Arsenic Trioxide (As_2O_3) - Induced Apoptosis and MAP Kinase Signaling Mechanism in Chronic Myelogenous Leukemia K562 Cells

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Moon-Jeong Shim

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CONTENTS

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ABBREVIATION

ATF	:	activating transcription factor
BSA	:	bovine serum albumin
Caspase	:	cysteine-dependent aspartate-directed protease
DMSO	:	dimethylsulfoxide
DNA	:	deoxyribonucleic acid
dNTP	:	deoxyribonucleotide triphosphate
DTT	:	dithiothreitol
EDTA	:	ethylenediaminetetraacetic acid
EDTA	:	ethylene glycol-bis (β-aminoethyl ether)N,N,N',N,-tetraacetic acid.
ELISA	:	enzyme-linked immunosorbent assay
ERK	:	extracellular signal-regulated kinase
FACS	:	fluorescent activated cell sorter
FCS	:	fetal calf serum
IC_{50}	:	50% inhibitory concentration
JNK	:	c-Jun N-terminal kinase
MAP	:	mitogen-activated protein
MEK	:	MAP kinase kinase
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide

PBS : phosphate-buffered saline

PI : propidium iodide

PMSF : phenylmethylsulfonyl fluoride

RT-PCR : reverse transcriptase-polymerase chain reaction

SAPK : stress-activated protein kinase

SDS-PAGE : sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TAE : tris-acetate EDTA

TBS : tris-buffered saline

Z-VAD-fmk: Z-Val-Ala-Asp(Ome)-fluoromethylketone

ABSTRACT

Arsenic Trioxide (As_2O_3) – Induced Apoptosis and MAP Kinase Signaling Mechanism in Chronic Myelogenous Leukemia K562 Cells

Arsenic trioxide (As_2O_3) has recently been demonstrated to be an effective inducer of apoptosis in patients with relapsed acute promyelocytic leukemia (APL) and in patients with APL in whom all-*trans*-retinoic acid and conventional chemotherapy failed. Chronic myelogenous leukemia (CML) cells are highly resistant to chemotherapeutic drugs. This resistance is mediated by the chimeric tyrosine kinase oncogene *bcr-abl*.

To determine if As_2O_3 might be useful for treatment of CML, the ability of As_2O_3 to induce apoptosis in K562 cells was examined. *In vitro* cytotoxicity of As_2O_3 was evaluated in K562 cells by MTT assay. The IC_{50} value for As_2O_3 was to be 10 μM. When analyzed by agarose

gel electrophoresis, the DNA fragmentation became evident after the incubation of the cells with 20 μM of As_2O_3 for 12 h and 24 h, respectively. Morphological changes and chromatin condensation of the cells undergoing apoptosis were also observed. After the treatment of 20 μM As_2O_3, activation of caspase-3 increased at 12 h and 24 h and decreased at 36 h and 48 h. In addition, pretreatment of Z-VAD-fmk, a specific inhibitor of caspase, decreased As_2O_3-induced cytotoxicity. And DNA flow cytometric analysis showed that As_2O_3 induce G2/M arrest in these cells. The effect of As_2O_3 on expression of Bcr-Abl oncoprotein was also examined. In result, the treatment of As_2O_3 downregulated not only the expression of Bcr-Abl oncoprotein but also *bcr-abl* mRNA levels, suggesting a transcriptional regulation.

Next, to examine how As_2O_3 affects the mitogen-activated protein (MAP) kinase signaling pathway, the relationship between MAP kinase response in K562 cells treated with As_2O_3 was investigated. As_2O_3 at 10 μM strongly induced the activation of p38, and JNK 1/2, while ERK 1/2 were inhibited. MEK 3/6, which acts on upstream of p38, and ATF-2, a transcription factor, were also activated after treatment of As_2O_3. In addition, pretreatment of SB203580, a specific inhibitor of p38, blocked As_2O_3–induced DNA fragmentation apoptotic cell death. These results suggest that As_2O_3 is able to induce

the apoptotic activity of K562 cells and its mechanism may be associated with down-regulation of Bcr-Abl oncoprotein, the activation of caspase-3 and p38 kinase. This result implicates that As_2O_3 may be a potential agent for treatment of CML.

Key words: Arsenic trioxide (As_2O_3), Chronic myelogenous leukemia (CML), Bcr-Abl, Apoptosis, Cytotoxicity, Caspase-3, Cell cycle, p38 MAP kinase

CHAPTER I

Arsenic Trioxide (As_2O_3) – Induced Apoptosis, Cell Cycle Arrest and Down Regulation of BCR-ABL in K562 Cells

I. INTRODUCTION

Arsenic compounds have been accepted as effective chemo-therapeutics for lung cancer, skin cancer and other diseases. The use of arsenic compounds as therapeutic drugs has a long history in Chinese traditional medicine. Fortunately, arsenic dose not produce any myelosuppression in most patients receiving arsenic trioxide (As_2O_3) treatment (Shen *et al.*, 1997). Recently, As_2O_3 has been demonstrated to be an effective drug in the treatment of acute promyelocytic leukemia (APL), via induction of differentiation and apoptosis (Shen *et al.*, 1997; Soignet *et al.*, 1998). The NB4 promyelocytic leukemia cell line has been extensively used as a model for many *in vitro* studies designed to understand the cellular and molecular effects underlying the mechanism of action of As_2O_3 (Mu *et al.*, 1994; Chen *et al.*, 1997; Wang *et al.*, 1998). As_2O_3 is usually considered a nongenotoxic agent and is assumed to act principally through an epigenetic effect by interfering with intracellular signaling molecules that lead to cell cycle progression, DNA repair, ubiquitination, tubulin polymerization, transcription factor activation, and oncogene expression (Cavigelli *et al.*, 1996; Hayes, 1997).

Clinical efficacy of As_2O_3 in APL has been confirmed even in patients

resistant to conventional chemotherapy (Soignet *et al.*, 1998). As_2O_3 has been successfully used intravenously, with limited toxicity, for the treatment of APL. Nine of ten patients who received only As_2O_3 did not develop thrombocytopenia or anemia. This suggests that therapeutic doses of As_2O_3 may not induce significant hematological toxicity, as do those of other anticancer therapies. Moreover, As_2O_3 produced a complete remission in patients who had relapses of APL that were resistant to all-trans-retinoic acid and chemotherapy, implying an alternative mode of action (Shen *et al.*, 1997; Soignet *et al.*, 1998).

Molecular mechanisms underlying cellular functions by As_2O_3, however, have been poorly investigated. The consensus of several reports is that As_2O_3 induces apoptosis in leukemia cells by activating genes for apoptosis (Chen *et al.*, 1996; Soignet *et al.*, 1998). Enhanced translocation of promyelocytic leukemia proteins to nuclear bodies, down regulation of Bcl-2 (Konig *et al.*, 1997; Akao *et al.*, 1998; Wang *et al.*, 1998), modification of glutathione redox system (Chen *et al.*, 1998; Dai *et al.*, 1999), caspase activation (Chen *et al.*, 1998; Soignet *et al.*, 1998) and cell cycle arrest (Bazarbachi *et al.*, 1999) have been suggested to participate in the process leading to cell death.

Recent reports have suggested that the apoptotic effect of As_2O_3 is not specific for APL cells, and newly recognized potential efficacy of this

chemotherapeutic agent for the treatment of other human malignancies and myeloproliferative syndromes is currently under study (Zhang *et al*., 1998; Bazarbachi *et al*., 1999; Rousselot *et al*., 1999).

Apoptosis is a highly regulated cell death that responds to a wide variety of stimuli, including developmental signals, cellular stress, disruption of cell cycle, and different kinds of chemicals (Ochi *et al*., 1996; Basu *et al*., 1998; Dragovich *et al*., 1998; Huang *et al*., 1998). However, the inhibition of programmed cell death, or apoptosis, is an important component of oncogenesis because its inhibition may provide a selective growth advantage to tumors (Thompson. 1995). In fact, the inhibition of apoptosis is thought to play a critical role in Bcr-Abl mediated leukemogenesis *in vivo* (Clarkson et al., 1993). Tumors develop not only from abnormal cell proliferation but also from reduced cell death due to inhibition of apoptosis (Bergamaschi *et al*., 1994). Possibility of modulating apoptosis of tumor cells suggests new strategies for improving chemotherapy (Steller, 1996).

Human chronic myelogenous leukemia (CML) is a malignancy of pluripotent hematopoietic cells. The initial chronic phase is characterized by excessive production of myeloid cells that retain a normal differentiation program. The chronic phase of CML is invariably followed by progression to an acute phase of disease termed “blast

crisis," which resembles acute leukemia. The presence of the Philadelphia (Ph) chromosome during chronic-phase CML provided the first indication of the molecular basis of the disease (Figure I-1) (Nowell and Hungerford. 1960). The Ph chromosome is formed as a result of reciprocal translocation t(9;22), which fuses parts of the *c-abl* gene, located on chromosome 9, to parts of the *bcr* gene on chromosome 22 (de Klein *et al.*, 1982; Groffen *et al.*, 1984). This results in transcription of chimeric 8.5 kb mRNA and translation of chimeric 210 kDa Bcr-Abl protein with enhanced tyrosine kinase activity compared to the normal 145 kDa Abl gene product (Figure I-2) (Konopka *et al.*, 1984; Kloetzer *et al.*, 1985), This abnormality occurs in virtually all cases of Ph chromosome positive CML. A typical clinical course of CML involves progression from a protracted chronic phase to an accelerated phase with duration of 1-1.5 years followed by a rapidly fatal blastic phase (Kantarjan *et al.*, 1993). In the chronic phase, the disease process is characterized by increased numbers of immature myeloid precursors in bone marrow, peripheral blood, spleen, and liver. Myeloid precursors retain an ability to differentiate terminally so that the hallmark of the disease is a marked granulocytosis. Transition from chronic phase to blast crisis is characterized by a dramatic increase in the number of blast cells in hematopoietic tissues. The ability of Bcr-Abl to initiate

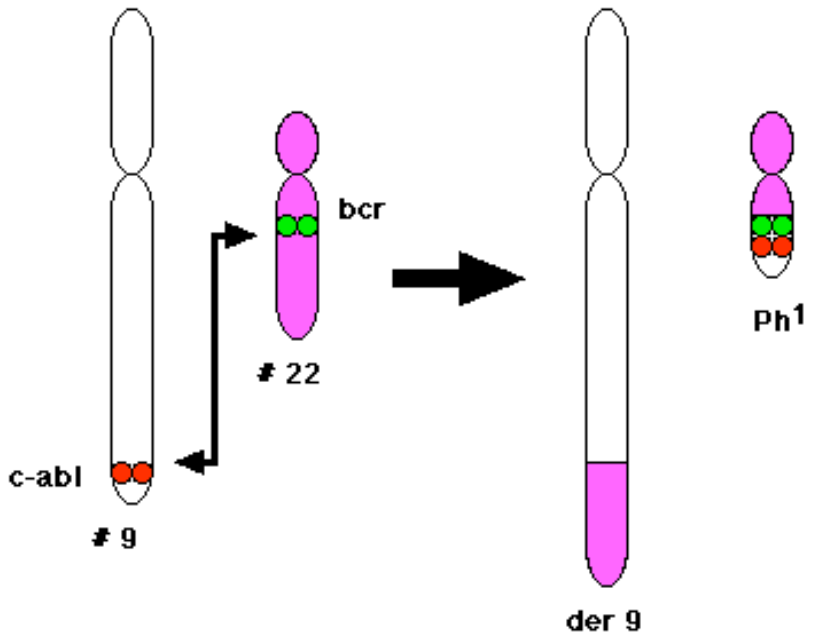


Figure I-1. Schematic view representing metaphase chromosomes. Reciprocal translocation between chromosome 9 and chromosome 22 forms an extra-long chromosome 9 ("der 9") and the Philadelphia chromosome (Ph) containing the fused *bcr-abl* gene.

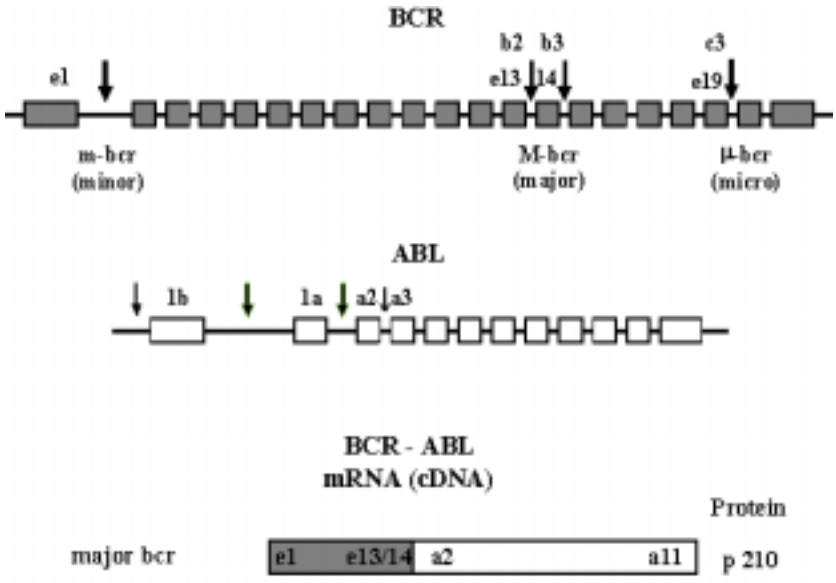


Figure I-2. Simplified scheme of the *bcr* and *abl* genes with indicated breakpoints. Possible *bcr-abl* transcripts and protein originating from individual breaks on the *bcr-abl* genes (Haskovec and Polak, 1999).

leukemogenesis has been established through extensive studies in cell culture and animal models (Daley *et al*., 1990; Heisterkamp *et al*., 1990; Voncken *et al*., 1995). Bcr-Abl is a deregulated tyrosine kinase that transforms both fibroblasts and hematopoietic cells in culture, and cells transformed by Bcr-Abl can form tumors in nude mice (Daley *et al*., 1988; Pendergast *et al*., 1993; Afar *et al*., 1994; Cortez *et al*., 1995). Bcr-Abl is an inhibitor of hematopoietic cell death normally caused by growth factor removal and DNA damaging agents *in vitro* (McGahon *et al*., 1994; Cortez *et al*., 1995). Such cells exihibit proliferative advantage over normal cells and loss of differentiation potential.

