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Original Communication

Dose-dependent biphasic effects of arsenic disulfide on differentiation and apoptosis of HL-60 cells

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ABSTRACT

We investigated the dose-dependent biphasic effects of arsenic disulfide (As_2S_2) on the differentiation and apoptosis of HL-60 cells. Cellular reactive oxygen species, glutathione, p38 mitogen-activated protein kinase (MAPK), as well as cell differentiation and apoptosis in HL-60 cells were assessed by flow cytometric analysis. The mean percentage of CD11b-positive cells induced by 8 μM As_2S_2 was more than 3 times higher than that induced by 16 μM As_2S_2. Eight μM As_2S_2 did not induce cell apoptosis, while 16 μM As_2S_2 induced apoptosis and decreased cell viability. Both 8 and 16 μM As_2S_2 increased cellular reactive oxygen species. Treatment with 16 μM As_2S_2 decreased the cellular glutathione levels at 1 h and 3 h after the exposure. The mitochondrial membrane potential depletions were observed in both 8 μM As_2S_2-induced differentiation and 16 μM As_2S_2-induced apoptosis. p38 MAPK inhibition enhanced As_2S_2-induced differentiation, but had little influence on As_2S_2-induced apoptosis. A moderate oxidative stress induced by 8 μM As_2S_2 can promote As_2S_2-induced differentiation, whereas more severe oxidative stress caused by glutathione depletion by 16 μM As_2S_2 reduced mitochondrial membrane potential, resulting in differentiation attenuation and apoptosis enhancement. p38 MAPK activation resulting from oxidative stress seems to be implicated in negative regulation of cell differentiation, rather than apoptosis, in As_2S_2-treated HL-60 cells.

KEYWORDS: arsenic disulfide, cell differentiation, HL-60 cells, mitochondrial membrane potential, p38 MAPK, reactive oxygen species

ABBREVIATIONS

As_2S_2, arsenic disulfide; MAPK, p38 mitogen-activated protein kinase; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5diphenyltetrazolium bromide; PML/RAR-α, promyelocytic leukemia/retinoic acid receptor protein

INTRODUCTION

Notable success has been reported in the treatment of acute promyelocytic leukemia with arsenic trioxide (As_2O_3) [1, 2]. The clinical use of arsenic disulfide (As_2S_2)-containing formulae to treat myeloid leukemia in China started as early as the beginning of the medical use of As_2O_3 [3-5]. Qinghuang Powder containing *QingDai* (Natural Indigo) and *Xiong huang* (Red Orpiment) in the formula was used to treat myeloid leukemia early from 1960s in our institute in China [4]. It was found that the therapeutic efficacy of Qinghuang Powder could be enhanced by increasing the content of *Xiong huang*, which contains > 90%

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As_2S_2, without strong adverse effects during administration even for more than 10 years [5, 6]. These experiences suggested that Qinghuang Powder is effective in the treatment of myeloid leukemia, and its therapeutic efficacy could be attributed to As_2S_2, the most important component of *Xiong huang*.

It has been suggested that As_2O_3 is able to induce both apoptosis and differentiation in leukemia cells depending on the dosage [7]. Studies carried out on a large number of leukemic cell lines have shown that this compound mainly exerts an apoptotic effect [8-11]. The action of As_2O_3-induced myeloid differentiation in acute promyelocytic leukemia model has been suggested to induce promyelocytic leukemia/retinoic acid receptor protein (PML/RAR-α) degradation [12, 13]. Recently, many results indicated that reactive oxygen species, intracellular glutathione, and p38 mitogen-activated protein kinase (MAPK) play roles in As_2O_3-induced apoptosis [14-20].

While knowledge on the action of As_2O_3 is thus increasing, similar aspects of As_2S_2 have not been sufficiently documented, although associations of reactive oxygen species and p38 MAPK pathways with As_4S_4-induced differentiation were reported in HL-60 cells [2, 21]. We suggested in our previous study that oxidative stress and oxidative stress-related activation of p38 MAPK have important roles in As_2S_2-induced differentiation and apoptosis of HL-60 cells [22]. Our previous data also suggested that moderate levels of oxidative stress induced by As_2S_2 positively contribute to HL-60 cell differentiation, and there might be dose-dependent biphasic effects of As_2S_2 on the cell differentiation and apoptosis [22]. Thus, in the present study, we further examined the role of p38 MAPK activation, reactive oxygen species generation, and intracellular glutathione levels in myeloid differentiation and apoptosis of HL-60 cells, induced by different concentrations of As_2S_2.

MATERIALS AND METHODS

Reagents

RPMI-1640 medium and fetal bovine serum were purchased from Gibco BRL, Grand Island, NY, USA. Cell proliferation kits I and II were obtained from Roche Diagnostics, Indianapolis, Ind, USA. As_2S_2, Rhodamine 123, 2',7'-dichlorofluorescein diacetate, and ortho-phthaldialdehyde were purchased from Sigma Aldrich, St. Louis, Mo, USA. Phycoerythrin-conjugated anti-human CD11b (Clone D12) and mouse IgG_1 isotype control were obtained from DakoCytomation, Denmark. p38 MAPK inhibitor SB203580 was purchased from Calbiochem, San Diego, CA, USA. Phycoerythrin-conjugated mouse anti-p38 MAPK (pT180/ pY182) and the Annexin V-fluorescein isothiocyanate apoptosis detection kit I were obtained from BD PharMingen, Tokyo, Japan. 10%-Formaldehyde neutral buffer was a product of Nacalai Tesque, Tokyo, Japan. As_2S_2 was dissolved in 1N NaOH, and HCl was used to adjust the pH to 7.35-7.45. The solution was passed through a 0.20 μm membrane, and the stock solutions were made using phosphate-buffered saline at a concentration of 5 mM, which was diluted with phosphate-buffered saline to working concentrations before use. 2',7'-dichlorodihydrofluorescein diacetate and Rhodamine 123 were dissolved in dimethyl-sulfoxide at 5 mM and 1 mg/ml, respectively, and stored at -20 °C. The ortho-phthaldialdehyde solution was freshly prepared in methanol at 10 mg/ml before use. SB203580 was dissolved in dimethylsulfoxide at a concentration of 20 mM, and stored at -20 °C until use.

