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Arsenic Trioxide Induces Apoptosis in Chronic Myelogenous Leukemia K562 Cells: Possible Involvement of p38 MAP Kinase

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Arsenic trioxide (As_2O_3) was recently demonstrated to be an effective inducer of apoptosis in patients with relapsed acute promyelocytic leukemia (APL) as well as in patients with APL in whom all-*trans*-retinoic acid and conventional chemotherapy failed. Chronic myelogenous leukemia cells are highly resistant to chemotherapeutic drugs. To determine if As_2O_3 might be useful for the treatment of chronic myelogenous leukemia, we examined the ability of As_2O_3 to induce apoptosis in K562 cells. *In vitro* cytotoxicity of As_2O_3 was evaluated in K562 cells by a MTT assay; the IC_{50} value for As_2O_3 was determined to be 10 μM. When analyzed by agarose gel electrophoresis, the DNA fragments became evident after incubation of the cells with 20 μM As_2O_3 for 24 h. We also found morphological changes and chromatin condensation of the cells undergoing apoptosis. Activation of caspase-3 was observed 6 h after treatment with 20 μM As_2O_3 by a Western blot analysis. Next, we examined the MAP kinase-signaling pathway of As_2O_3-induced apoptosis in K562 cells. As_2O_3 at 10 μM strongly induced the activation of p38 and JNK 1/2, while ERK 1/2 was inhibited. In addition, pretreatment of SB203580, a specific inhibitor of p38, inhibited As_2O_3 induced apoptotic cell death. These results suggest that As_2O_3 is able to induce the apoptotic activity in K562 cells, and its apoptotic mechanism may be associated with the activation of p38.

Keywords: Apoptosis, Arsenic trioxide, Chronic myelogenous leukemia, p38 MAP kinase

Introduction

Arsenic trioxide (As_2O_3) is 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 that are designed to more fully understand the cellular and molecular effects that underlie the mechanism of action of As_2O_3 (Mu *et al*., 1994; Chen *et al*., 1997; Wang *et al*., 1998). The clinical efficacy of As_2O_3 in APL has also been confirmed, even in patients that are resistant to conventional chemotherapy (Soignet *et al*., 1998). Recent reports suggest that the apoptotic effect of As_2O_3 is not specific for APL cells. The newly recognized potential efficacy of this chemotherapeutic agent for the treatment of other human malignancies and myeloproliferative syndromes is currently being studied (Zhang *et al*., 1998; Bazarbachi *et al*., 1999; Rousselot *et al*., 1999).

Human chronic myelogenous leukemia (CML) is a malignancy of pluripotent hematopoietic cells that is caused by the dysregulated activity of the tyrosine kinase that is encoded by the chimeric *bcr-abl* oncogene (Witte *et al*., 1999). CML cells are highly resistant to chemotherapeutic drugs and very difficult to treat (Amarante-Mendes *et al*., 1998); therefore, anticancer drugs that are active against this type of leukemia are urgently needed.

Several years ago, 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; So *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. As_2O_3 induced apoptosis in NB4 cells that were cloned from a relapsed patient with APL by inducing the loss of the PML/RARα protein (Shao *et al*., 1998) and suppressing the expression of the Bcl-2 protein (Chen *et al*., 1996). Several studies 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). However, the mechanism for arsenics relative selectivity is still unclear. It is probably related to the biological property and phenotype of the cells, arsenics

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metabolism 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. To more clearly define the cellular mechanism through which As_2O_3 is capable of inducing apoptosis, we investigated intracellular signaling pathways that lead to apoptosis in response to As_2O_3 in human leukemia K562 cells.

In the present study, we demonstrated that apoptosis is induced in CML cell lines with significant cytotoxicity. Also, the activation of p38 kinase signaling pathways markedly affects the induction of apoptosis and may provide a useful rationale for the treatment of chronic myelogenous leukemia.

Materials and Methods

Materials As_2O_3 was purchased from the Sigma Chemical Co. (St. Louis, USA). As_2O_3 was dissolved in sodium hydroxide (NaOH) at a concentration of 1 mM/L and diluted to a working solution before use. The maximum concentration of NaOH in culture had no influence on the cell growth in the cell lines. DMSO, NaOH, MTT, PBS, RNase, SB203580, and Hoechst 33258 were purchased from the Sigma Chemical Co. CycleTest-Plus DNA reagent KIT was purchased from Becton Dickinson (Mountain View, USA). Anti-phospho-p38, anti-phospho-ERK1/2, anti-cleaved caspase 3 were from New England Biolab. Anti-phospho-JNK was from Santa Cruz Biotechnology.