The human CML-blast crisis-derived K562 cells expressing both the $p210^{bcr\text{-}abl}$ and $p145^{abl}$ proteins (Lozzio et al., 1975; Campbell et al., 1993) are particularly resistant to induction of apoptosis by various agents including diphtheria toxin, camptothecin, Ara-C, etoposide, paclitaxel, staurosporine and anti-Fas antibodies (Chang *et al*., 1989; Kaufmann *et al*., 1993; McGahon *et al*., 1995; Dubrez *et al*., 1995; Ray *et al*., 1996). Effects of arsenic were limited mainly to APL cells (Kitamura *et al*., 1997). Because the expression of multidrug resistance gene (mdr-1/P-glycoprotein) is common in CML cells, CML usually is resistant to many anticancer chemotherapeutic drugs and has a poor prognosis (Sato *et al.*, 1995; Amarante-Mendes *et al*., 1998; San Miguel

et al., 1998). Therefore, anticancer drugs that are active against this type of leukemia are urgently demanding.

The present study aimed to investigate (1) effects of As_2O_3 on cytotoxicity, cell cycle, and apoptosis in K562 Bcr-Abl-positive leukemic cell, and (2) a relationship between the expression of the translocation product Bcr-Abl and As_2O_3-induced apoptosis.

II. MATERIALS AND METHODS

Materials

As_2O_3 was purchased from Sigma Chemical Co. (St. Louis, USA). As_2O_3 was dissolved in 1.65 M/l sodium hydroxide (NaOH) to make a stock solution of 1 mM/l, which was serially diluted in RPMI 1640. The maximum concentration of NaOH in culture had no influence on the cell growth in the cell lines. DMSO, NaOH, MTT, PBS, Rnase, Hoechst 33258 and SeaKem gold agarose were purchased from Sigma Chemical Co. CycleTest-Plus DNA reagent KIT was purchased from Becton Dickinson (Mountain View, USA). Caspase inhibitor (Z-VAD-fmk) was from Enzyme System Products (Livermore, USA). Anti-cleaved caspase 3 was from New England Biolab (NEB, Beverly, USA); anti-Bcr/Abl was from Santa Cruz biotechnology (Santa Cruz, USA). All other chemicals and reagents were the highest grade commercially available.

Cell culture and *in vitro* cytotoxicity assay

Human chronic myelogenous leukemia K562 cells were maintained in RPMI 1640 (Gibco BRL, Grand Island, USA) medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL). The cells in logarithmic growth were seeded at 1×10^5 cells/ml for studies

performed in duplicate. They were grown at 37°C in a humidified atmosphere of 5% CO_2. Cytotoxicity was measured by the microculture tetrazolium (MTT) method. The cells were resuspended in 100 μl RPMI 1640 at 1×10^4 cells/ml after verifying cell viability by trypan blue dye (Sigma Chemical Co.) exclusion assay. 100 μl of cell suspension were distributed into each well of 96 well plates (Nunc, Inc., Roskilde, Denmark). After the treated cells were incubated for 24 h, 50 μl MTT (1 mg/ml) was added into each well, and the plates were incubated for 4 h. To dissolve formazan, 150 μl DMSO was added and the absorbance values of each well at 540 nm were read by using an ELISA Reader (Molecular Device, Menlo Park, USA). The IC_{50} values were determined by plotting the drug concentration versus the survival ratio of the treated cells. Assays were performed at least three times, and data shown are representative of those assays.

Electron microscopy

After treatment with or without As_2O_3, 5×10^4 cells were washed with PBS (pH 7.4) and resuspended in the same buffer. And the cells to be processed for electron microscopy (JEM 1200 EX-II, JEOL, Japan) were centrifuged at $400 \times g$, fixed with 2.5% glutaraldehyde in PBS for 2 h, washed in 0.1 M Caocodylate (pH 7.4) and fixed with 0.1% OsO_4 in 0.1

M Caocodylate for 1.5 h. After fixation, the cells were washed with in 0.1 M Caocodylate and then dehydrated in graded ethanol. Next, the cells were impregnated with propylene oxide and embedded in Polybed 812 (Polyscience, Inc., Warrington, USA). After incubation at 60℃, the cells were stained with uranyl acetate and lead citrate.

Chromatin condensation by fluorescence microscope

Cells were incubated with 5 μg/ml Hoechst 33258 for 30 min at 37℃. An aliquot of cells was transferred to a microscope slide and fitted with a coverslip, and DNA was visualized with a fluorescence microscope. The number of apoptotic cells was determined by staining with Hoechst 33258 and the cells with condensed chromatin and fragmented nuclei were scored as apoptotic. At least 200 cells were scored from each group, and data were expressed as the percentage of cells with condensed chromatin.

DNA fragmentation analysis

Cells (5×10^6) which were treated for 24 h were washed twice in a solution of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl and then lysed with 500 μl lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 20 mM EDTA). Lysates were centrifuged by 1,000 $\times$ g for 10

min. and the supernatants were incubated for 3 h at 37°C with 100 µl, 1% SDS 10 µl, TE/RNase (RNase 10 mg/ml, 10 mM Tris-HCl, pH 7.5, 15 mM NaCl), 50 µl proteinase K (1 mg/ml, Sigma). Then, the DNA was extracted with phenol / chloroform / isoamylalcohol (25:24:1, Sigma, USA). After precipitation, pellets were resuspended in 30 µl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Each DNA sample was electrophoresed through a 1.8% agarose gel (TAE buffer 35 ml, 0.63 g SeaKem gold agarose) containing ethidium bromide and DNA bands were visualized by UV light.

Cell cycle analysis by flow cytometry

The cells treated with different concentrations of As_2O_3 and for a variety of time periods and controls were collected and fixed in 70% ethanol and stored at –20°C before use. After resuspension, cells were washed with PBS and treated with Cycle TEST^{TM} Plus DNA Reagent Kit (Becton Dickinson). The cells were resuspened in 250 µl of 1.12% sodium citrate buffer (pH 8.4) together with 12.5 µg of RNase at 37°C for 30 min. Then the cellular DNA was stained by 250 µl of propidium iodide (50 µg/ml) for 30 min at room temperature. The stained cells were analyzed with FACScan flow cytometer (Becton Dickinson) for relative DNA content based on increased red fluorescence. Each of the

G0/G1, S, G2/M phases of the cell cycle was calculated using the RFIT program.

Preparation of cell lysates

The cells were washed twice with PBS. Washed cells were lysed in lysis buffer (10 mM HEPES pH 7.9, 0.5% Triton X-100, 1 mM NaF, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 1 mM PMSF) and then the lysate was centrifuged at 15,000 × g for 10 min. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard. The supernatant was used for Western blot analysis.

Western blot analysis

A cell lysate containing 50 μg of protein was fractionated by SDS-PAGE on the appropriate percent polyacrylamide gel and then proteins were transferred to nitrocellulose membranes at a constant current of 220 mA for 90 min. The membrane was blocked with 5% skim milk in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.15% Tween-20) for 1 h at room temperature and subsequently probed overnight at 4℃ with anti-cleaved caspase-3, anti-Bcr/Abl primary antibodies at a 1:1,000 dilution in TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl). After the

membrane was washed three times with TBST, it was incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (NEB) at a 1:10,000, 1:2,000 dilution in TBS. After washing of the membrane with TBST, bands of protein on the membrane were detected with an enhanced chemiluminescence detection method by immersing the blots for 1 min. with a 1:1 mixture of chemiluminescence reagents A and B (Amersham, Buckinghamshire, UK).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

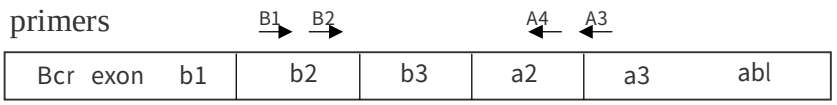
After incubation with As_2O_3, cells were counted and frozen at –70℃ in PBS. Total RNA was extracted from thawed samples using the method of Chomczynski and Saachi (1987). A typical 20 μl reaction for the conversion of RNA to cDNA contained up to 10 μl RNA, 0.5 μl reverse transcriptase (20 U/μl, Takara, Japan), 2 μl each dNTP (200 μM/l, Takara, Japan), 0.5 μl RNase inhibitor (35 U/μl, MBI Fermentas, Latvia), 2 μl random hexamer (333 μM, MBI, USA) and 4 μl RT-PCR buffer (20 mM KCl, 25 mM Tris-HCl, 3 mM $MgCl_2$, 5 mM dithiothreiton, pH 8.3). The reaction mixture was incubated for 1 h at 37℃ and subsequently stopped by heating to 95℃ for 5 min. After the

incubation the reaction volume was divided into two equal parts. The *bcr-abl* transcripts were detected by RT-PCR using specific primers. The location of each primer within *bcr-abl* mRNAs is shown in Figure I-3. After reverse transcription, amplification of *bcr-abl* mRNA was performed with the primers shown in Table I-1, using nested PCR.

The first PCR reaction contained 0.5 μl *Taq* DNA polymerase (2 U/μl, Takara, Japan), 2 μl primer mix (B1, A3) (10 pM), 2 μl dNTP (200 μM), 10 μl of the cDNA reaction, reaction buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, pH 8.3) in a total volume of 50 μl. As an internal control primers A1 and A3 replaced primers B1 and A3. PCR amplification was performed in a DNA thermalcycler (Perkin-Elmer Cetus, USA). In a programmable heating block, 26 cycles were carried out for 1 min at 94℃, 1 min at 61℃ and 1 min at 72℃. The last step at 72℃ was extended to 7 min. After completion of the PCR mixture were diluted 1:100.

The second round of PCR was performed using the nested internal primers B2 and A4, or A2 and A4 for the control reaction, 5 μl of the diluted first PCR round and 2 μl of dNTP and 0.5 μl *Taq* DNA polymerase in the same buffer as for the first PCR. After 30 cycles, each reaction mixture was size separated by gel electrophoresis, ethidium bromide stained and photographed.

RT-PCR of major *bcr-abl* rearrangement



RT-PCR of normal *c-abl* as an internal RNA control

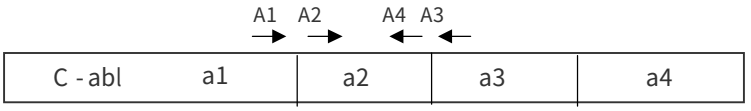


Figure I-3. Locations of primers

Table I-1. Sequences of primers

Primers	Sequence
A1	5’ ATC TGC CTG AAG CTG GTG GGC T 3’
A2	5’ AGT GAA GCC GCT CGT TGG AAC TCC AA 3’
A3	5’ TGA TTA TAG CCT AAG ACC CGG A 3’
A4	5’ ATC TCC ACT GGC CAC AAA ATC ATA CA 3’
B1	5’ GAA GTG TTT CAG AAG CTT CTC C 3’
B2	5’ TGG AGC TGC AGA TGC TGA CCA ACT CG 3’

Data presentation

All assays were set up in triplicate, and the results were expressed as mean ± SD. Statistical significance for chemical or temporal effect was examined by independent pairs Student's *t*-test at P = 0.05 or 0.01.

III. RESULTS

Cytotoxic effects of As_2O_3

Recent reports indicate a broad spectrum of antileukemic activity for As_2O_3 (Wang *et al*., 1998; Bazarbachi *et al*., 1999; Rousselo *et al*., 1999). The cytotoxic effect of As_2O_3 on human CML cell line K562 was examined by treating with different concentrations of As_2O_3 for 24 h, 48 h, and 72 h respectively. K562 cells were exposed to As_2O_3, ranging from 1 to 80 μM, and cytotoxicity was evaluated by MTT assay. With the increasing concentrations of As_2O_3, the numbers of dead cells were increased. The IC_{50} value for As_2O_3 was to be 10 μM, as shown in Figure I-4. Chen *et al*. (1997) reported that at concentrations of 0.5 – 2 μM, As_2O_3 triggers the apoptosis of both NB4 and cultured primary APL cells derived from patients. The cytotoxicity of K562 cells was shown at higher As_2O_3 concentration than that of NB4 cells. The experiments clearly showed that As_2O_3 induced the cytotoxicity in the K562 CML cell line in a dose-dependent manner.