Cell culture

HL-60 cells were obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA. The cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in continuous logarithmic growth between 2 x 10^5 and 1 x 10^6 cells/ml at 37 °C in a humidified 5% CO_2 incubator.

Cell proliferation assay

The leukemia cells were washed and resuspended in the above medium to 5 × 10^5 cells/ml. Then, 196 μl of the cell suspension was placed in each well of a 96-well flat-bottomed plate. Four μl of phosphate-buffered saline containing As_2S_2 was added to yield final concentrations of 0.5, 2, 8, and 16 μM. Four μl of phosphate-buffered saline was added to the control wells. The cells were incubated for 48 h in an atmosphere containing

5% CO_2 at 37 °C in a humidified chamber. After the incubation period, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) labeling reagent (final concentration 0.5 mg/ml) was added to each well and the plate was placed on a microshaker for 10 s, after which the cells were further incubated for 4 h in a humidified atmosphere. Subsequently, 100 µl of solubilizing solution was added to each well and mixed, after which the plate was left overnight in an incubator in humidified atmosphere. The spectrophotometric absorbance of the samples was measured on a microplate reader (LS-PLATE manager 2001, Japan) at 570 nm.

Assessment of myeloid differentiation

After HL-60 cells (1×10^6/ml) were treated with the indicated concentrations of As_2S_2, the cells were washed twice and resuspended in 100 µl phosphate-buffered saline containing 2% fetal bovine serum. Then 10 µl phycoerythrin-conjugated CD11b antibody solution was added, and the cells were incubated in the dark at 4 °C for 30 min. After the incubation, the cells were resuspended in 400 µl phosphate-buffered saline containing 1% fetal bovine serum, and subsequently the cells were analyzed by a FACSCalibur™ flow cytometry (BD Biosciences, Mountain View, CA, USA). Isotypic mouse IgG_1 was used to set threshold parameters. A total of 30,000 non-gated cells were analyzed. For the assessment of differentiation induced by H_2O_2 combined with As_2S_2, cells were pre-incubated with H_2O_2 for 1 h before treatment with As_2S_2 for 48 h. For the assessment of effects of SB203580 on As_2S_2-induced differentiation, cells were incubated with As_2S_2 combined with SB203580 for 72 h, using 0.05% dimethylsulfoxide as vehicle in the control wells.

Apoptosis and viability assays

After HL-60 cells (1×10^6/ml) were treated with As_2S_2, the cells were washed twice in cold phosphate-buffered saline, and resuspended in binding buffer at a density of 1×10^6 cells/ml. Then, 1×10^5 cells were stained with 5 µl Annexin V-fluorescein isothiocyanate and 5 µl propidium iodide solution for 15 min at 4 °C in the dark. Then, 400 µl of binding buffer was added, and the cells were analyzed by flow cytometer within 1 h of staining. A total of 30,000 non-gated cells were analyzed. Cell viability was determined by propidium iodide exclusion assay. Apoptotic cells were identified by Annexin V-staining. For the assessment of apoptosis-inducing effect of H_2O_2 combined with As_2S_2, cells were pre-incubated with H_2O_2 for 1 h before treatment with As_2S_2 for 72 h. For the assessment of the effects of SB203580 on As_2S_2-induced apoptosis, cells were incubated with As_2S_2 combined with SB203580 for 72 h, using 0.05% dimethylsulfoxide as vehicle in the control wells.

Measurement of cellular reactive oxygen species

Cellular reactive oxygen species were measured using 2',7'-dichlorodihydrofluorescein diacetate. Viable cells can deacetylate the agent to 2',7'-dichlorodihydrofluorescein, which is not fluorescent but can react with H_2O_2 to produce highly fluorescent dichlorofluorescein. The cytofluorometric measurement of dichlorofluorescein provides an index of cellular reactive oxygen species level. At the end of treatment with As_2S_2, the cells in each well were collected, washed twice in phosphate-buffered saline and incubated in 1 ml phosphate-buffered saline containing 10 µM 2',7'-dichloro-dihydrofluorescein diacetate at 37 °C for 30 min. The cells were then washed and resuspended in 0.5 ml phosphate-buffered saline. The mean fluorescence intensity of dichlorofluorescein green fluorescence from 10,000 cells was detected by flow cytometry. The amount of reactive oxygen species in the treated cells as compared to that in control cells was represented by relative mean fluorescence intensity, which is defined as: sample mean fluorescence intensity/control mean fluorescence intensity (%).