Cell culture and *in vitro* cytotoxicity assay Human chronic myelogenous leukemia K562 cells were maintained in a RPMI 1640 medium that was supplemented with 10% fetal bovine serum. All of the cells 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 a trypan blue dye exclusion assay. One hundred µl of cell suspension were distributed into each well of a 96-well plate. After the treated cells were incubated for 24 h, 50 µl MTT (1 mg/ml, Sigma) was added into each well. 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 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.

Morphological features of apoptosis After treatment with or without As_2O_3, 5×10^4, the cells were washed with PBS (pH 7.4) and resuspended in the same buffer. The cells that were 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, 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°C, the cells were cut and stained with uranyl acetate and lead citrate.

DNA fragmentation analysis The 5×10^6 cells that were treated for 24 h were washed twice in a solution of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 100 mM NaCl. They were then lysed with a 500 µl lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 20 mM EDTA). The lysates were centrifuged by $1000 \times g$ for 10 min. 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), and 50 µl proteinase K (1 mg/ml, Sigma, USA). Then, the DNA was extracted with phenol/chloroform/isoamylalcohol (25 : 24 : 1, Sigma, USA). After precipitation, the pellets were resuspended in a 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) that contained ethidium bromide. The DNA bands were visualized by UV light.

Chromatin condensation The cells were incubated with 5 µg/ml Hoechst 33258 (Sigma, USA) for 30 min at 37°C. An aliquot of the cells was transferred to a microscope slide and fitted with a coverslip. DNA was visualized with a fluorescent microscope. The cells that exhibited 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.

Cell cycle analysis The cells that were treated with 20 µm of As_2O_3 for a variety of time periods and controls were collected and fixed in 70% ethanol and stored at –20°C before use. After resuspension, the cells were washed with PBS and treated with Cycle TEST™ Plus DNA Reagent Kit (Becton Dickinson). The cells were resuspened in 250 µl of a 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 ml of propidium iodide (50 µg/ml) for 30 min at room temperature. The stained cells were analyzed with a FACScan flow cytometer (Becton Dickinson) for relative DNA content, based on increased red fluorescence. Each of the G1, S, G2/M phases of the cell cycle was calculated using the RFIT program.

Preparation of cell lysates and Western blot analysis The cells were washed twice with PBS. The washed cells were lysed in a 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), then the lysate was centrifuged at $15{,}000 \times g$ for 10 min. The protein concentration was determined by Bradford's method (1976) using bovine serum albumin as a standard. The supernatant was used for a Western blot analysis. A cell lysate that contained 50 µg of the protein was fractionated by SDS-PAGE on the appropriate percent polyacrylamide gel. Then the 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. It was subsequently probed overnight at 4°C with anti-phospho-p38 antibody, anti-phospho-ERK1/2 (New England Biolabs, Beverly, USA), anti-cleaved caspase-3, and anti-phospho-JNK (Santa Cruz Biotechnology, Santa Cruz, USA) 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 and 1 : 2,000 dilution in TBS. After washing the membrane with TBST, the protein bands on the membrane were detected with an enhanced chemiluminescence detection method by immersing the blots for 1 min in a 1 : 1 mixture of chemiluminescence reagents A and B (Amersham, Buckinghamshire, England). They were then exposed to Kodak film for a few minutes.

Statistical analysis All of the assays were set up in triplicate. The results were expressed as the mean ± SD. Statistical analysis was determined by a Student's *t*-test.

Results and Discussion

Cytotoxic effects of As_2O_3 We examined the cytotoxicity effect of As_2O_3 on the human leukemia cell line K562 by treating with different concentrations of As_2O_3 for 24 h and 48 h. K562 cells were exposed to As_2O_3, ranging from 1 to 80 μM, and cytotoxicity was determined by a MTT assay. Cell death increased with increased concentrations of As_2O_3. The IC_{50} value for As_2O_3 was 10 μM (Fig. 1). Chen *et al.* (1997) reported that at concentrations of 0.5-2 μM, As_2O_3 triggers the apoptosis of both NB4 and the cultured primary APL cells that are derived from patients. The cytotoxicity of K562 cells was shown at higher As_2O_3 concentrations than that of NB4 cells. The experiments clearly showed that As_2O_3 induced cytotoxicity in the K562 CML cell line in a dose-dependent manner.