Effects of As_2O_3 on morphological changes

Apoptotic mechanisms are influenced with drugs and cell-type-specific and, are associated with the perturbation of mitochondrial

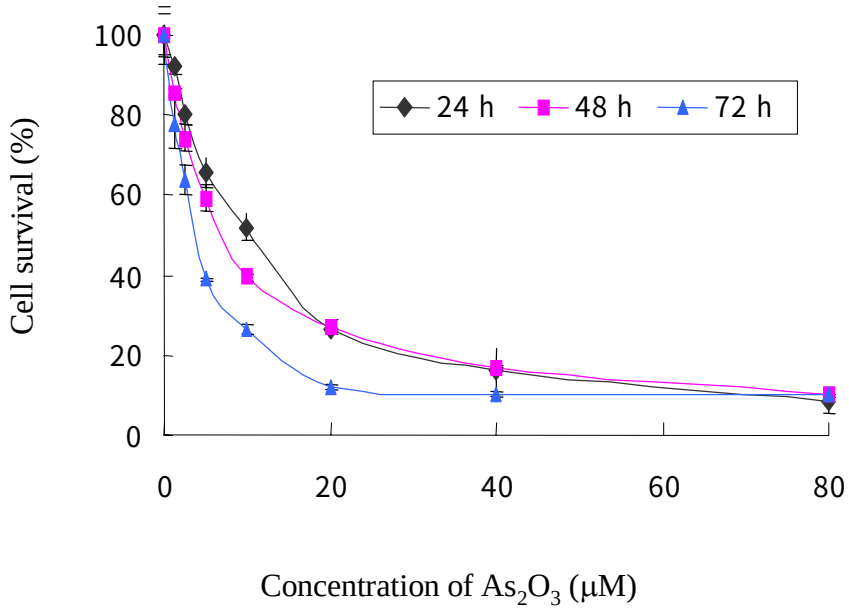


Figure I-4. Cytotoxic effects of As_2O_3 on K562 cells. The cells were incubated with various concentrations of As_2O_3 for 24 h, 48 h, and 72 h. Cytotoxicity was measured by MTT assay. The results are presented as mean ± SD for triplicate.

functions. This process results in the activation of caspase and the fragmentation of DNA, coupled with characteristic morphologic changes. When the condensed nuclei were fixed with glutaraldehyde, and examined by a thin section electron microscope, the treatment of the K562 cells with As_2O_3 resulted in morphological changes that are consistent with the process of apoptosis. Morphological changes in apoptosis, including blebbing of the plasma membrane, chromatin condensation, fragmentation of nuclei and formation of apoptotic bodies were observed. Initially in the nucleus, a rim of heterochromatin appeared at the nuclear periphery, the nucleolus simultaneously disappeared, and then the nuclei subsequently fragmented. Finally, packagings of the nuclear fragments into multiple membrane-enclosed apoptotic bodies were found (Figure I-5).

Next, the morphological study by Hoechst 33258 staining showed the characteristic phenotypes of apoptosis such as chromatin condensation and nuclear segmentation. Treatment of leukemia K562 cells with As_2O_3 at concentrations above 10 μM for 24 h resulted in condensation of the chromatin and fragmentation of nuclei as detected after with Hoechst 33258 (Figure I-6).

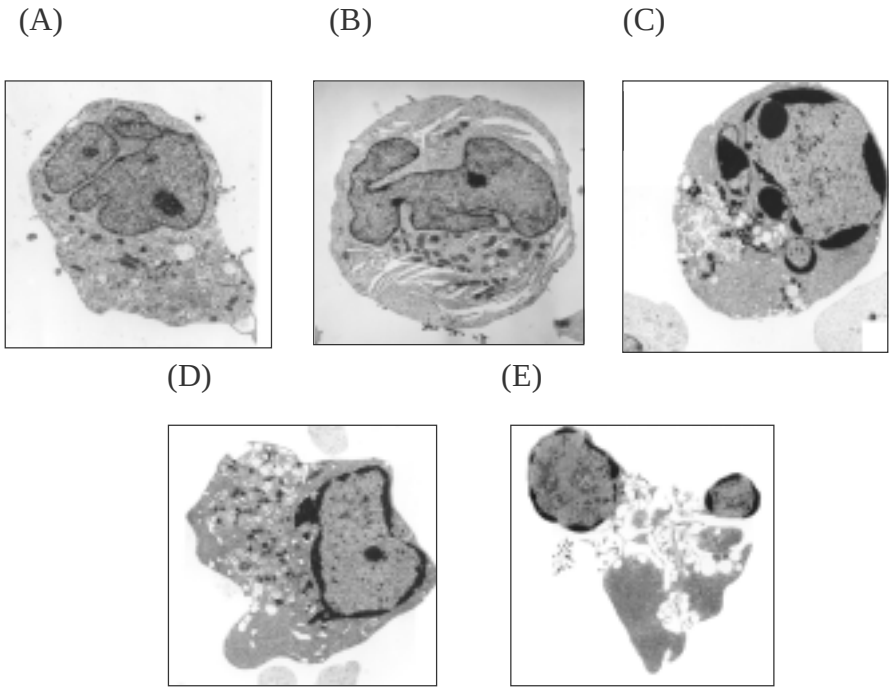
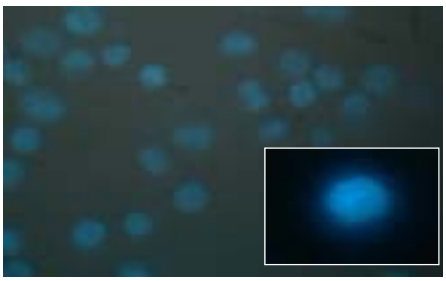
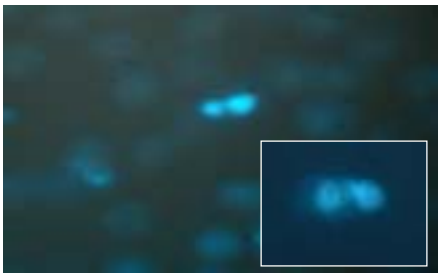


Figure I-5. Electron micrographs of K562 cells treated with As_2O_3. The cells were treated with the control vehicle (A), 10 μM As_2O_3 for 24 h (B), or 20 μM As_2O_3 for 24 h (C), for 36 h (D), for 48 h (E), stained with uranyl acetate and lead citrate, and analyzed under an electron microscope (x 4,000).

(A)



(B)



(C)

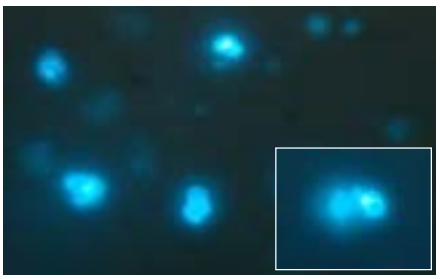


Figure I-6. Fluorescence microscopic appearance of Hoechst 33258-stained nuclei of As_2O_3–treated cells. The cells were treated with a control vehicle (A), 10 μM As_2O_3 for 24 h (B), 20 μM As_2O_3 for 24 h (C), stained with Hoechst 33258, and analyzed under a fluorescence microscope (x100).

Effect on the change of chromatin condensation

To examine further the effect of As_2O_3 on apoptosis, Apoptotic cells were investigated under a fluorescence microscope. The percentages of apoptotic cells in K562 cultured cells were determined by monitoring nuclear fragmentation and chromatin condensation after staining with Hoechst 33258. In brief, the apoptotic cells increased in a time-and dose-dependent manner. Approximately 25% of K562 cells underwent apoptosis at concentration of 20 μM As_2O_3 for 24 h (Figure I-7). This result supports that As_2O_3 induces apoptosis in K562 cells.

DNA fragmentation characteristic of apoptosis

Although K562 cells are particularly resistant to apoptosis against the various agents, K562 cells were sensitized to drug-induced apoptosis by the treatment with antisense oligonucleotides against the *bcr-abl* gene (McGahon *et al*., 1994). Therefore it was further investigated whether the cytotoxic effect of As_2O_3 was by apoptosis. In this study, the apoptotic effect of As_2O_3 was examined the apoptotic response as judged by monitoring the appearance of a DNA fragmentation by gel electrophoresis. A characteristic pattern of nucleosomal DNA fragmentation, which is the biochemical hallmark of apoptosis, was detected after 24 h exposure to 20 μM of As_2O_3 (Figure I-8) and also

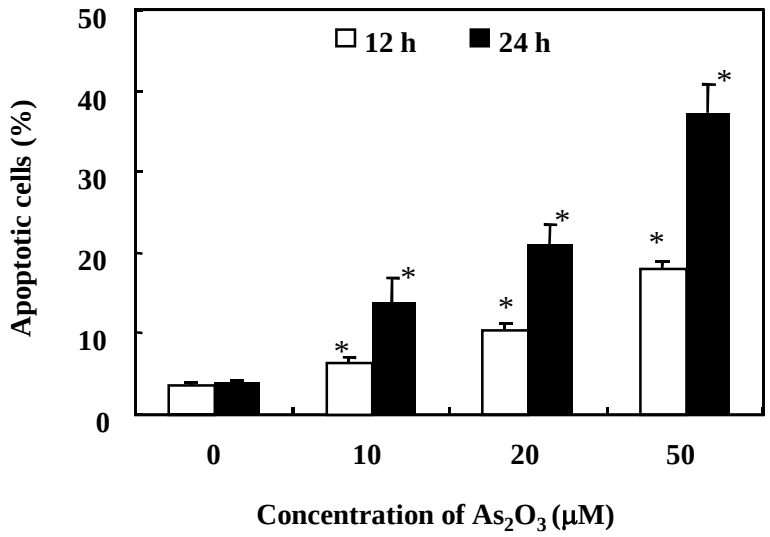


Figure I-7. Apoptotic K562 cells by As_2O_3. The cells were treated with indicated concentration of As_2O_3 and were stained with Hoechst 33258. Apoptotic cells with condensed and fragmented nuclei were counted under a fluorescence microscope (x100). The results are presented as mean ± SD for triplicate * $p < 0.01$ vs control

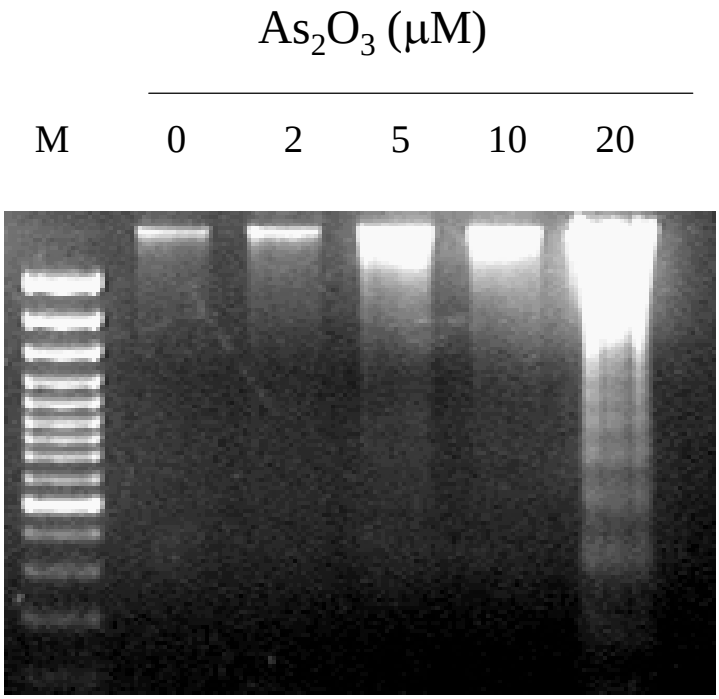


Figure I-8. Dose-dependent induction of DNA fragmentation in As_2O_3-treated K562 cells. The cells were treated with various concentrations of As_2O_3 for 24 h. Extracted DNA was fractionated by electrophoresis and stained by ethidium bromide. Lane M, DNA marker.

observed in the treated cells of 20 μM As_2O_3 for 12 h and 24 h, respectively (Figure I-9).

Activation of caspase-3 by As_2O_3

Caspases, especially caspase-3 (CPP-32) plays a key role in the induction of apoptosis. To determine whether apoptosis induced by As_2O_3 regulated by caspase-3, expression level of cleaved caspase-3 during apoptosis was examined by Western blot analysis. Caspases are synthesized initially as an inactive precursor of 32 kDa subsequent and proteolytic processing generates the two subunits of 17 and 12 kDa that form the active protease (Nicholson *et al.*, 1995). Cleaved of caspase-3 antibody detects only the large fragment of activated caspase-3 (17 kDa) that results from cleavage after Asp 175. Activation of cleaved caspase-3 (17 kDa) was observed 12 h after treatment with 20 μM As_2O_3, reached a maximum 24 h and then decreased (Figure I-10). This finding indicated that the caspase-3 might be associated with As_2O_3-induced apoptosis in K562 cells.

Effect of caspase inhibitor on the cytotoxicity of As_2O_3

Although caspase activation is hallmark of apoptosis, inhibition of caspase dose not always prevent cells from undergoing apoptosis,

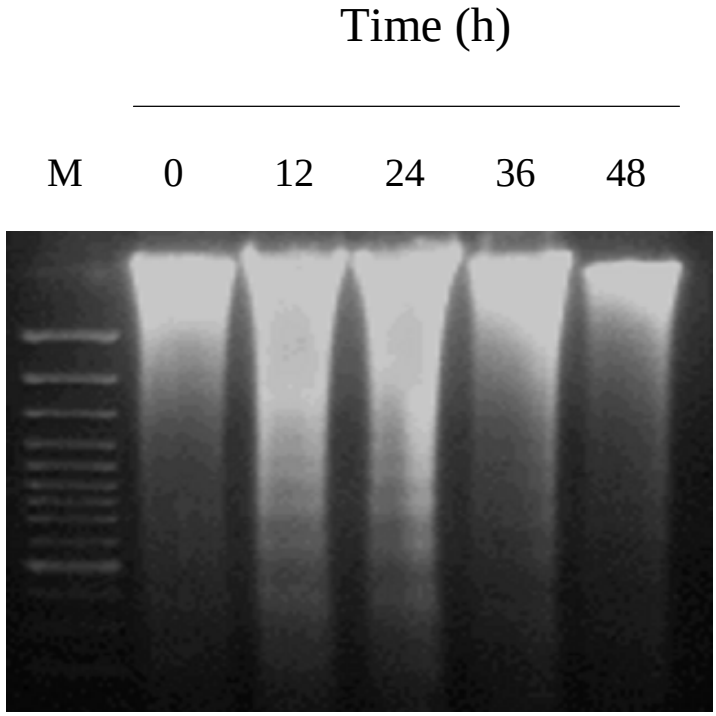


Figure I-9. Induction of DNA fragmentation in As_2O_3-treated K562 cells. The cells were treated with 20 μM of As_2O_3 for indicated times. Extracted DNA was fractionated by electrophoresis and stained by ethidium bromide. Lane M, DNA marker.