Determination of cellular glutathione concentration

The temporal change in cellular glutathione amounts was evaluated using ortho-phthaldialdehyde, which can react with glutathione to yield a highly fluorescent product. Cultured cells were washed twice by phosphate-buffered saline and then suspended in 900 µl phosphate-buffered saline. Then, 100 µl of 10 mg/ml ortho-phthaldialdehyde dissolved in methanol was added, and the cells were incubated at 37 °C for 30 min. After being washed by phosphate-buffered saline, the cells were resuspended in 500 µl phosphate-buffered saline and kept on ice. Mean fluorescence

intensity thus produced from 10,000 cells was measured by flow cytometry. The cellular glutathione amounts were represented by relative mean fluorescence intensity.

Analysis of activated p38 MAPK

Activated p38 MAPK was detected by flow cytometry using phycoerythrin-labeled antibody specific to phosphorylated (activated) p38 MAPK. Cells (1×10^6/ml) treated with As_2S_2 were collected by centrifugation and washed with phosphate-buffered saline, and fixed for 10 min at 37 °C in phosphate-buffered saline containing 2% formaldehyde neutral buffer. The cells were centrifuged and resuspended in 90% methanol, and then incubated for 30 min on ice. The cells were washed twice and resuspended with 100 µl phosphate-buffered saline. Subsequently, phycoerythrin-labeled antibody against the phosphorylated p38 MAPK was added, and the cells were incubated for 1 h at room temperature. The cells were resuspended in 400 µl phosphate-buffered saline containing 1% formaldehyde neutral buffer, and analyzed by flow cytometry.

Determination of mitochondrial membrane potential

The mitochondrial membrane potential was assessed using the cationic lipophilic dye Rhodamine 123. Cells (1×10^6/ml) treated with As_2S_2 were collected and washed twice in phosphate-buffered saline, and incubated in 1 ml phosphate-buffered saline containing 10 µg/ml Rhodamine 123 at 37 °C for 30 min. The cells were washed and resuspended in 500 µl phosphate-buffered saline. Subsequently, 30,000 cells were analyzed with flow cytometry, and mitochondrial membrane potential is represented by relative mean fluorescence intensity.

Statistics

Data were shown as the mean ± standard deviation of three independent experiments. Differences were estimated by one-way ANOVA. A value of $p < 0.05$ was considered to be significant.

RESULTS

Effects of As_2S_2 on cell proliferation, differentiation and apoptosis

HL-60 cells were continuously treated with As_2S_2 at concentrations of 8 and 16 µM for 72 h, and cell proliferation was measured in an MTT assay. Eight µM As_2S_2 did not affect, while 16 µM As_2S_2 significantly inhibited, proliferation of HL-60 cells ($p < 0.05$) (Fig. 1).

Differentiated cells were detected by cell-surface expression of CD11b. After treatment of cells with As_2S_2 at concentrations of 8 and 16 µM for 48 h, the percentage of CD11b-positive cells significantly increased to 17 and 5.5 times of control, respectively (Fig. 2) ($p < 0.05$), and the mean percentage of CD11b-positive cells induced by 8 µM was more than 3 times higher than that induced by 16 µM As_2S_2 (Fig. 2) ($p < 0.05$). The degrees of CD11b expression peaked at 48 h in cells treated with both 8 and 16 µM As_2S_2 for 24-72 h (Fig. 2).

After treatment for 24 to 72 h, 16 µM As_2S_2 significantly induced apoptosis and decreased cell viability in a time-dependent manner ($p < 0.05$) (Fig. 3A, B) whereas As_2S_2 at 8 µM conversely decreased apoptosis and rather increased cell viability compared to the control (Fig. 3A, B).

Cellular reactive oxygen species generated by As_2S_2 treatment

After incubation of cells for 0.5 to 24 h in the presence of 8 or 16 µM As_2S_2, a notable amount of cellular reactive oxygen species was observed within a few hours. The amounts of cellular reactive oxygen species rapidly increased to more than 120% of control at 0.5 h and sustained to 3 h (Fig. 4A). Of note, the levels of cellular reactive oxygen species generated by treatment with 16 µM As_2S_2 were significantly higher than those generated by treatment with 8 µM As_2S_2 at 3 h (Fig. 4B).

Contribution of oxidative stress to As_2S_2-induced cell differentiation and apoptosis

The effects of H_2O_2 on As_2S_2-induced differentiation were assessed by measuring percentages of CD11b-positive cells after treatment with As_2S_2 and H_2O_2 alone or in combination of these agents for 48 h. H_2O_2 alone at 20 µM significantly promoted myeloid cell differentiation, as compared to control (Fig. 5A) ($p < 0.05$). Treatment of cells with 8 and 16 µM As_2S_2 increased the percentages of CD11b-positive cells (Fig. 5A), as already described in Fig. 2, whereas preincubation of the cells with 20 µM H_2O_2 for 1 h attenuated the differentiation-inducing activity of As_2S_2 (Fig. 5A) ($p < 0.05$).

