD-fmk for 12 hours and followed by 1 μM As_2O_3 for another 24 hours. The percentage of apoptosis was determined by acridine orange staining. The results are expressed as mean $\pm$ SE from three different experiments.
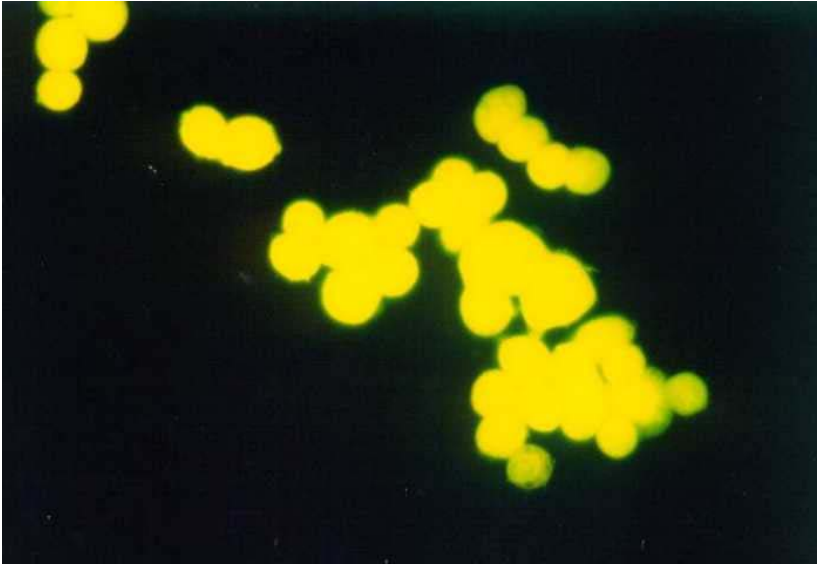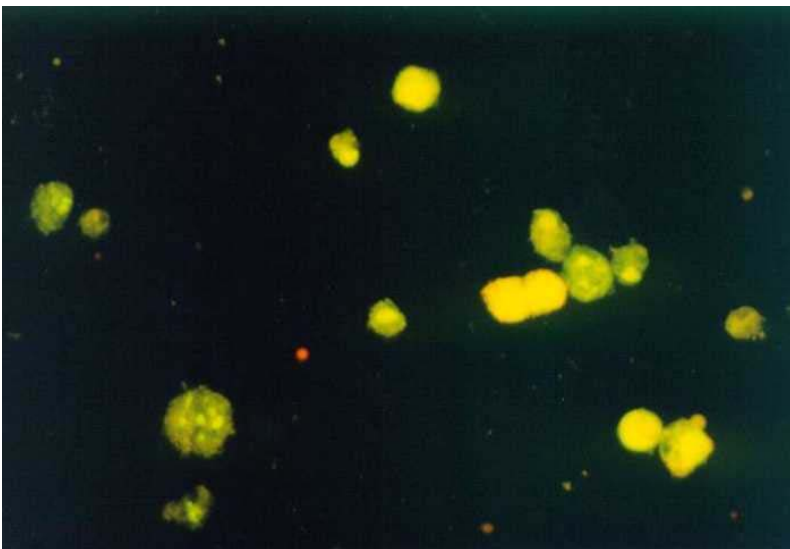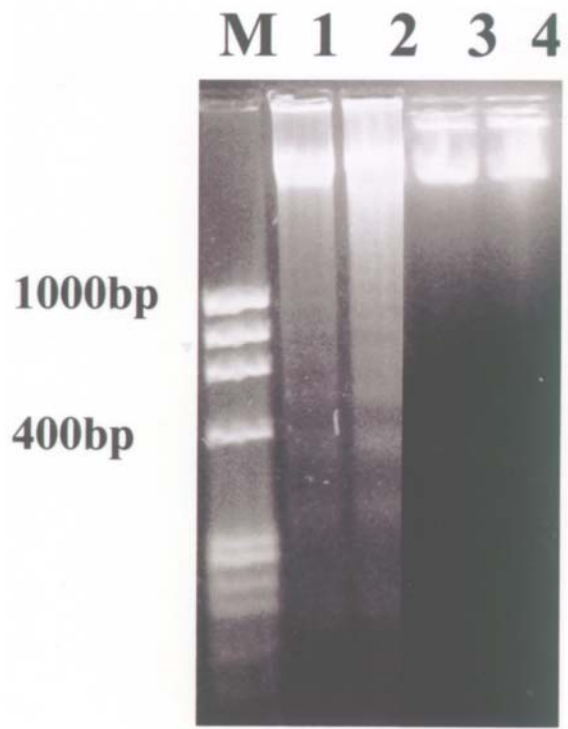
Figure 1a

Figure 1b

Figure 2

Figure 3

a

Cell Number

G0/G1=58.5%

S=33.2%

G2/M=8.4%

Apop=3.8%

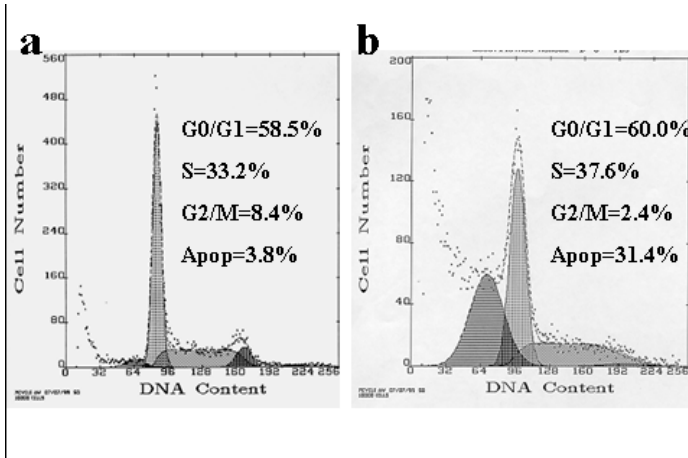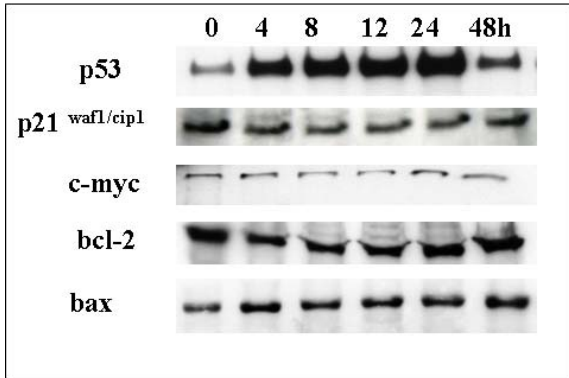DNA Content

b

Cell Number

G0/G1=60.0%

S=37.6%

G2/M=2.4%

Apop=31.4%

DNA Content

Figure 4

Figure 5

Figure 8

Figure 9

A

| As$_2$O$_3$ 1μM | (-) | (-) | (+) | (+) |
| zVAD-fmk 100μM | (-) | (+) | (+) | (-) |

——32kDa

——17kDa

B

| As$_2$O$_3$ 1μM | (-) | (-) | (+) | (+) |
| DEVD-fmk 100μM | (-) | (+) | (+) | (-) |

—32kDa

—17kDa