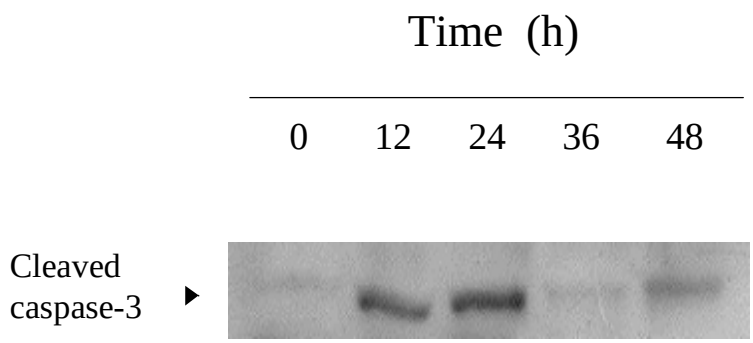


Figure I-10. Activation of cleaved caspase-3 by As_2O_3. The cells were harvested at the indicated times after incubation with 20 μM of As_2O_3. The whole cell lysates were prepared and subjected to Western blot analysis with an antibody specific to cleaved caspase-3 (17 kDa).

suggesting the existence of a caspase-independent pathway. To confirm the essential role of caspase-3 in As_2O_3-induced apoptosis, effect of a pan-inhibitor of caspase family, Z-VAD-fmk, on cytotoxicity was analyzed. And, cytotoxicity was evaluated by MTT method after pretreatment 25 μM Z- VAD-fmk before treatment of As_2O_3 for 24 h. In result, the cytotoxicity was decreased than nontreated cell (Figure I-11).

Effect on cell cycle phase distribution

Recently, it was reported that the apoptotic signal pathway is related with the arrested phase of the cell cycle (Molnar *et al*., 1997). To investigate the anti-proliferative mechanisms of As_2O_3, the cell cycle phase distribution was analyzed by flow cytometry. The cells were incubated in the absence or presence of As_2O_3 at 37℃ for 12 h, 24 h, 48 h, and 72 h. As shown in Table I-2, 46.0% of control cells were in G0/G1 phase, 50.9% were in S phase, and 3.2% were in G2/M phase. The cells treated with 10 μM As_2O_3 for 72 h showed an accumulation of cells in the G2/M phase. The cells in G2/M phase were 31.3% (Figure I-12). The G2/M arrest induced by As_2O_3 in relation to time in K562 cells was examined (Figure I-13). Next, the cell cycle phase distribution of cells treated with different concentrations of As_2O_3 for 48 h was analyzed (Figure I-14). The result showed that As_2O_3 also arrested the

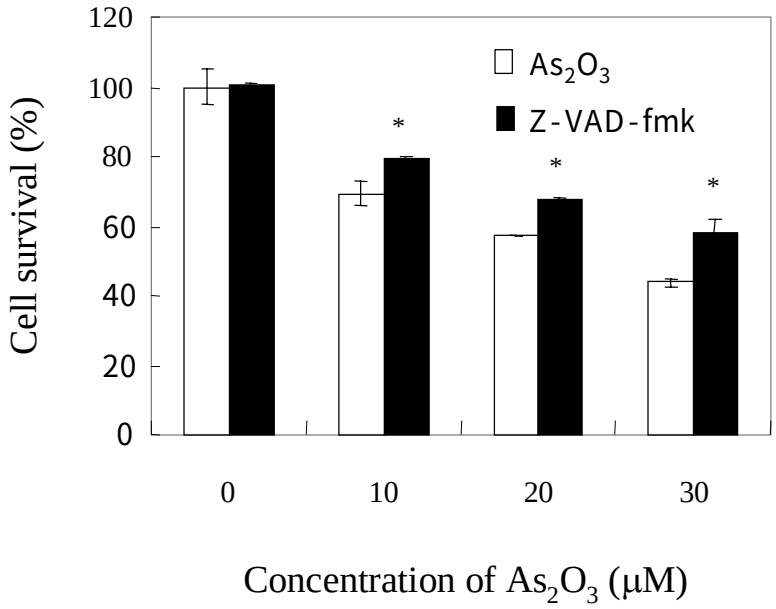


Figure I-11. Effect of caspase inhibitor, Z-VAD-fmk, on cytotoxicity in As_2O_3-treated K562 cells. The cells were treated with 25 μM Z-VAD-fmk for 1 h and then treated with the indicated concentrations of As_2O_3 for 24 h. Cytotoxicity was measured by MTT assay. * The datum in presence of Z-VAD-fmk is significantly different from that in the absence of Z-VAD-fmk ($p < 0.05$)

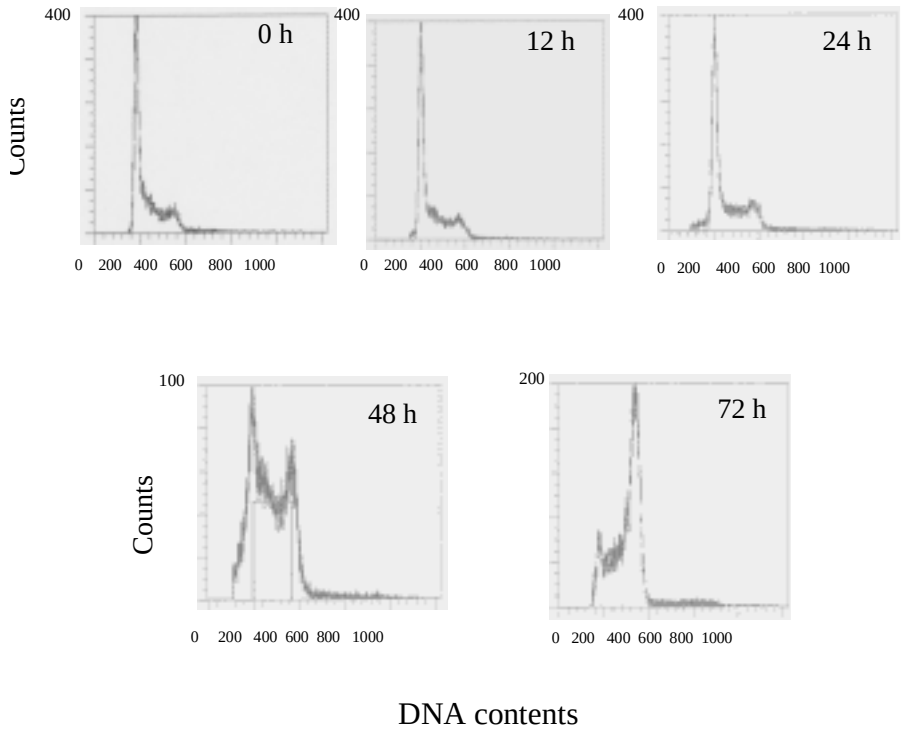


Figure I-12. FACS analysis of K562 cells treated with As_2O_3. The cells were treated with 10 μM for indicated times, and then the cells were fixed 70% ethanol, stained with PI, and DNA content was measured by flow cytometry.

Table I-2. Time-dependent alteration in cell cycle induced by As_2O_3. The cells were incubated with 10 μM of As_2O_3 for indicated times, stained with propidium iodide, and assayed by flow cytometry.

	% Cells		
Time	G0/G1	S	G2/M
0	46.0	50.9	3.20
12	46.2	50.6	3.30
24	48.8	43.4	7.80
48	28.2	56.3	15.5
72	14.0	54.7	31.3

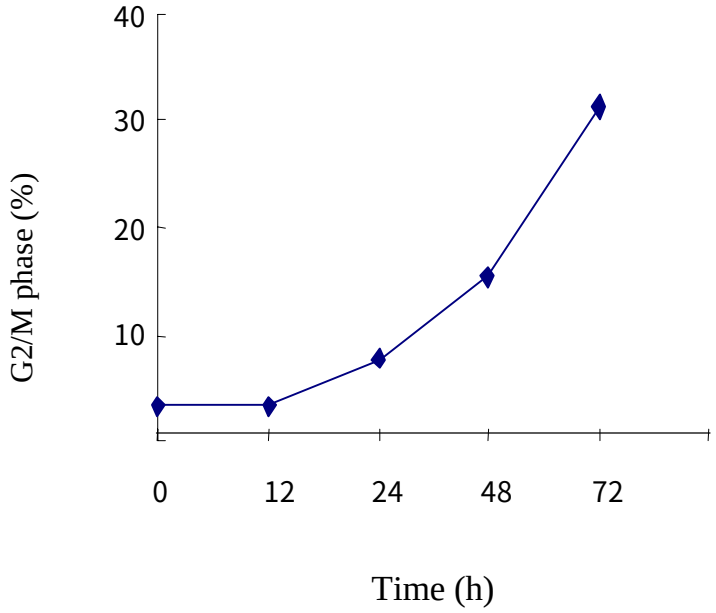


Figure I-13. Time-dependent alteration in G2/M phase induced by As_2O_3. Cells were incubated with 10 μM of As_2O_3 for indicated times, stained with propidium iodide, and assayed by flow cytometry.

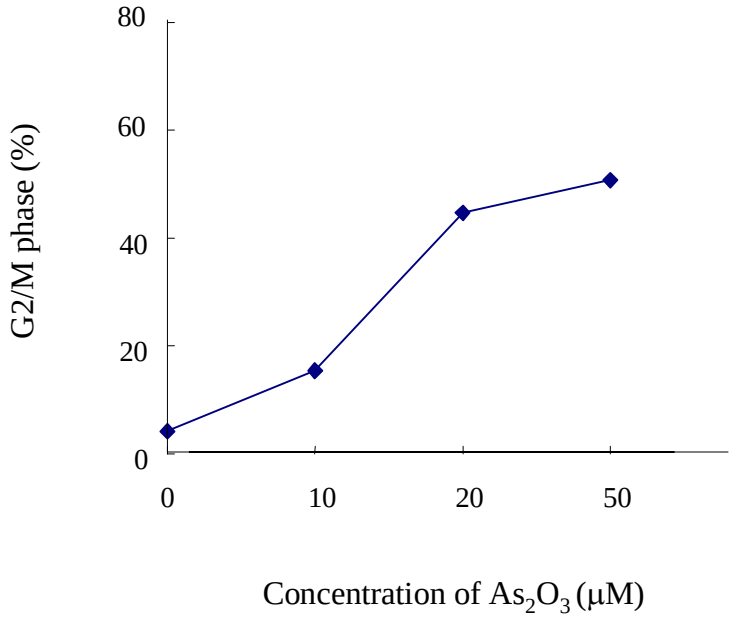


Figure I-14. Dose-dependent alteration in cell cycle induced by As_2O_3. The cells were incubated with the indicated concentration of As_2O_3 for 48 h, stained with propidium iodide, and assayed by flow cytometry.

cells in the G2/M phase. Perkins *et al*. (2000) recently reported that As_2O_3 increased the percentage of K562 cells accumulated in the G2/M phase of the cell cycle. On the contrary, in U937 cells, the growth inhibition was not correlated with arrest in a specific cell cycle phase (Jing *et al*., 1999). In this study, it was found that treatment with As_2O_3 significantly increased the percentages of cells accumulated in the G_2/M phase of the cell cycle in a time-, dose-dependent manner. These results indicated that As_2O_3 inhibited the cellular proliferation of K562 cells via G2/M phase arrest of the cell cycle.

Effect of As_2O_3 on the Bcr-Abl expression

Oncogenic Bcr-Abl has been attributed antiapoptotic activity, which appears to promote the development and drug resistance of CML. In this study, the effect of As_2O_3 on the Bcr-Abl expression was investigated by Western blot analysis. Significant reduction in $p210^{Bcr\text{-}Abl}$ in K562 cells was clearly observed after incubation with As_2O_3 (Figure I-15). It was found that treatment with As_2O_3 induced down regulation of Bcr-Abl expression levels. The decline in Bcr-Abl levels may explain As_2O_3 induced apoptosis in K562 cells. Next, it was investigated whether the As_2O_3-induced down regulation of Bcr-Abl is transcriptionally or post-transcriptionally regulated.

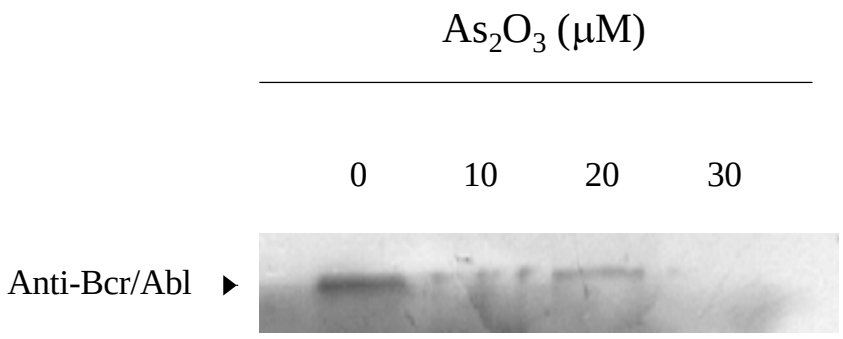


Figure I-15. Effect of As_2O_3 on $p210^{BCR-ABL}$ expression in K562 cells. The cells were harvested at the indicated concentration of As_2O_3 for 24 h. The whole cell lysates were prepared and subjected to Western blot analysis with an antibody specific to Bcr/Abl.

Effect of As_2O_3 on *bcr-abl* transcripts expression

Fusion of *bcr* and *abl* genes results in the expression of leukemia-specific chimeric mRNAs. Ph chromosomes of CML and acute lymphoblastic leukemia (ALL) are indistinguishable by cytogenetics. Molecular genetic studies however showed that the *bcr* gene region of the chromosomal breakpoint is located in a more 5'-direction in about 50% of ALL cases, while the *abl* sequence remains the same as in CML (Hermans *et al*., 1987; Clark *et al*., 1988). At the genomic level, these breakpoints are clustered in the 3' end of the first intron of bcr (Chen *et al*., 1989). In CML, the breakpoints are clustered in the midportion of the *bcr* gene, termed M-*bcr* (major breakpoint cluster region). This type of translocation generates 8.5 kb fusion *bcr-abl* mRNA, resulting in a 210 kD protein (Groffen *et al*., 1984; Heisterkamp *et al*., 1985). This *bcr-abl* mRNA is the target for the PCR (Mullis *et al*., 1987; Saiki *et al*., 1988). After transcription of the mRNA, the complementary DNA (cDNA) was amplified with the heat-stable *Taq* polymerase in two rounds with different primer sets. The PCR technique has evolved into a valuable tool in diagnosing the Ph translocation in CML by detecting *bcr-abl* mRNA (Kawasaki *et al*., 1988; Lange *et al*., 1989; Lee *et al*., 1989; Roth *et al*., 1989).