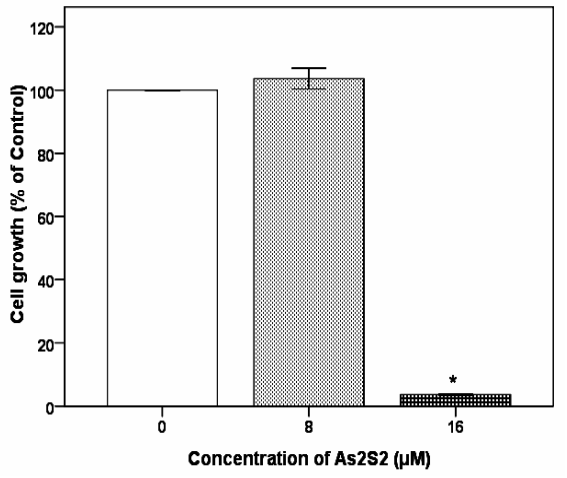


Fig. 1. Effects of As_2S_2 on *in vitro* growth of HL-60 cells. Cells were treated with 8 or 16 μM As_2S_2 for 72 h, and cell proliferation was determined by an MTT assay. Data are the mean ± standard deviation of 3 independent experiments. *$p < 0.05$ as compared with control (0 μM).

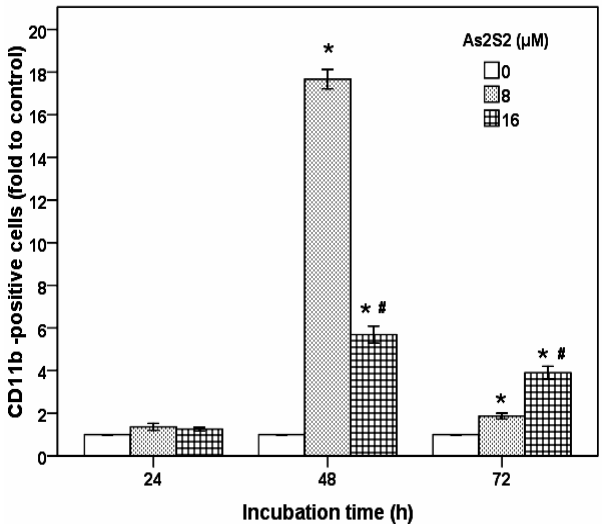
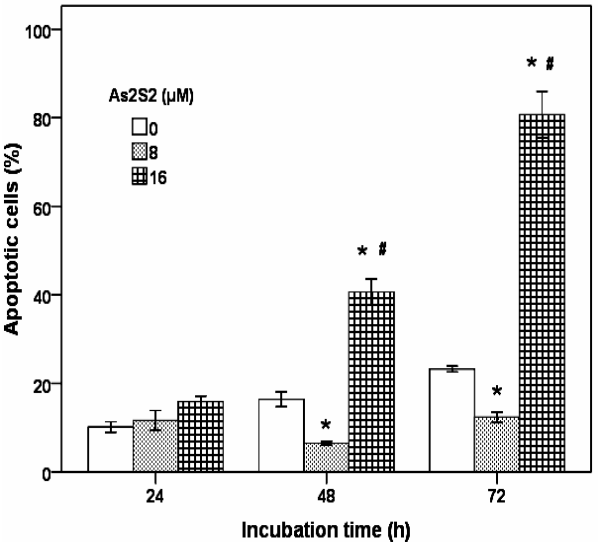


Fig. 2. As_2S_2-induced differentiation in HL-60 cells. Numbers of CD11b-positive cells (folds to control) are shown after treatment with As_2S_2 at concentrations of 8 and 16 μM for 24 to 72 h. Data are the mean ± standard deviation of 3 independent experiments. *$p < 0.05$ as compared with control group (0 μM As_2S_2); $^{\#}p < 0.05$ as compared to cells treated with 8 μM As_2S_2.



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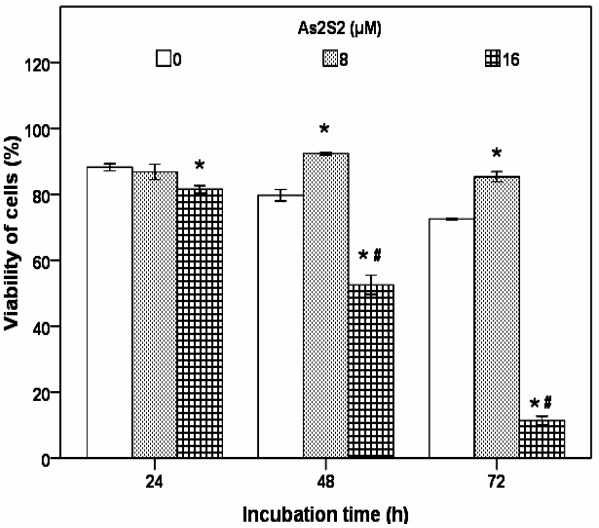


B

Fig. 3. Mean percentages of apoptotic cells (A) and viable cells (B) in HL-60 cells treated with 8 or 16 μM As_2S_2 for 24-72 h. Data are the mean ± standard deviation of 3 independent experiments. *$p < 0.05$ as compared with control group (0 μM As_2S_2). $^{\#}p < 0.05$ as compared to cells treated with 8 μM As_2S_2.