Induction of apoptosis by As_2O_3 The K562 cells are particularly resistant to apoptosis against various agents and have a poor prognosis. However, the consensus of several reports recently is that As_2O_3 induces apoptosis in leukemia cells. Enhanced translocation of promyelocytic leukemia proteins to nuclear bodies, modification of the glutathione redox system, caspase activation, and cell cycle arrest may participate in the process that leads to cell death in the APL cell line (Chen *et al*., 1996; Soignet *et al*., 1998; Bazarbachi *et al*., 1999). Therefore, we continued to investigate whether or not the cytotoxic effect is caused by apoptosis in K562 cells. In order to determine the apoptotic effect of As_2O_3, we examined the apoptotic response, as judged by the appearance of a DNA ladder, by gel electrophoresis. A characteristic pattern of nucleosomal DNA fragmentation, which is the biochemical hallmark of apoptosis, was detected 24 h after exposure to 20 μM of As_2O_3 (Fig. 2A). The amount of nucleosomal DNA fragments gradually increased with the concentration of indent As_2O_3. Treatment of leukemia K562 cells with As_2O_3 at concentrations above 10 μM also resulted in the condensation of the chromatin and fragmentation of nuclei, detected after staining with Hoechst 33258 (Fig. 2B). This result supports the conclusion that As_2O_3 induces apoptosis in K562 cells.

To investigate the apoptotic morphological changes after an As_2O_3 treatment, the condensed nuclei were fixed with glutaraldehyde and examined by a thin section of EM. The treatment of the K562 cells with As_2O_3 resulted in

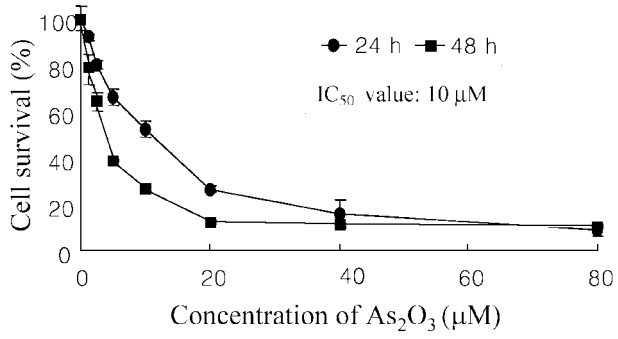

Fig. 1. Cytotoxic effects of As_2O_3 on K562 cells. The cells were incubated with various concentrations of As_2O_3 for 24 h and 48 h. Cytotoxicity was measured by a MTT assay. The results are presented as mean ± SD for triplicate, and the bar represents the standard deviation.