In the present study, it was evaluated the effect of As_2O_3 on *bcr-abl*

fusion gene expression by RT-PCR for *bcr-abl* mRNA levels by As_2O_3. The results revealed the *bcr-abl* rearrangement was not detected after treatment with concentration more than 20 μM As_2O_3 for 24 h (Figure I-16) and that As_2O_3 affected the transcription of the *bcr-abl* fusion gene. These results support that down regulation of *bcr-abl* fusion gene is mediated via As_2O_3-induced apoptosis.

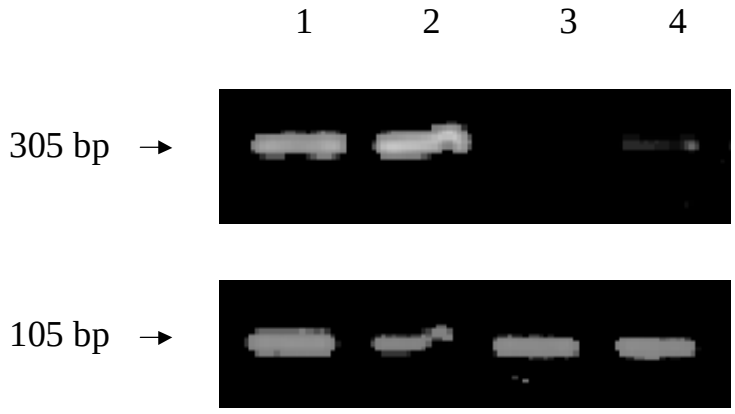


Figure I-16. Detection of the *bcr-abl* transcripts in K562 cells by RT-PCR. (*top*) Lane 1, Control; Lane 2, 10 μM As_2O_3; Lane 3, 20 μM As_2O_3; Lane 4, 30 μM As_2O_3 ; (*bottom*) Internal controls for Lane 1-4 respectively.

IV. DISCUSSION

Leukemia arises in hematopoietic progenitor cells and is characterized by impaired or blocked differentiation, uncontrolled proliferation, and resistance to apoptosis (Hiddemann and Griesinger, 1993). Leukemia can be effectively treated using sole or combinations of chemotherapeutic agents, such as taxol, that induce apoptosis (Kerr *et al*., 1994). A notable exception is CML, which is highly resistant to most commonly utilized chemotherapeutic drugs, including taxol (Green *et al*., 1994; Bedi *et al*., 1995). There are several molecular mechanisms that have been implicated as being involved in Bcr-Abl-mediated resistance to the intrinsic or mitochondrial pathway to apoptosis (Skorski *et al*., 1997; Amarante-Mendes *et al*., 1998). Increased Bcl-x_L levels and Akt kinase activity, observed in Bcr-Abl-transformed cells, may contribute toward the inhibition of either the cytosolic accumulation of cytochrome c or activities of effector caspases that may mediate the resistance against the intrinsic pathway to apoptosis (Yang *et al*., 1997; Datta *et al*., 1999).

Apoptosis is an active form of cell death that plays an essential role in physiological and pathological conditions throughout development and adult life multicellular organisms, eliminating damaged cells or cells with defects in key-regulated processes such as growth (Kerr *et al*.,

1972; Wyllie *et al*., 1980; Ellis and Horvitz, 1986). At the morphological level, it is characterized by membrane blebbing, cell shrinkage, chromatin condensation, nuclear/cytoplasmic fragmentation, and formation of dense bodies that are quickly removed via phagocytosis by neighboring cells. Apoptosis can be induced through receptor-mediated mechanisms and as a consequence of stresses, such as growth factor withdrawal or exposures to cytotoxic drugs (Frish and Francis, 1994; Xia *et al*., 1995; Muschel *et al*., 1998). Once triggered, apoptotic program involves activation of a series of biochemical events comprising most of the times the release of proteins from the mitochondria into the cytoplasm and the nucleus. The best-characterized execution pathway of apoptosis involves release of cytochrome c that leads, in sequence, to activation of caspases, proteolytic degradation of specific substrates, activation of nucleases, and internulceosomal DNA fragmentation. There is evidence that apoptosis plays a role in response of leukemia patients to chemotherapy. There is probably an association between therapy-induced apoptosis and therapeutic efficacy (Dive *et al*., 1992; Sachs *et al*., 1993; Kerr *et al*., 1994).

As_2O_3 is able to induce growth inhibition and apoptosis of human myeloid leukemia cells resistant to multiple apoptotic stimuli. In these cells, apoptosis and multi-drug resistance were shown to be secondary

to diverse mechanisms, including the expression of Bcr-Abl (Bhalla *et al*., 1985; Huang *et al*., 1997; Ibrado *et al*., 1996; Amarante-Mendes *et al*., 1998; Li *et al*., 1999). In the present study, molecular events during apoptosis induced by As_2O_3 were determined. As_2O_3 inhibited growth of and induced apoptosis in K562 leukemia cells in a dose-dependent manner. DNA fragmentation and chromatin condensation, a hallmark of apoptosis, were observed after treatment of As_2O_3. The morphological alterations were confirmed with an electron and fluorescence microscope. The percentages of apoptotic cells also induced by treatment with above 10 μM As_2O_3 for 24 h were significantly higher than untreated cells. It has become apparent that the mechanism of action of As_2O_3 is closely related to its ability to induce apoptosis.

Members of the caspase family play key roles in the execution phase of apoptosis. These cysteine proteases are synthesized as inactive pro-enzymes, which are activated by different apoptotic stimuli, leading to specific cleavage of a range of cellular protein substrates (Alnemri *et al*., 1997). These substrates include enzymes involved in genome surveillance and DNA repair, such as poly (ADP)-ribose polymerase (PARP). Among the family of 10 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

(Datta *et al.*, 1996; Schlegel *et al.*, 1996; Soignet *et al.*, 1996). The importance of caspase-3 has been further confirmed by a study showing that caspase-3 knockout mice suffered from severe developmental abnormalities attributed to the disturbed regulation of apoptosis (Kueda *et al.*, 1996). Caspase-3 is involved in As_2O_3-induced apoptosis in APL, B-cell leukemia cell lines and neuroblastoma cell lines (Akao *et al.*, 1998; 1999). In this study, to address whether As_2O_3-induced apoptosis is mediated by caspase-3 activity, the same samples were probed with a specific antibody. It was shown that As_2O_3-induced apoptosis is controlled through caspase-3 activation. However, it is likely that As_2O_3 could also induce apoptosis partly *via* a caspase-independent pathway. Recently, it was reported that caspase-3 is not involved in As_2O_3-induced apoptosis of Bcr-Abl positive BV173 cells (Puccetti *et al.*, 2000). Therefore, the role of caspase-3 like activity in As_2O_3-induced apoptosis is controversially discussed.

The cell cycle is controlled through the balance between factors promoting progression and suppressive mechanisms. In general, cancers result from activation of cell cycle progression together with loss of the suppressive mechanism. K562 cells possess the *bcr-abl* fusion gene and also show inactivation of tumor suppresser gene, p53, p15 and p16 (Gale and Canaani, 1984; Law *et al.*, 1993; Otsuki *et al.*, 1995). Such changes

will confer more aggressive biological characteristics. Apoptosis at least partly results from alteration of the cell cycle, since growth stimulation under unfavorable growth conditions can induce apoptosis in various kinds of cells. Nishii *et al*. (1996) have shown, using BaF3 cells transfected with Bcr-Abl may be responsible for the prolonged G2 arrest following radiation. Because mitotic catastrophe shares several features with apoptosis, including dissolution of the nuclear lamina, chromosomal condensation and DNA fragmentation, it is conceivable that apoptosis may result from a similar failure of coupling the G2/M transition to completion of DNA repair or replication. Cortez *et al.* (1997) demonstrated that 32D cells stably expressing Bcr-Abl protein fail to induce cell cycle arrest following growth factor starvation. It has been indicated that antiproliferative action of As_2O_3 was linked to a G1 phase arrest in lymphoid neoplasms and a G2/M phase arrest in NB4 cells and U937 cells (Ma *et al*., 1998; Zhang *et al*., 1998; Park *et al*., 2001). Recent studies (e.g. Bazarbachi *et al*., 1999) revealed that INFα and As_2O_3 have dramatic synergistic effects to induce cell cycle arrest and apoptosis, specifically in HTLV-1 infected cells. Interestingly, As_2O_3 induced G1 arrest, while IFNα alone had little effect on these cells. In this study, As_2O_3 mediated G2/M phase arrest and also correlates with the onset of apoptosis.

The Ph chromosome is a cytogenetic feature in 90 – 95% of CML (Rowley, 1980). Several strategies have been developed to target either the mRNA or protein encoded by the *bcr-abl* fusion gene, which is pathogenetically responsible for the malignant phenotype of CML (Skorski *et al*., 1994; Cobaleda and Sanchez-Garcia, 2000). In this study, however, treatment with As_2O_3 significantly downregulated not only Bcr-Abl oncoprotein but also *bcr-abl* fusion gene mRNA levels. These findings are consistent with a previous report demonstrating that the abrogation of Bcr-Abl expression by antisense oligonucleotides selectively eliminates CML blast cells (le Courte *et al*., 1999). In addition, abrogation of Bcr-Abl activity by a relatively specific tyrosine kinase inhibitor has been recently shown to cause *in vitro* and *in vivo* eradication of human Bcr-Abl positive leukemia cells. Collectively, these findings point to As_2O_3-mediated down regulation of Bcr-Abl as the key perturbation responsible for facilitating the apoptosis of K562 cells. This is the first report that As_2O_3-induced Bcr-Abl down regulation is transcriptionally regulated.

Taken together, As_2O_3 has a cytotoxic effect against K562 Bcr-Abl positive cells via inducing G2/M phase arrest as well as triggering the apoptosis through caspase-3 activation and Bcr-Abl down-regulation. Therefore, it seems that combination of down regulation of Bcr-Abl and

activation of caspase-3 might be essential to As_2O_3-induced apoptosis of CML cells. The potential use of As_2O_3 in treatment of leukemia deserves further exploration.

CHAPTER II

The Involvement of p38 MAP kinase in Apoptosis Induced by As_2O_3 in K562 Cells

I. INTRODUCTION

The Philadelphia (Ph) chromosome is the hallmark of chronic myeloid leukemia (CML) (Heisterkamp *et al.*, 1985; Ben-Neriah *et al.*, 1986). To date, $p210^{bcr\text{-}abl}$, the gene product of the resultant *bcr-abl* hybrid gene is one of the most intensively studied signaling proteins in cancer research. Non-oncogenic c-Abl is a member of the family of non-receptor protein tyrosine kinases. The elevated Abl tyrosine kinase activity is regarded as the powerhouse behind the induction of CML. The inappropriate high levels of tyrosine kinase activity appear to send an unregulated proliferation signal to the nucleus of the leukemic cell (Konopka *et al.*, 1985). The oncoprotein is associated with enhanced expression of several major downstream effectors such as Ras (Sawyers *et al.*, 1995), phosphoinositide 3-kinase (PI-3K) and protein kinase B (Skorski *et al.*, 1997). One of the most excessively studied signaling pathways to date is the Ras pathway, the center of which is the GDP/GTP-regulated Ras protein (Barbacid *et al.*, 1987). Activated by a variety of cytokine and growth factor receptors (Satoh *et al.*, 1992; Medema and Bos, 1993), Ras seemed to be an attractive mediator of Bcr-Abl-induced signal transduction. Ras is essential to Bcr-Abl-mediated blockade of apoptosis in hematopoietic cells and its activation

is also necessary for cellular transformation in fibroblasts (Pendergast *et al*., 1993; Cortez *et al*., 1995). Also, Bcr-Abl-expressing hematopoietic cells are able to proliferate in a cytokine-independent manner (Carlesso *et al*., 1994; Laneuville *et al*., 1994; Cortez *et al*., 1995) These observations suggest that Bcr-Abl may not only function to inhibit apoptosis but may also contribute to leukemogenesis by inducing cellular proliferation. A summation of these functions may accelerate oncogenesis (Cortez *et al*., 1995). Subsequently, a serine/threonine kinase cascade involving mitogen-activated protein (MAP) kinases is activated.