The effects of H_2O_2 on As_2S_2-induced apoptosis were assessed by Annexin V-positive cells after treatment with As_2S_2 and H_2O_2 alone or in combination for 72 h. H_2O_2 alone at 20 μM significantly increased the percentages of apoptotic cells, as compared to control (Fig. 5B) ($p<0.05$). When cells were pre-incubated with 20 μM H_2O_2 for 1 h, followed by combined treatment with 8 or 16 μM As_2S_2, the percentages

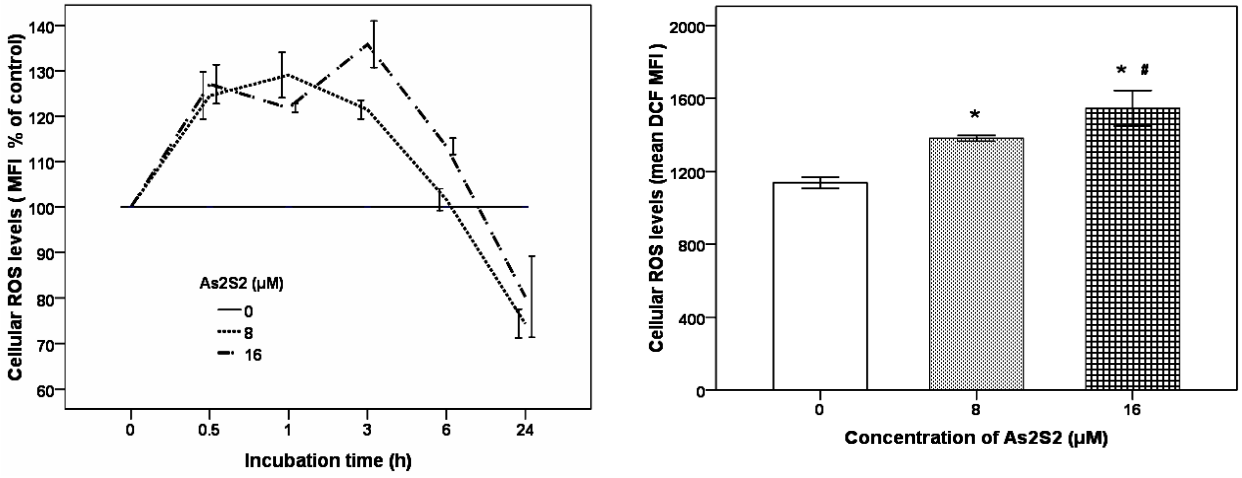


Fig. 4. Generation of cellular reactive oxygen species in As_2S_2-treated HL-60 cells. (A) Changes in amounts of reactive oxygen species in cells after treatment with 8 or 16 μM As_2S_2 for 0.5 to 24 h. The amounts of reactive oxygen species were presented as the relative mean fluorescence intensity, which were defined as: (sample mean fluorescence intensity/control mean fluorescence intensity) × 100%. (B) The amounts of reactive oxygen species in cells after treatment with 8 or 16 μM As_2S_2 for 3 h. The data are the mean fluorescence intensity related to dichlorofluorescein produced in cells by reactive oxygen species. Data are the mean ± standard deviation of 3 independent experiments. $^*p < 0.05$ as compared with control (0 μM As_2S_2); $^{\#}p < 0.05$ as compared to cells treated with 8 μM.

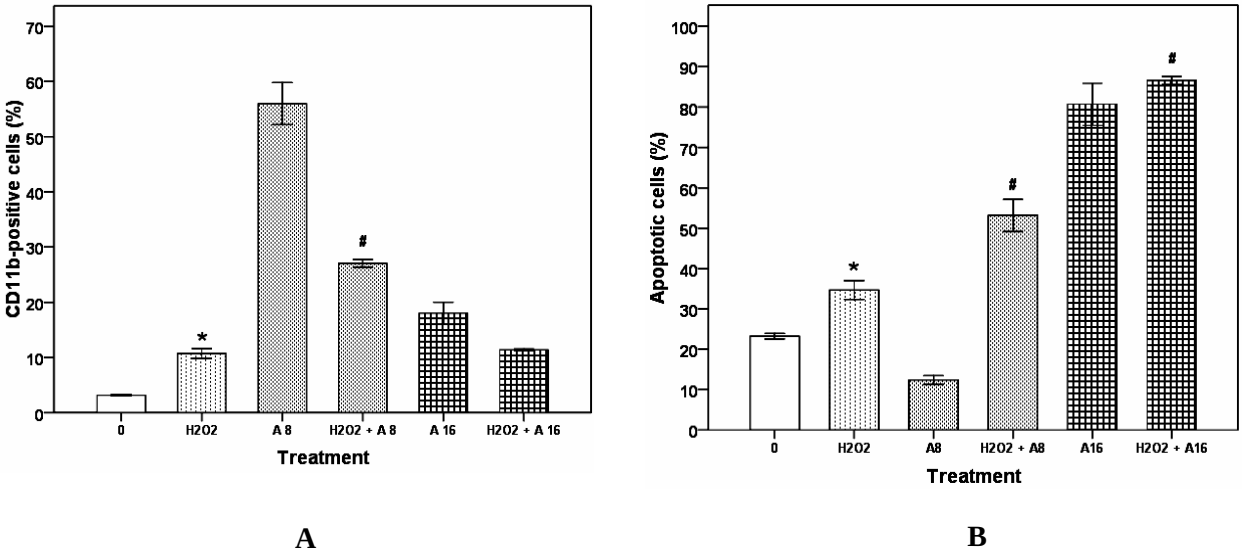


Fig. 5. Effects of hydrogen peroxide (H_2O_2) on As_2S_2-induced differentiation (A) and apoptosis (B) in HL-60 cells. Cells were treated with 20 μM H_2O_2, 8 μM As_2S_2 (A8), 16 μM As_2S_2 (A16), or pre-incubated with 20 μM H_2O_2 for 1 h followed by treatment with H_2O_2 plus A8, as well as H_2O_2 plus A16. After the treatment for 48 h (differentiation) or 72 h (apoptosis), the cells were stained with anti-CD11b antibody or Annexin-V, and then analyzed with flow cytometry. Data are the mean ± standard deviation of 3 independent experiments. Means were compared by 1-way ANOVA. $^*p < 0.05$ as compared with control (without H_2O_2 and As_2S_2); $^{\#}p < 0.05$ as compared to cells treated with As_2S_2 alone.

of Annexin V-positive cells significantly increased (Fig. 5B) ($p < 0.05$).