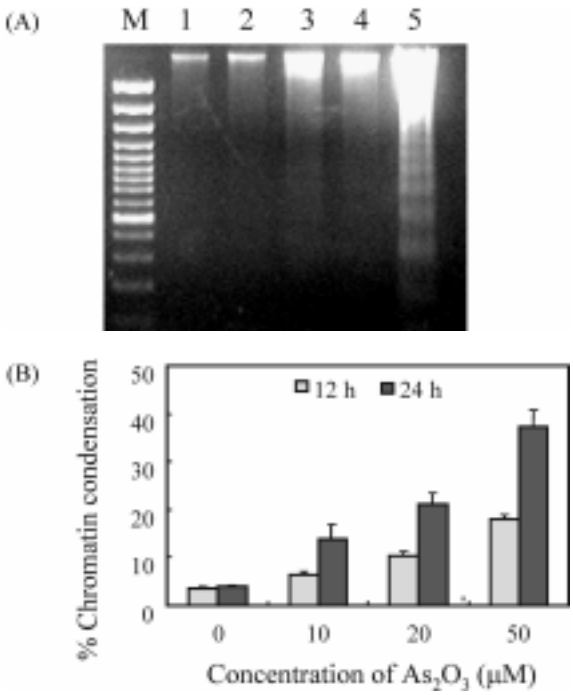

Fig. 2. Effect of As_2O_3 on the induction of apoptosis and DNA fragmentation in K562 cells. (A) The cells were treated with various concentrations of As_2O_3 for 24 h. Extracted DNA was fractionated by electrophoresis and stained by ethidium bromide. Lane M, DNA marker; Lane 1, control; Lane 2, 2 μM As_2O_3; Lane 3, 5 μM As_2O_3; Lane 4, 10 μM As_2O_3; Lane 5, 20 μM As_2O_3. (B) The cells were treated with the indicated concentration for 12 h and 24 h and stained with Hoechst 33258. The cells with condensed and fragmented nuclei were counted as apoptotic under a fluorescence microscope (× 100).

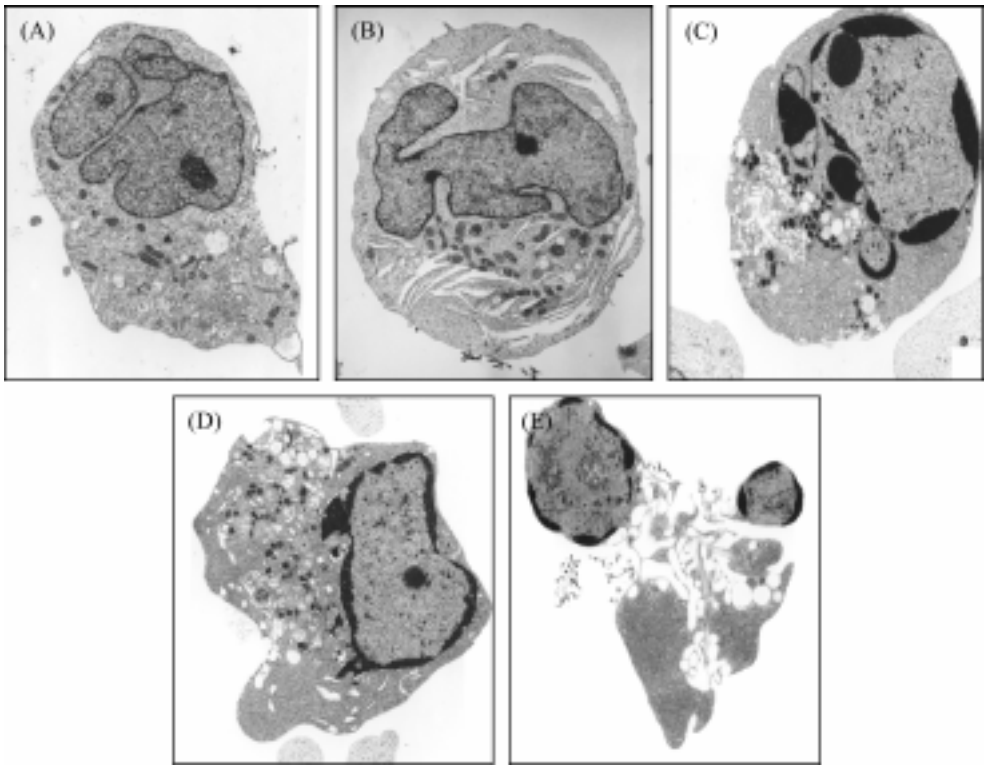

Fig. 3. Effect of As_2O_3 treatment on the morphology of K562 cells. The cells were treated with the control vehicle (A), 10 μM As_2O_3 for 24 h (B), or 20 μM As_2O_3 for 24 h (C), 36 h (D), 48 h (E), stained with uranyl acetate and lead citrate, then analyzed under an electron microscope (× 4,000).

morphological changes that are consistent with the process of apoptosis. Morphological changes that are consistent with the process of apoptosis (including blebbing of the plasma membrane, chromatin condensation, fragmentation of nuclei and formation of apoptotic bodies) were observed after 24 h at a concentration of higher than 10 μM (Fig. 3).