In mammalian cells, three distict MAP kinase cascades have been identified, extracellular signal-regulated kinases (ERK), c-jun amino-terminal kinases (JNK) or stress-activated protein kinases (SAPK), p38 MAP kinase (p38) (Boulton *et al*., 1991; Seger *et al*., 1991; Derijard *et al*., 1994; Kyriakis *et al*., 1994). These cascades have become prototypes for the study of structurally related but functionally distinct pathways (Figure II-1). The classical MAP kinases (ERK1/2) are activated by a variety of cell growth and differentiation stimuli (Ray *et al*, 1987; Marshall, 1995) and play a central role in mitogenic signaling (Pages *et al*., 1993). The p38 and JNK cascades are primarily activated by various

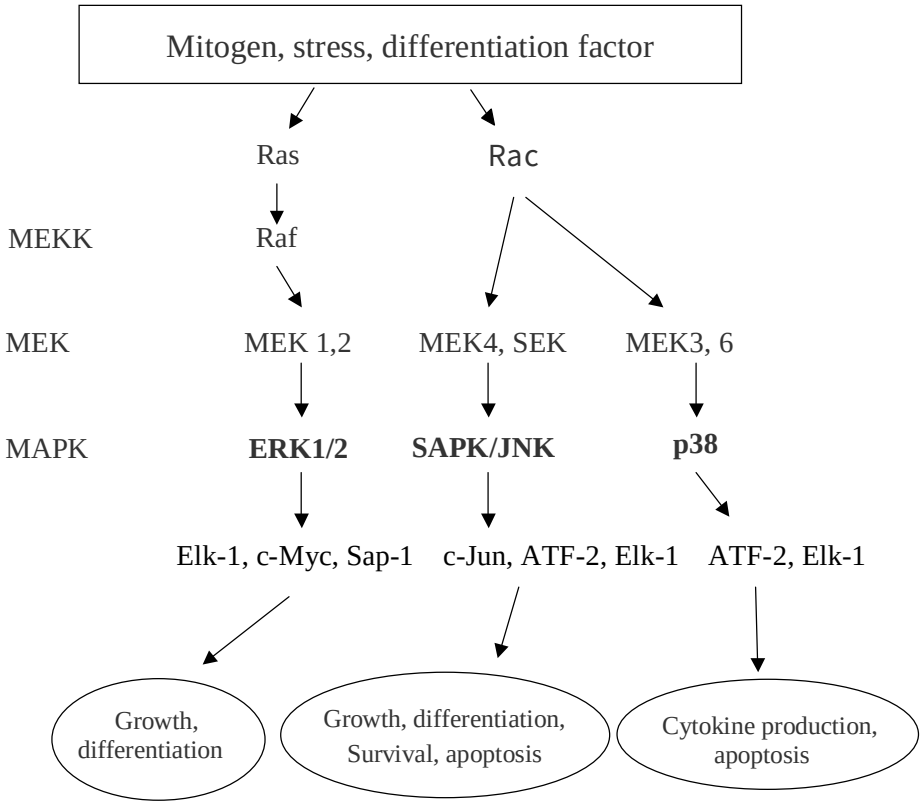


Figure II-1. The Mitogen-Activated Protein (MAP) kinases in multiple signaling pathways.

environmental stresses (osmotic shock, ultraviolet radiation, heat shock, x-ray radiation, hydrogen peroxide) and by the proinflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) (Freshney *et al*., 1994; Yan *et al*., 1994; Raingeaud *et al*., 1995; Verheij *et al*., 1996).

There are some evidences that the Bcr-Abl tyrosine kinase activates mitogenic signaling pathways including Ras, ERK, and JNK pathways as a primary consequence of expression (Cortez *et al*., 1997), and Ras-dependent signaling pathway is associated with the antiapoptotic effect of Bcr-Abl (Sakai *et al*., 1994; Cortez *et al*., 1996). However, the precise signaling events that elicit the antiapoptotic effects of Bcr-Abl are still unclear. Recently, it has been shown that PI 3-kinase dose not play any role in Bcr-Abl-mediated resistance to apoptosis (Marante-Mendes *et al*., 1997). It has been several years since it was suggested that apoptosis might be enhanced by the disruption of survival-associated MAP kinase signal transduction (Eastman *et al*., 1995; Kim *et al*., 1999; Lee *et al*., 2001). This offers an attractive hypothesis for the rational design of therapeutic chemicals that could be used in conjunction with current chemotherapeutic agents.

Recently, it was reported that As_2O_3 induced apoptosis in NB4 cells cloned from a relapsed patient with APL, by inducing loss of PML/RAR

α protein (Shao *et al.*, 1998), by suppressing the Bcl-2 protein expression (Chen *et al.*, 1996). Another biological activity of As_2O_3 is related to its effects on sulfhydryl-rich proteins, including enzymes that affect protein tyrosine phosphorylation (Cavigelli *et al.*, 1996; Chen *et al.*, 1998). Such alterations have been associated with its effects on cytokine-mediated signal transduction.

Several studies have reported on the ability of As_2O_3 to increase the activity of JNK, p38 in other cell lines (Kawasaki *et al.*, 1996; Iwama *et al.*, 2001), meanwhile, to decrease the activity of ERK 1 and ERK 2 in U937 leukemia cells (Iwama *et al.*, 2001). It was also reported previously that As_2O_3 caused the activation of both JNK and p38 but did not affect ERK in HeLa cells (Cavigelli *et al.*, 1996) and in NIH 3T3 cells (Lim *et al.*, 1998). However, the mechanism for the relative selectivity of As_2O_3 is not yet clear. Probably, it is related to the biological property and phenotype of the cells, the metabolism of As_2O_3 by the cells and target differences in different cells. The precise role that MAP kinases play in the regulation of As_2O_3-induced apoptosis in K562 cells is still unclear. In order to define more clearly the cellular mechanism through which As_2O_3 is capable of inducing apoptosis, it is investigated MAP kinase signaling pathways that lead to apoptosis in response to As_2O_3 in human leukemic K562 cells.

II. MATERIALS AND METHODS

Materials

Anti-phospho-p38, anti-phospho-ERK1/2, anti-cleaved caspase 3 were from New England Biolab; anti-phospho-JNK, anti-phospho-MEK3/6 and anti-phospho-ATF-2 were from Santa Cruz biotechnology. p38 inhibitor (SB203580) was purchased from Sigma Chemical Co. All other chemicals and reagents were the highest grade commercially available.

Preparation of cell lysates and Western blot analysis

Exponentially growing cells were starved for 24 h and then were exposed to 10 μM As_2O_3 for 0.5, 1, 2 and 3 h. And to analyze the effects of SB203580 on As_2O_3–treated cells, the starved cells were exposed to 10 μM SB203580 for 1 h and then were treated with 10 μM As_2O_3 for 0.5, 1, 2 and 3 h. After incubation, the treated cells were washed twice with PBS. Washed cells were lysed in lysis buffer (10 mM HEPES pH 7.9, 0.5% Triton X-100, 1 mM NaF, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 1 mM PMSF) and then the lysate was centrifuged at 15,000 × g for 10 min. And for Western blot method, the same procedure as chapter 1 was used. Anti-phospho p38, anti-phospho ERK 1/2, anti-phospho JNK 1/2, anti-

phospho-MEK 3/6 and anti-phospho-ATF-2 antibodies were used instead of anti-cleaved caspase-3 antibody. The expression of β-actin was used as a loading control.

DNA fragmentation analysis

Cells (5×10^6) which were treated with different concentrations of As_2O_3 for 24 h were harvested and washed twice as previously. And to analyze the effects of SB203580 on As_2O_3–treated cells, the cells were exposed to 10 μM SB203580 for 1 h and then were treated with various concentrations of As_2O_3 for 24 h. Next, DNA fragmentation was analyzed as described in chapter 1.

Chromatin condensation by fluorescence microscope

The cells were treated with 10 μM SB203580 for 1 h and then were treated with 10, 20 and 50 μM As_2O_3 for 24 h. The treated cells were incubated with 5 μg/ml Hoechst 33258 (Sigma, USA) for 30 min at 37℃. And for morphological examination of chromatin condensation, the same procedure with previous chapter was used.

Cell cycle analysis by flow cytometry

The Cells were incubated with As_2O_3 (10 μM) in the absence or presence of SB203580 (10 μM) for 24 h, 48 h and 72 h, stained with propidium iodide, and assayed by flow cytometry. This analysis was also done as described in chapter 1.

Data presentation

All assays were set up in triplicate, and results were expressed as the mean ± SD. Statistical significance for chemical or temporal effect was examined by independent pairs Student's *t*-test at P = 0.05 or 0.01.

III. RESULTS

Activation of p38 kinase and JNK 1/2

Many forms of cellular stress, including treatment with anticancer drugs, have been shown to modulate MAP kinase signaling pathways and induce apoptosis. However, the role of these signaling pathways in cell death has not been fully established, and contradictory evidence exists. During the course of apoptosis induced by stress such as UV irradiation, ceramide, anticancer drugs, and crosslinking of membrane IgM, activation of SAPK and p38 kinase are associated with or required for apoptosis (Butterfield *et al.*, 1997; Graves *et al.*, 1996; Juo *et al.*, 1997). Therefore, the activities of p38 and JNK were examined by Western blot analysis with phospho-specific antibodies that recognize the active form of each kinase. When K562 cells were treated with 10 μM As_2O_3, marked activation of p38 was observed 1 h after treatment and then its activity decreased (Figure II-2).

And it was examined that activation of JNK 1/2 was also induced in K562 cells for 3 h after treatment of cells with As_2O_3 (Figure II-3). This characteristic of activation of JNK is quite similar to those reported when mouse epidermal JB6 cells are treated with sodium arsenite (Lim *et al.*, 1998). Recently, it has been reported the pathways of JNKs are

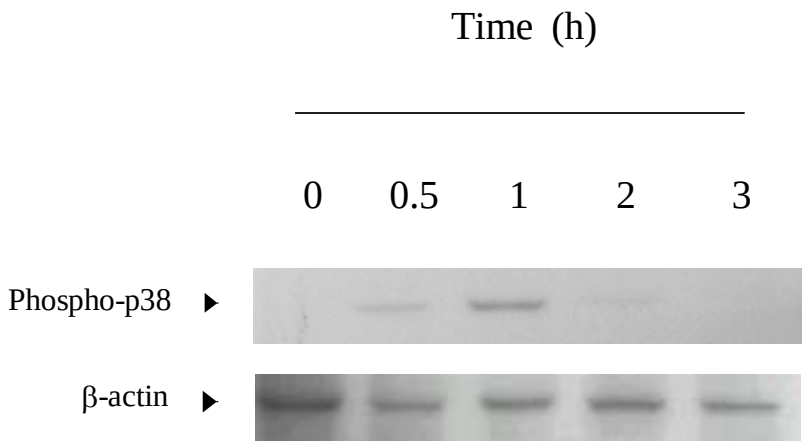


Figure II-2. Activation of p38 kinase by As_2O_3 in K562 cells. The cells were harvested at the indicated times after incubation with 10 μM of As_2O_3, and after cell lysis, total lysates were resolved by SDS-PAGE and Western blotted with an antibody against the phosphorylated form of p38.

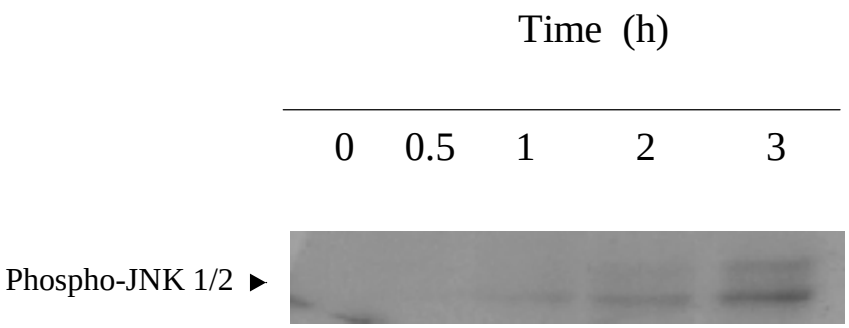


Figure II-3. Activation of JNK 1/2 by As_2O_3 in K562 cells. The cells were harvested at the indicated times after incubation with 10 μM of As_2O_3, after cell lysis, and total lysates were resolved by SDS-PAGE and Western blotted with an antibody against the phosphorylated form of JNK 1/2.

implicated in induction of apoptosis. The evidence for requirement of JNKs in apoptosis derives from studies where overexpression of JNKs results in apoptosis in some cells, and the antisense inhibition or the use of dominant-negative constructs attenuates apoptotic response (Basu *et al*., 1998).

Inhibition of ERK 1/2

It has been proposed that a balance between the ERK, JNK and p38 pathways might play an important role in the regulation of apoptosis (Xia *et al*., 1995), since activation of JNK and p38 promotes apoptosis, whereas the activation of ERK prevents it. Recently, it has been reported that activation of the JNK and p38 kinase and concurrent inhibition of ERK are critical for NGF withdrawal-induced apoptosis in rat PC-12 pheochromocytoma cells (Xia *et al.*, 1995).

Therefore, phosphorylated forms of ERK 1/2 were detected by Western blot analysis. In this study, in contrast to the marked activation of JNK and p38, it was observed that ERK 1/2 was inhibited after treatment of As_2O_3 (Figure II-4).

Activation of MEK 3/6 and ATF-2 by As_2O_3

p38 kinase, one member of MAP kinase family, is specifically

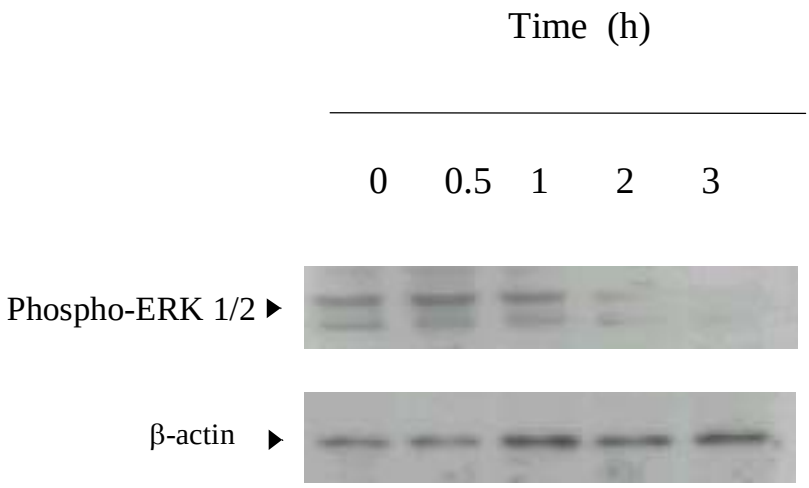


Figure II-4. Inhibition of ERK 1/2 by As_2O_3 in K562 cells. The cells were harvested at the indicated times after incubation with 10 μM of As_2O_3, after cell lysis, and total lysates were resolved by SDS-PAGE and Western blotted with an antibody against the phosphorylated forms of ERK 1/2.

activated by two immediate upstream regulators, MAP kinase kinase (MEK) 3 and MEK 6 (Moriguchi *et al*., 1996; Han *et al*., 1996). MEK 3 and MEK 6, like other MAP kinase family members, are activated by the dual phosphorylation on two Ser/Thr residues. Involvement of MEK 3/6, which acts on upstream of p38 pathway, in As_2O_3-induced apoptosis was studied. And it was examined As_2O_3 significantly activated MEK 3/6 at 1 h after treatment (Figure II-5). Next, to study the possible regulation of nuclear target by As_2O_3, the activity of transcription factor, ATF-2, was examined by Western blot analysis. In result, As_2O_3 activated ATF-2 at 1 h after treatment (Figure II-6). In this study, it was found the activation of MEK 3 and MEK 6 / p38 / ATF-2 pathway is involved in the signal transduction of As_2O_3-induced apoptosis.