Cellular glutathione level

Cellular glutathione amounts in cells treated with 8 or 16 μM As_2S_2 for 0.5-24 h transiently increased to 110% of control at 0.5 h, then rapidly declined and dropped to the minimum at 3 h (Fig. 6A). At this incubation time, the cellular glutathione amounts in cells treated with 16 μM As_2S_2 decreased to 66% of control ($p < 0.05$), which were less than those in cells treated with 8 μM As_2S_2 (88% of control, $p < 0.05$) (Fig. 6B) ($p < 0.05$). These changes in cellular glutathione amounts after treatment with As_2S_2 appeared to be negatively correlated with the changes in the amounts of reactive oxygen species, as shown in Fig. 4.

Activation of p38 MAPK

After incubation of cells with As_2S_2 at concentrations of 8 and 16 μM for 1 to 72 h, the cellular amounts of phosphorylated (activated) p38 MAPK gradually increased from 6 to 72 h, and rose to 128% and 145%, respectively, at 72 h with respect to the control (Fig. 7A). Of note, the amounts of the activated p38 MAPK weakly decreased to 82% of control in cells treated with 8 μM As_2S_2 for 1 h, whereas they significantly increased to 125% of control in cells treated with 16 μM (Fig. 7B) ($p < 0.05$).

Loss of mitochondrial membrane potential

The relative values of mitochondrial membrane potential significantly declined to 65% and 53% of the control in cells treated with 8 and 16 μM As_2S_2, respectively, for 48 h (Fig. 8A) ($p < 0.05$). Furthermore, the loss of mitochondrial membrane potential in cells treated with 16 μM As_2S_2 was more apparent than that in cells treated with 8 μM As_2S_2 at 48 h (Fig. 8B) ($p < 0.05$). Similar to our previous report [22], our experimental results also demonstrated that the mitochondrial membrane potential decreased to the minimum in cells treated with 8 μM As_2S_2 for 48 h (Fig. 8A), and conversely, the level of CD11b-positive cells increased to the maximum (Fig. 2). In contrast to these observations using 8 μM As_2S_2, the mitochondrial membrane potential decreased to the lowest level (25% of the control) in cells treated with 16 μM for 72 h (Fig. 8A), accompanied with the highest percentage level of apoptotic cells (Fig. 8C), which was also shown in Fig. 3A.

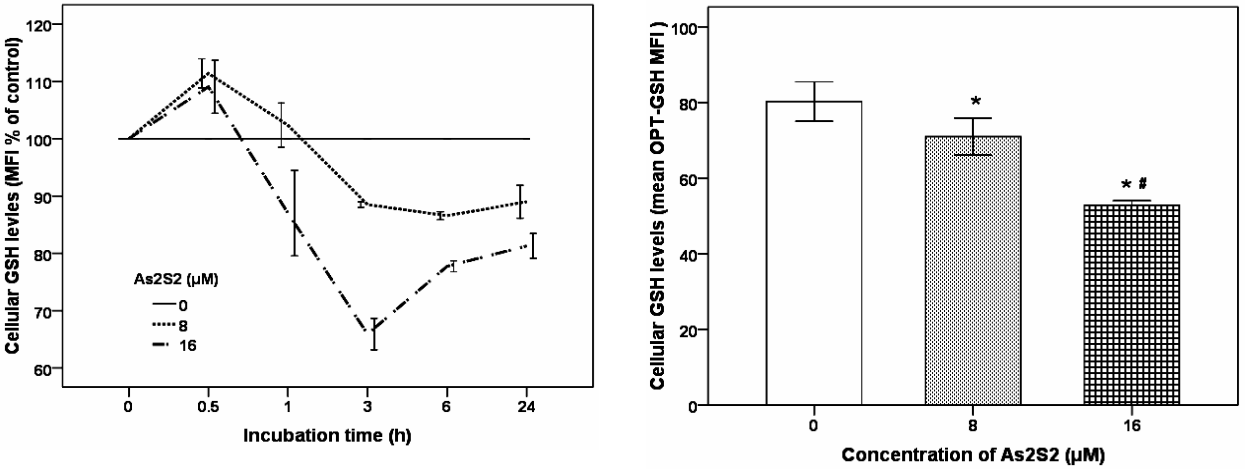
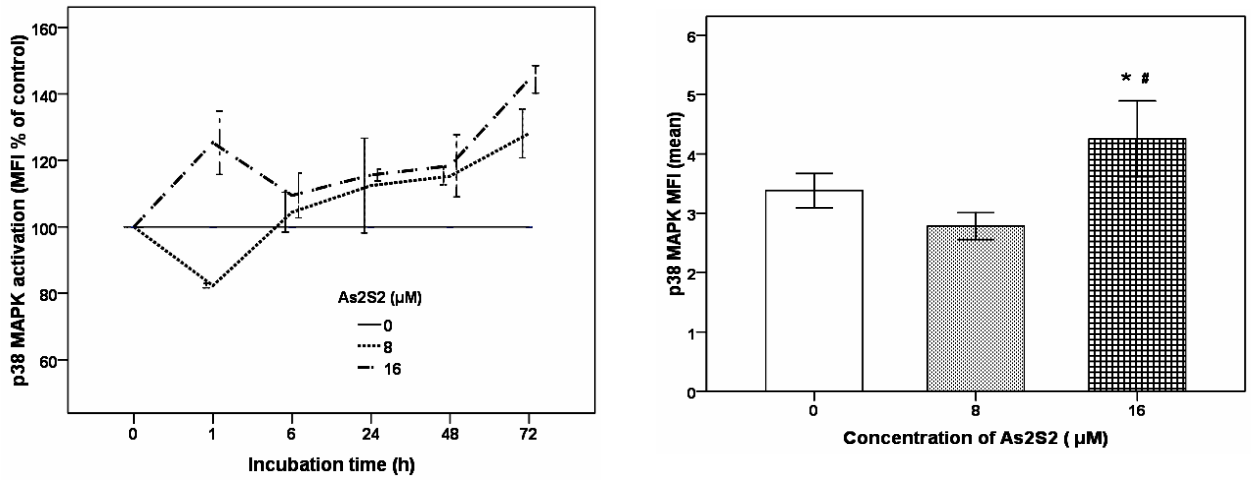