Caspases, especially caspase-3 (CPP-32) that plays an essential role in the induction of apoptosis, is synthesized initially as an inactive precursor of 32 kDa. Proteolytic processing is required in order to generate the two subunits of 17 and 12 kDa that form the active protease. The cleaved caspase-3 antibody detects only the large fragment of activated caspase-3 (17 kDa) that results from cleavage after Asp175. To determine whether or not apoptosis that is induced by As_2O_3 was regulated by caspase-3, we examined the expression of cleaved caspase-3 during apoptosis by a Western blot analysis. After treatment, activation of cleaved caspase-3 (17 kDa) was observed 6 h after the start of treatment with 20 μM As_2O_3 (Fig. 4). These finding indicate that caspase-3 was involved in As_2O_3-induced apoptosis in K562 cells. However, further studies will be needed to investigate whether or not the activation of caspase-3 might be directly associated with the induction of apoptosis with a specific inhibitor of caspase-3.

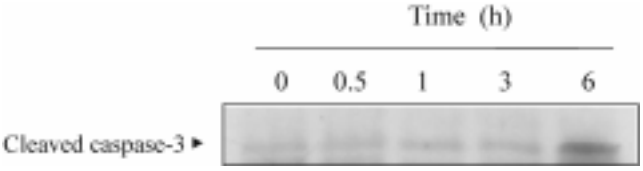

Fig. 4. Western blot analysis of cleaved caspase-3 in K562 cells. The cells were harvested at the indicated times after incubation with 20 μM of As_2O_3. The whole cell lysates were prepared and subjected to a Western blot analysis with an antibody specific to cleaved caspase-3.

Effect on cell cycle phase distribution It was recently reported that the apoptotic signal pathway is related to 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 of cells that were treated with different concentrations of As_2O_3 was analyzed by flow cytometry. As shown in Table 1, 46.0% of the control cells were in the G0/G1 phase, 50.9% were in the S phase, and 3.2% were in the G2/M phase. The cells that were treated with 10 μM As_2O_3 for 48 h showed an accumulation of cells in the G2/M phase of the cell cycle, and 15.5% of the cells were in the G2/M phase. Perkins *et al*. (2000) recently reported that As_2O_3 increased the percentage of K562 cells that accumulated in the G2/M phase of the cell cycle. These results were consistent with our data. We found that As_2O_3 treatment

Table 1. Time-dependent alteration of 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.

Time	% Cells		
	G0/G1	S	G2/M
0	46.0	50.9	3.2
12	46.2	50.6	3.3
24	48.8	43.4	7.8
48	28.2	56.3	15.5

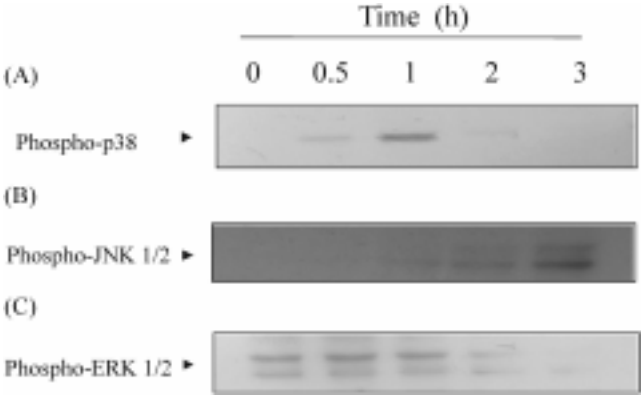

Fig. 5. Effect of As_2O_3 on mitogen-activated protein kinases in K562 cells. (A) Activation of p38 by the treatment of As_2O_3. The cells were harvested at the indicated times after incubation with 10 μM of As_2O_3. The whole cell lysates were prepared and subjected to a Western blot analysis with anti-phospho-p38. (B) Activation of JNK 1/2 by the treatment of As_2O_3. The cells were harvested as noted previously. Activated forms of JNK 1/2 were detected with anti-phospho-JNK 1/2. (C) Inhibition of ERK 1/2 by the treatment of As_2O_3. The cells were harvested as noted previously. Activated forms of ERK 1/2 were detected with anti-phospho-ERK 1/2.

increased the percentage of cells that accumulated in the G2/M phase of the cell cycle.