Inhibition of DNA fragmentation by SB203580

Although the As_2O_3–induced apoptosis in K562 cells was accompanied by the activation of JNK and p38 kinase, it is obscure which pathway is important for As_2O_3–induced apoptosis. To examine whether activation of p38 by As_2O_3 might be associated with the induction of apoptosis in K562 cells, SB203580, a specific inhibitor of p38, was treated to the cells. In the presence of SB203580, it was

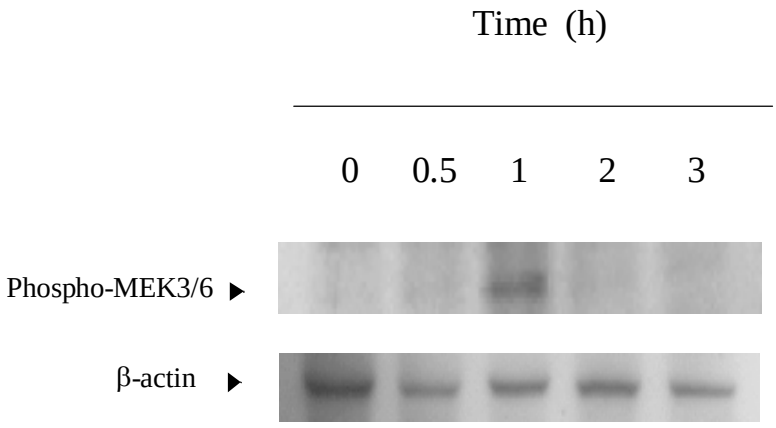


Figure II-5. Activation of MEK 3/6 by As_2O_3 in K562 cells. The cells were harvested at the indicated times after incubation with 10 μM of As_2O_3, and after cell lysis, total lysates were resolved by SDS-PAGE and Western blotted with an antibody against the phosphorylated forms of MEK 3/6.

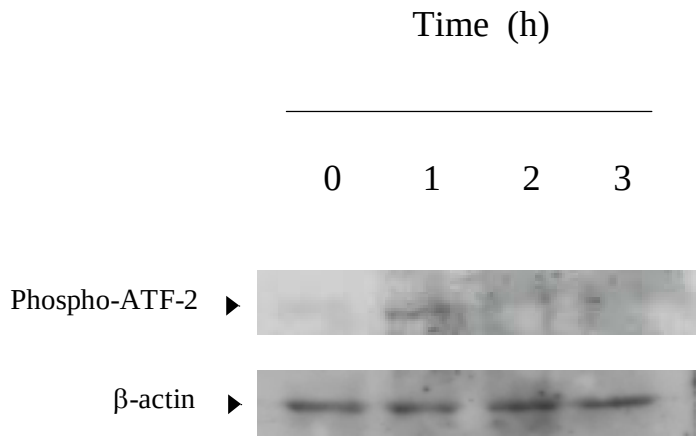


Figure II-6. Activation of ATF-2 by As_2O_3 in K562 cells. The cells were harvested at the indicated times after incubation with 10 μM of As_2O_3, and after cell lysis, total lysates were resolved by SDS-PAGE and Western blotted with an antibody against the phosphorylated form of ATF-2.

confirmed that the activation of p38 was blocked during As_2O_3 treatment (Figure II-7). It was previously examined that the amount of nucleosomal DNA fragmentation gradually increased with the time of exposure to As_2O_3. To confirm that p38 has relation to As_2O_3-induced apoptosis, SB203580 was added to the medium at a concentration of 10 μM, before treatment with As_2O_3. In result, the formation of nucleosomal DNA fragmentation induced by As_2O_3 was significantly blocked by SB203580 (Figure II-8). This finding suggests that the pathway of p38 plays an essential role in As_2O_3-induced apoptosis.

Inhibition of apoptotic cell death by SB203580

It was also examined that p38 kinase participates in As_2O_3-induced apoptosis. In the presence of SB203580, severe nuclear condensation and fragmentation were inhibited in approximately 35-45% compared to that of the nontreated cells (Figure II-9). It was found that inhibition of p38 phosphorylation significantly reduced nuclear apoptosis. The role of p38 activity in the alterations of nuclear morphology was confirmed in K562 cells. And cytotoxicity was evaluated by MTT method after pretreatment 10 μM SB203580 before treatment of As_2O_3 for 24 h. In result, cytotoxicity was decreased in the SB203580 treated cells than nontreated cell (Figure II-10). These results suggest that As_2O_3-induced apoptosis was

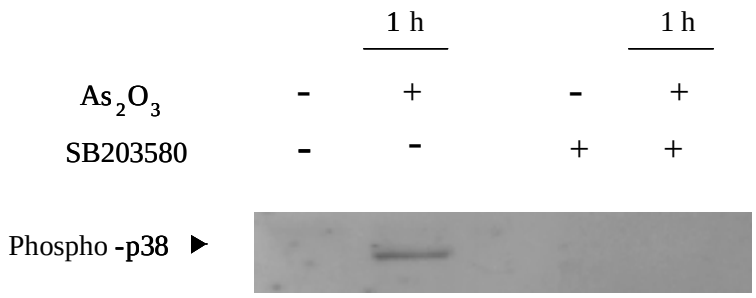


Figure II-7. Inhibition of p38 activation by SB203580, a specific inhibitor of p38. The cells were treated with or without 10 μM SB203580 for 1 h and harvested at the indicated times after incubation with 10 μM of As_2O_3. The whole cell lysates were prepared and subjected to Western blot analysis with phospho-p38 antibody.

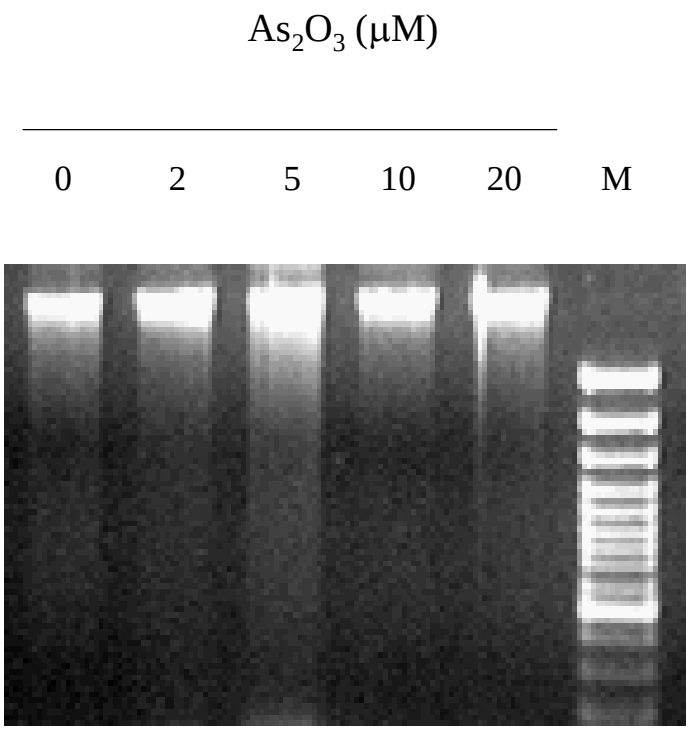


Figure II-8. Inhibition of As_2O_3-induced DNA fragmentation by SB203580. K562 cells were treated with 10 μM SB203580 for 1 h and then individually treated with various concentration of As_2O_3 for 24 h. Lane M, DNA marker.

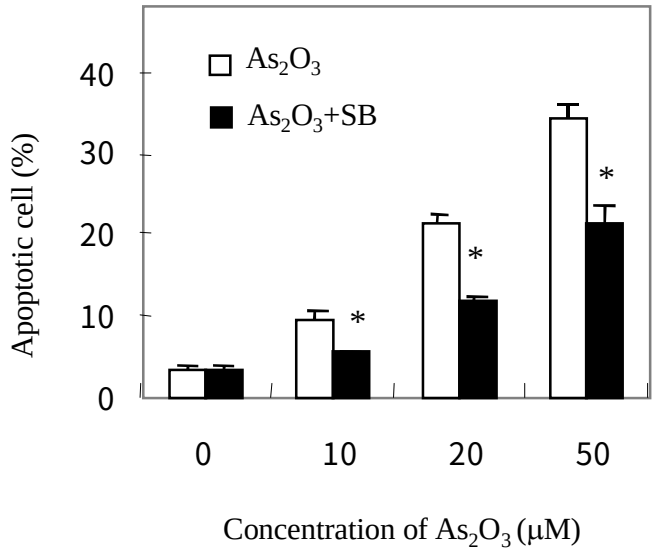


Figure II-9. Effect of SB203580 on As_2O_3-induced apoptosis. The cells were treated with 10 μM SB203580 for 1 h and then treated with indicated concentration for 24 h, and stained with Hoechst 33258, and cells with condensed chromatin and fragmented nuclei were counted as apoptotic under a fluorescence microscope (× 100). * The datum in presence of SB203580 is significantly different from that in the absence of SB203580 ($p < 0.05$).

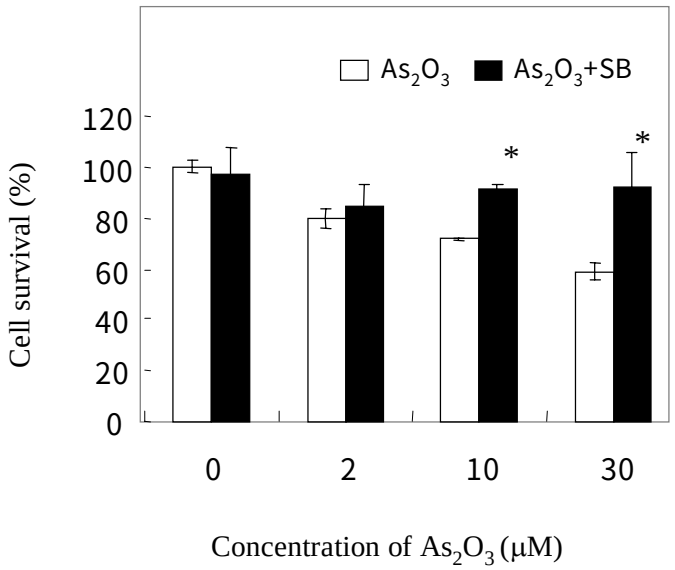


Figure II-10. Effect of SB203580 on cytotoxicity in As_2O_3-treated K562 cells. The cells were treated with 10 μM SB203580 for 1 h and then treated with the indicated concentrations of As_2O_3 for 24 h. Cytotoxicity was measured by MTT assay.* The datum in presence of SB203580 is significantly different from that in the absejnce of SB203580 ($p < 0.05$)

inhibited by SB203580, indicating a requirement for p38 kinase signaling in the apoptosis induced by As_2O_3.

Inhibition of caspase-3 by SB203580

To confirm the essential role of p38 in the activation of caspase-3 induced by As_2O_3, the effect of SB203580 on the expression of caspase-3 in K562 cells was analyzed. In result, SB203580 inhibited the activation of caspase-3 (Figure II-11). For this, it was confirmed that p38 kinase is involved in As_2O_3-induced apoptosis once more.

Effect of SB203580 on cell cycle

To investigate whether a p38 inhibitor would prevent As_2O_3–induced G2/M arrest and apoptosis, the cells were treated for 24 h, 48 h, and 72 h with 10 μM As_2O_3 alone or in the presence of 10 μM SB203580. As shown in Figure II-12, about 31.3% of the cells had arrested at G2/M phase after being treated with As_2O_3 alone and about 20.6% of the cells treated with SB203580 combination with As_2O_3 (Table II-1). This data suggest that p38 is involved in As_2O_3-induced G2/M arrest, but the activation of p38 by itself is not sufficient to induce apoptosis.

In the present study, it is implied that the activation of p38 kinase may be related to apoptotic signal transduction and affects the G2/M phase arrest by As_2O_3.

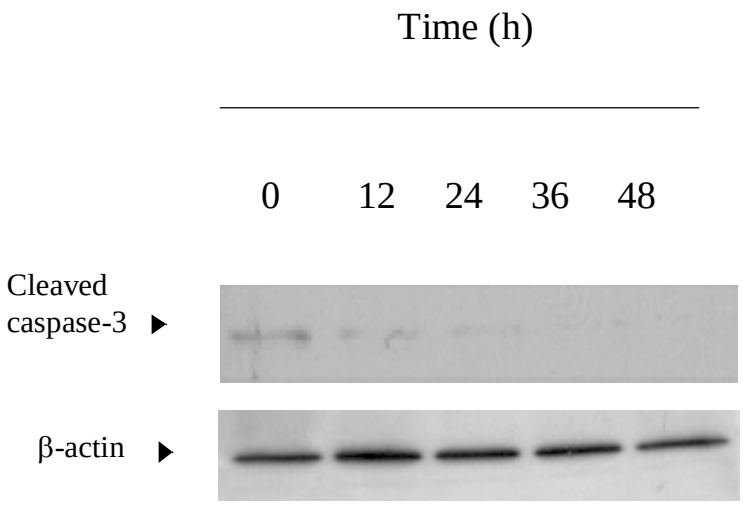


Figure II-11. Inhibition of caspase-3 by SB203580 in As_2O_3 treated K562 cells. The cells were treated with 10 μM SB203580 for 1 h and then with indicated times after incubation with 20 μM of As_2O_3, and after cell lysis, total lysates were resolved by SDS-PAGE and Western blotted with an antibody against the cleaved caspase-3.