Fig. 6. Changes in cellular glutathione amounts in HL-60 cells after treatment with 8 and 16 μM As_2S_2. (A) Changes in glutathione amounts in cells treated with 8 and 16 μM As_2S_2 for 0.5 to 24 h, as compared to the control group. (B) The levels of glutathione in cells treated with As_2S_2 for 3 h. Data are expressed as the mean fluorescence intensity of orthophthaldialdehyde-glutathione. Bars indicate the mean ± standard deviation of 3 independent experiments. *$p < 0.05$ as compared with control (0 μM As_2S_2). $^{\#}p < 0.05$ as compared to cells treated with 8 μM As_2S_2.



A **B**

Fig. 7. Changes in activated p38 MAPK in HL-60 cells treated with 8 and 16 μM As_2S_2. The activated p38 MAPK was detected by phycoerythrin-labeled anti-phosphorylated p38 MAPK antibody. (A) Changes in the amounts of activated p38 MAPK in cells treated with 8 and 16 μM As_2S_2 for 1 to 72 h, which were defined as: (sample mean fluorescence intensity/control mean fluorescence intensity) × 100%. (B) Changes in the amounts of activated p38 MAPK in cells treated with 8 and 16 μM As_2S_2 for 1 h. Data are the mean ± standard deviation of 3 independent experiments. $^*p < 0.05$ as compared with control (0 μM As_2S_2). $^{\#}p < 0.05$ as compared to cells treated with 8 μM As_2S_2.

Effects of p38 MAPK inhibition on As_2S_2-induced apoptosis

As shown in Fig. 2, when the cells were treated with 8 μM As_2S_2 for 24-72 h, the ratio of CD11b-positive cells increased to the maximum at 48 h, while the ratio significantly decreased at 72 h. At this time, the amounts of activated p38 MAPK rose to the maximum (Fig. 7A). Treatment with a combination of 8 μM As_2S_2 and 10 μM SB203580, a specific p38 MAPK inhibitor, for 72 h was found to increase the CD11b-positive cell ratio compared to treatment with each alone, as shown in Fig. 9A, the observation of which was consistent with our previous data [22]. When the cells were treated with 16 μM As_2S_2 for 24-72 h, the percentages of apoptotic cells gradually increased as shown in Fig. 3A and Fig. 8C, and the increase in the amount of activated p38 MAPK was simultaneously observed. Then, the effects of p38 MAPK activation on apoptosis induced by 16 μM As_2S_2 were assessed. Almost no difference was observed between the combination treatment of 16 μM As_2S_2/10 μM SB203580 and the treatment with 16 μM As_2S_2 alone (Fig. 9B).

DISCUSSION

In the present study we showed, a dose-dependent biphasic effects of arsenic disulfide on the differentiation and apoptosis of HL-60 cells. As_2O_3 is known to affect multiple cellular functions via different molecular targets, such as PML-RARalpha fusion protein, mitochondria and MAPK. [7]. In details, As_2O_3 tends to promote differentiation at low concentrations while it induces apoptosis at higher concentrations in leukemic cells *in vitro* and *in vivo*. Studies carried out on a large number of leukemic cell lines have shown that this compound mainly exerts an apoptotic effect [8-11]. The exploration of the mechanism underlying As_2O_3-induced myeloid differentiation in acute promyelocytic leukemia was mainly focused on PML/RAR-α protein degradation [12, 13]. Differentiated cells induced by treatment with As_4S_4 have been observed in HL-60 cells [2, 18]. In these studies, 2 to 8 μM of As_4S_4 induced cell differentiation as assessed by CD11b expression, without incurring apoptosis. In our present study, 8 and 16 μM As_2S_2 significantly induced HL-60 cell differentiation by 17 and 5.5 times that of the control, respectively