Effects on the MAP kinase activity Many forms of cellular stress, including treatment with anticancer drugs, have been shown to modulate MAP kinase signaling pathways and induce apoptosis. The classical MAP kinases (ERK 1/2) are activated by a variety of cell growth and differentiation stimuli, and play a central role in mitogenic signaling (Marshall *et al*., 1995; Kim *et al*., 2001). During the course of apoptosis that is induced by stress (such as UV irradiation, ceramide, anticancer drugs, and crosslinking of membrane IgM), the activation of SAPK and p38 kinase are associated with or required for apoptosis (Graves *et al*., 1996; Butterfield *et al*., 1997; Juo *et al*., 1997). However, the role of these signaling pathways in cell death has not yet been fully established, and contradictory evidence exists. Also, there is

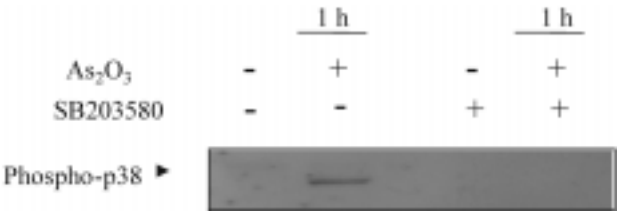

Fig. 6. The Inhibition of phospho-p38 activation by SB203580, a specific inhibitor of p38. The cells were treated with or without 10 μM SB203580 for 1 h, and harvested at the indicated times after incubation with 10 μM of As_2O_3. The whole cell lysates were prepared and subjected to a Western blot analysis with anti-phospho-p38.

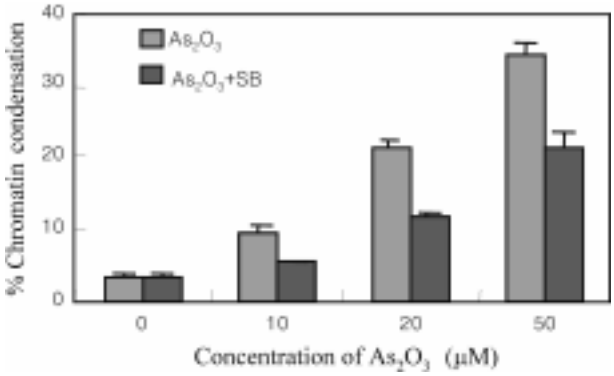

Fig. 7. Effect of SB203580 on As_2O_3-induced apoptosis. The cells were treated with the indicated concentrations for 24 h and stained with Hoechst 33258. The cells with condensed chromatin and fragmented nuclei were counted as apoptotic under a fluorescence microscope (× 100).

only minimal data about the MAP kinase signaling mechanism of As_2O_3 action on the CML cell line. Therefore, the activities of ERK, JNK, and p38 were examined by a Western blot analysis with phospho-specific antibodies that recognize the active form of each kinase. Marked activation of p38 and JNK was observed by treatment with 10 μM As_2O_3 in K562 cells (Fig. 5A). These characteristics of the activation of JNK and p38 are quite similar to those that were reported when mouse fibroblast NIH3T3 cells were treated with sodium arsenite (Lim *et al*., 1998). In contrast to the marked activation of JNK and p38, we observed a decrease in the levels of the activated forms of ERK 1/2 upon treatment of the cells with As_2O_3 (Fig. 5C). It was reported recently that the activation of the JNK and p38 kinase, as well as the concurrent inhibition of ERK, are critical for NGF withdrawal-induced apoptosis in rat PC-12 cells (Xia *et al*., 1995).

Inhibition of apoptotic cell death by SB203580 Although the As_2O_3-induced apoptosis in K562 cells was accompanied by the activation of JNK and p38 kinase, it was unclear which pathway was associated with As_2O_3-induced apoptosis. To

examine whether or not the activation of p38 by As_2O_3 might be associated with the induction of apoptosis in K562 cells, we treated the cells with SB203580, a specific inhibitor of p38. In the presence of SB203580, we confirmed that the activation of p38 kinase was blocked during As_2O_3 treatment (Fig. 6). Severe nuclear condensation and fragmentation were inhibited in approximately 38-45% of the non-treated cells (Fig. 7). Inhibiting p38 significantly reduced nuclear apoptosis. These results suggest that the activation of p38 is associated with As_2O_3-induced apoptosis.

In summary, we demonstrated that the As_2O_3 induced cytotoxicity and cell cycle arrest at the G2/M phase were correlated with apoptosis. The induction of apoptosis by As_2O_3 was mediated by the activation of caspase-3 and p38. These findings suggest that the potential use of As_2O_3 in the treatment of chronic myelogenous leukemia deserves further exploration.

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