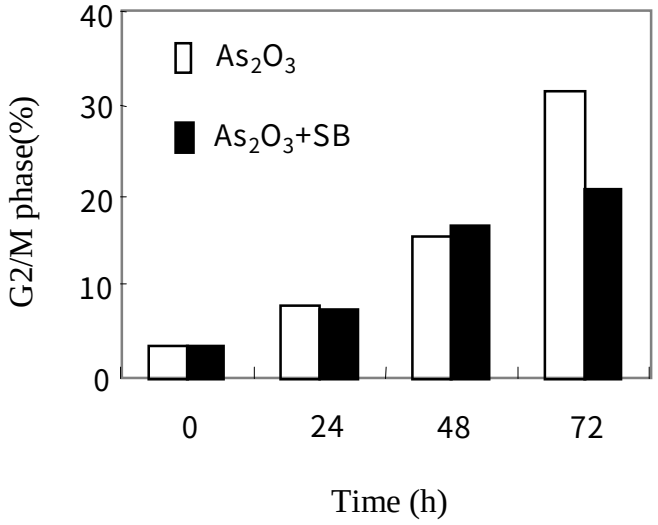


Figure II-12. Effect of SB203580 on the alteration of G2/M phase by As_2O_3 in K562 cells. The cells were incubated with As_2O_3 (10 μM) in the absence or presence of SB203580 (10 μM) for 24 h, 48 h and 72 h, stained with propidium iodide, and assayed by flow cytometry.

Table II-1. Effect of SB203580 on the alteration of cell cycle by As_2O_3 in K562 cells. The cells were incubated with As_2O_3 (10 μM) in the absence or presence of SB203580 (10 μM) for 24 h, 48 h and 72 h, stained with propidium iodide, and assayed by flow cytometry.

Sample	24 h (%)			48 h (%)			72 h (%)		
	G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M
Control	46.0	50.9	3.20	45.3	51.4	3.30	46.6	50.0	3.40
As_2O_3	48.8	43.4	7.80	28.2	56.3	15.5	14.0	54.7	31.3
SB203580	48.3	48.2	3.40	53.6	45.5	0.80	55.2	40.6	4.20
As_2O_3+SB	47.9	44.7	7.30	13.5	69.9	16.6	28.7	50.7	20.6

IV. DISCUSSION

In the last few years, a large body of evidence has implicated the MAP kinase family of proline-directed serine/threonine kinases in the regulation of apoptosis. Three MAP kinase family members have been characterized thus far. Each MAP kinase is activated through a similar but selective pathway of kinases. A number of studies indicate that the activation of JNK/SAPK and p38 may play decisive roles in the control of apoptotic cell death (Xia *et al*., 1995; Chen *et al*., 1996; Verheij *et al*., 1996; Luo *et al*., 1998). Involvement of these stress-activated protein kinases has been proposed in the apoptosis that is induced in differentiaed PC12 cells by deprivation of NGF, in U937 cells by ceramide (Verheij *et al*., 1996), in mouse pam 212 keratinocytes by cisplatin (Sanchez-Perez, 1998), and in Jurkat T cells by UV and γ irradiation (Chen et al., 1996), Assefa *et al* (1994), reached a similar conclusion, showing that the JNK and p38 pathways protect HeLa cells from apoptosis induced by photodynamic therapy with hypericin. Apoptosis is a form of cell death culminates in the activation of caspases and nucleases that serve to degrade protein and genomic DNA within the cell. Most cancer chemotherapeutic agents have been reported to induce apoptosis. However, the signal transduction mechanisms that

regulate apoptosis have yet to be clearly defined.

The abnormal *bcr-abl* oncogene encodes the constitutively active Bcr-Abl tyrosine kinase, which plays an essential role in the pathogenesis of the disease via phosphorylation of protein substrates and activation of multiple downstream mitogenic pathways (Daley *et al.*, 1990; Sattler and Salgi, 1998). It is well known that As_2O_3 interferes with the phosphorylation of tyrosine. As_2O_3 might thus block phosphorylation of proteins associated with signal transduction. Therefore, Western blot analysis examined the time course of the amounts of proteins and phosphorylated tyrosines with anti-phosphotyrosine antibody. It was found that none of them showed any significant change in amount (data not shown).

In order to define more clearly the cellular mechanism through which As_2O_3 is capable of inducing apoptosis, MAP kinases signaling pathway that lead to apoptosis in response to As_2O_3 in K562 cells was investigated. These data indicate that As_2O_3 activates the MEK 3/6, p38 kinase and ATF-2 pathway in the induction of apoptosis in K562 cells. Several studies have reported on the ability of As_2O_3 to increases the activity of JNK, p38 in other cell lines. It was reported previously that arsenite caused the activation of both JNK and p38 but did not affect ERK in HeLa cells (Cavigelli *et al.*, 1996) and in NIH3T3 cells (Lim *et*

al., 1998). In fibroblast Rat-1 cells and PC12 cells, 400 μM of sodium arsenite activated ERK, JNK and p38 (Liu *et al*., 1998). A higher concentration, 500 μM, of sodium arsenite also activated ERK1/2 in a p38-dependent manner during transformation of human HEK293 embryonic kidney cells (Ludwig *et al*., 1998).

Futhermore, in studies to determine the effects of p38 on down regulation of Bcr-Abl, it was found that inhibition of p38 activation using the p38 inhibitor SB203580 did not affect on the expression of Bcr-Abl (data not shown). Thus, the As_2O_3-inducible p38 kinase pathway and down-regulation of Bcr-Abl oncoprotein appear to function independently of each other in K562 cells and Bcr-Abl may act on upstream of the p38 kinase.

Western blot analysis confirmed that the apoptotic process elicited by the exposure to As_2O_3 was executed through the activation of caspase-3. The time course of caspase-3 activation corresponded to that of nucleosomal DNA fragmentation. In the present study, it was demonstrated that the formation of nucleosomal DNA fragmentation, severe nuclear condensation and fragmentation, and caspase-3 expression induced by As_2O_3 were significantly blocked by SB203580. These findings suggest that the activation of p38 appeared to be required for the induction of apoptosis by As_2O_3 since SB203580, significantly inhibited As_2O_3-

induced apoptosis.

In this study, it was also examined whether inhibition of p38 kinase was mediated in the inhibition of the cytotoxic effects and cell cycle arrest induced by As_2O_3. But these results suggest that activation of p38 by itself is not sufficient to induce apoptosis but renders cells susceptible to apoptosis upon exposure to As_2O_3. Accordingly, activation of the p38 kinase pathway by As_2O_3 may have additional effects that mediate cytotoxic responses such as regulation of signals that modify cell cycle progression in CML cells. Thus, the p38 kinase pathway may participate in the generation of anti-leukemic effect of As_2O_3 and provide a useful rationale for treatment CML.

CONCLUSIONS

I. Arsenic Trioxide (As_2O_3) - Induced Apoptosis, Cell Cycle Arrest and Down Regulation of BCR-ABL in K562 Cells

1. As_2O_3 induced cytotoxicity in the human chronic myelogenous leukemia (CML)-blast crisis-derived K562 cell line.

2. Morphological changes of the cells undergoing apoptosis were confirmed with an electron microscope, and chromatin condensational changes were also observed with Hoechst 33258 staining.

3. The percentages of apoptotic cells increased in a time- and dose-dependent manner.

4. DNA fragmentation, which is the biochemical hallmark of apoptosis, was detected 12 h and 24 h after exposure to 20 μM As_2O_3.

5. Activation of caspase-3 increased at 12 h and 24 h, and decreased at 36 h and 48 h after treatment with 20 μM As_2O_3.

6. Pretreatment of 25 μM Z-VAD-fmk, a specific inhibitor of caspase, decreased As_2O_3-induced cytotoxicity.

7. As_2O_3 significantly increased the percentages of the cells accumulated in the G2/M phase of the cell cycle in a time- and dose-dependent manner.

8. Not only the expression of Bcr-Abl oncoprotein but also *bcr-abl* mRNA levels decreased significantly in As_2O_3-treated cells, suggesting a transcriptional regulation.

These results suggest that As_2O_3 induces apoptosis through caspase-3 activation and Bcr-Abl down regulation in K562 cells, and may provide a useful rationale for CML treatment.

II. The Involvement of p38 MAP kinase in Apoptosis Induced by As_2O_3 in K562 Cells

1. As_2O_3 strongly induced activation of p38 and c-jun N-terminal kinases (JNK 1/2) by treatment with 10 μM, while extracellular signal-regulated protein kinases (ERK 1/2) were inhibited.

2. Not only MEK 3/6, which acts on upstream of p38, but also ATF-2, a transcription factor, was activated after treatment of As_2O_3.

3. Pretreatment of SB203580, a specific inhibitor of p38, inhibited As_2O_3-induced DNA fragmentation and apoptotic cell death.

4. Activation of caspase-3 induced by As_2O_3 was significantly blocked by SB203580.

These results suggest that As_2O_3-induced apoptotic mechanism may be associated with signal transduction via p38 kinase in K562 cells.

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국문 요약

K562 만성 골수성 백혈병 세포에서 Arsenic trioxide에 의한 Apoptosis 및 작용기전에 관한 연구

만성 골수성 백혈병 (CML)은 모든 성숙단계의 골수구계 세포의 증식과 세포유전학적으로 필라델피아 염색체를 특징적으로 나타내는 조혈모세포 질환으로 CML 세포주인 K562세포는 *bcr-abl* 융합유전자에 의한 tyrosine kinase의 높은 활성에 의해 발생되는 백혈병 세포이다. 여러 자극에 대해 상당히 높은 apoptosis 내성을 가지고 있어 치료에 또한 어려움이 많은 질환 중의 하나이다.

최근 arsenic trioxide (As_2O_3)는 all-*trans* retinoic acid에 내성을 보이거나, 재발된 급성 전골수성 백혈병 환자의 치료에 있어서 좋은 효과를 보였음을 보고한 이래로 다른 악성 질환 및 골수증식성 질환 등에서도 실험이 진행 중에 있다. 본 연구에서는 *bcr-abl* 융합유전자를 가진 K562세포를 이용하여 As_2O_3가 apoptosis에 미치는 영향과 mitogen-activated protein (MAP) kinase와 관련된 작용기전에 대해 알아보고자 하였다.

우선 K562세포에 대한 As_2O_3의 세포 독성능에 대해 살펴 본

결과 24시간 배양시 50% 세포독성을 나타내는 IC_{50}은 10 μM로 나타났으며, 전자현미경 및 형광현미경을 통해 핵의 분절, 염색질 응축, apoptotic body 등 apoptosis로 진행되는 세포의 형태학적인 변화를 관찰하였다. Apoptosis의 특징인 DNA 분절이 20 μM의 As_2O_3처리 12시간과 24시간에서 형성됨을 1.8% agarose 전기영동을 통해 확인하였다.

또한 As_2O_3 처리에 의한 caspase-3의 활성 증가를 관찰하였고, caspase 억제제인 Z-VAD-fmk를 함께 처리한 경우 As_2O_3만 처리한 군에 비해 세포독성이 감소하였고 DNA 분절능이 억제된 결과를 통해 caspase-3가 apoptosis mediator로서 작용한다는 것을 확인하였으며, As_2O_3가 세포주기에 영향을 미치는지 알아보고자 시간별, 농도별로 처리하여 유세포 분석을 통해 세포주기 변화를 측정한 결과, G2/M 세포의 비율이 높게 나타남을 확인하였다. 그리고, Western blot 분석을 통해 As_2O_3에 의한 Bcr-Abl oncoprotein 발현변화를 관찰한 결과, 대조군에 비해 As_2O_3 처리시 감소되었음을 확인하였고, RT-PCR에 의한 *bcr-abl* 융합유전자 검출에서도 대조군과 10 μM 처리군에서는 305 bp의 위치에서 band가 관찰되었으나, As_2O_3 20 μM 처리 후에는 거의 관찰되지 않음을 통해, As_2O_3가 Bcr-Abl 단백질의 down-regulation에 관여함을 확인하였다.

다음으로 As_2O_3에 의해 유도되는 apoptosis와 관련된 신호전달과정 중 MAP kinase와 관련하여 알아보았다. As_2O_3처리에 의한 apoptosis과정 중 p38, 그의 upstream으로 작용하는 MEK 3/6,

그리고 transcription factor인 ATF-2의 활성이 증가되었음을 Western blot 분석을 통해 확인하였다. P38의 inhibitor인 SB203580의 전처리시 As_2O_3로 유도되는 DNA 분절능의 억제와 apoptotic cell death가 억제됨을 확인함으로써 apoptosis와 p38 MAP kinase의 관련성을 뒷받침하였다. 그러나, SB203580 처리 후 세포 독성능과 세포주기 변화에 미치는 영향을 살펴본 결과 매우 의미있게 감소되지 않음을 보아서 As_2O_3에 의한 apoptosis에 있어서 p38 kinase만으로는 충분하지 못하며 다른 요인이 작용할 것이라 보며 이에 대한 추후 연구가 계속되어야 될 것 이다.

위의 실험 결과를 통해 K562 CML 세포주는 As_2O_3에 의해 apoptosis가 유도되었으며, 이 과정에서 caspase-3의 활성증가와 Bcr-Abl 단백질의 down-regulation 및 mRNA의 소실 등을 확인하였다. 그리고 이와 관련된 신호전달기전은 MAP kinase family 중에서 p38 kinase pathway가 관여한다는 것을 알 수 있었다. 따라서 As_2O_3에 의한 apoptosis 기전을 응용함으로써 CML에 있어서 새로운 치료제의 가능성에 관한 정보의 제공과 함께 임상적 응용에도 도움을 줄 것으로 생각된다.

핵심어: Arsenic trioxide (As_2O_3), 만성 골수성 백혈병 (CML), Bcr-Abl, Apoptosis, 세포 독성능, caspase-3, 세포주기, 신호전달, p38 MAP kinase