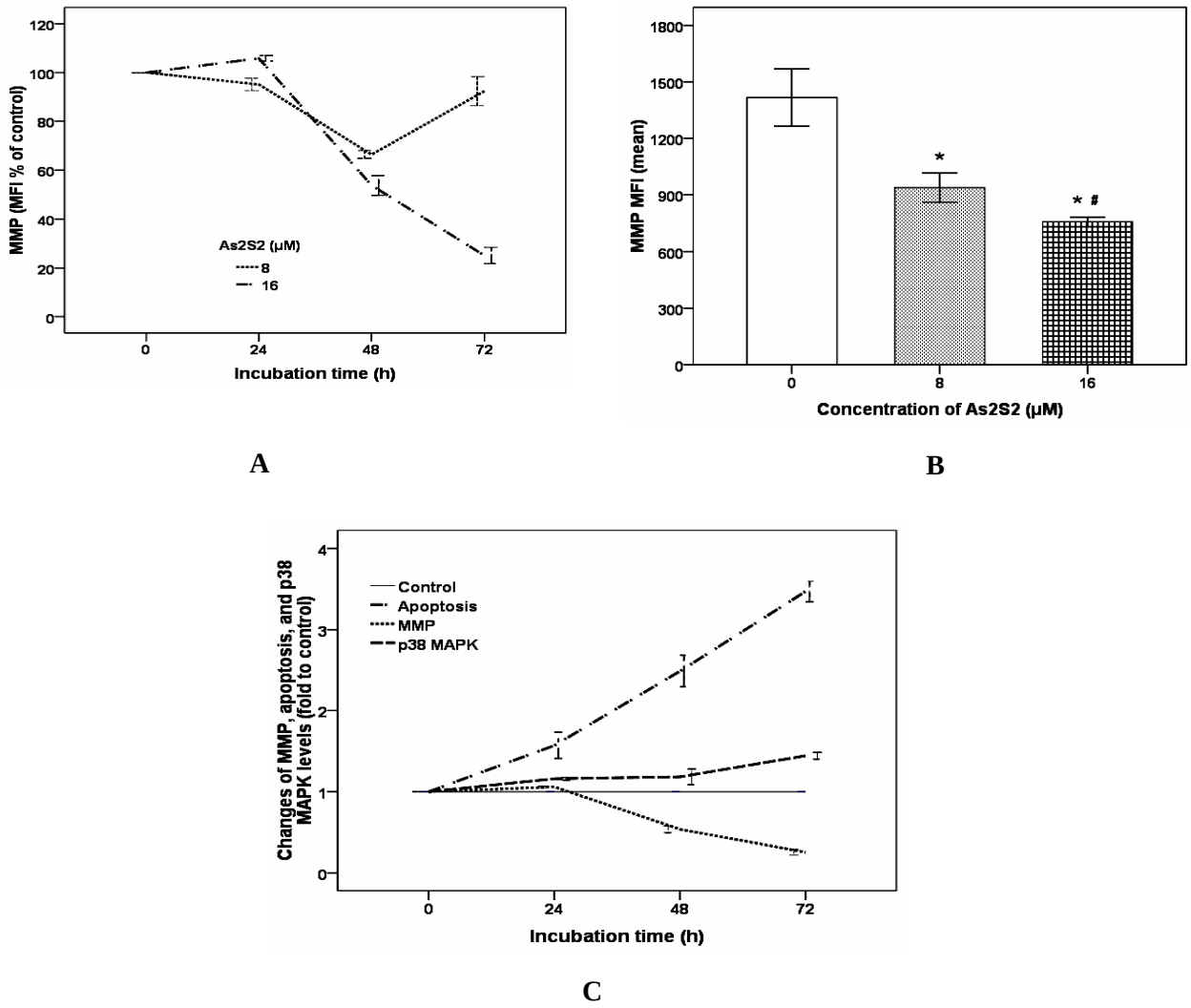


Fig. 8. Loss of mitochondrial membrane potential related to As_2S_2-induced differentiation and apoptosis in HL-60 cells. (A) Changes in mitochondrial membrane potential in cells treated with As_2S_2 at concentrations of 8 and 16 μM for 24 to 72 h, which were defined as: (sample mean fluorescence intensity/control mean fluorescence intensity) × 100%. (B) Loss of mitochondrial membrane potential in cells treated with 8 and 16 μM As_2S_2 for 48 h. Data are the mean ± standard deviation of 3 independent experiments. $^*p < 0.05$ as compared with control (0 μM As_2S_2). $^{\#}p < 0.05$ as compared to cells treated with 8 μM As_2S_2. (C) Changes in mitochondrial membrane potential, as compared with those of cell apoptosis and activated p38 MAPK amounts, after the cell treatment with 16 μM As_2S_2 for 24 to 72 h. The data for the mitochondrial membrane potential, the expression level of CD11b expression, and the amounts of activated p38 MAPK are presented as the relative mean fluorescence intensity.

(Fig. 2), and the percentage of CD11b-positive cells in cells treated with 8 μM As_2S_2 was more than 3 times higher than that in cells treated with 16 μM As_2S_2 (Fig. 2). Interestingly, 8 μM As_2S_2 attenuated cell apoptosis and increased the viability of HL-60 cells, whereas 16 μM As_2S_2 significantly promoted cell apoptosis and decreased cell viability in a time-dependent manner (Fig. 3).

Reactive oxygen species play critical roles in cell proliferation, differentiation and apoptosis by eliciting a variety of cellular responses. Low concentrations of reactive oxygen species promote cell survival and proliferation; however, once the reactive oxygen species exceed the elimination capacity of antioxidants including glutathione, the cells suffer the so-called “oxidative stress”,

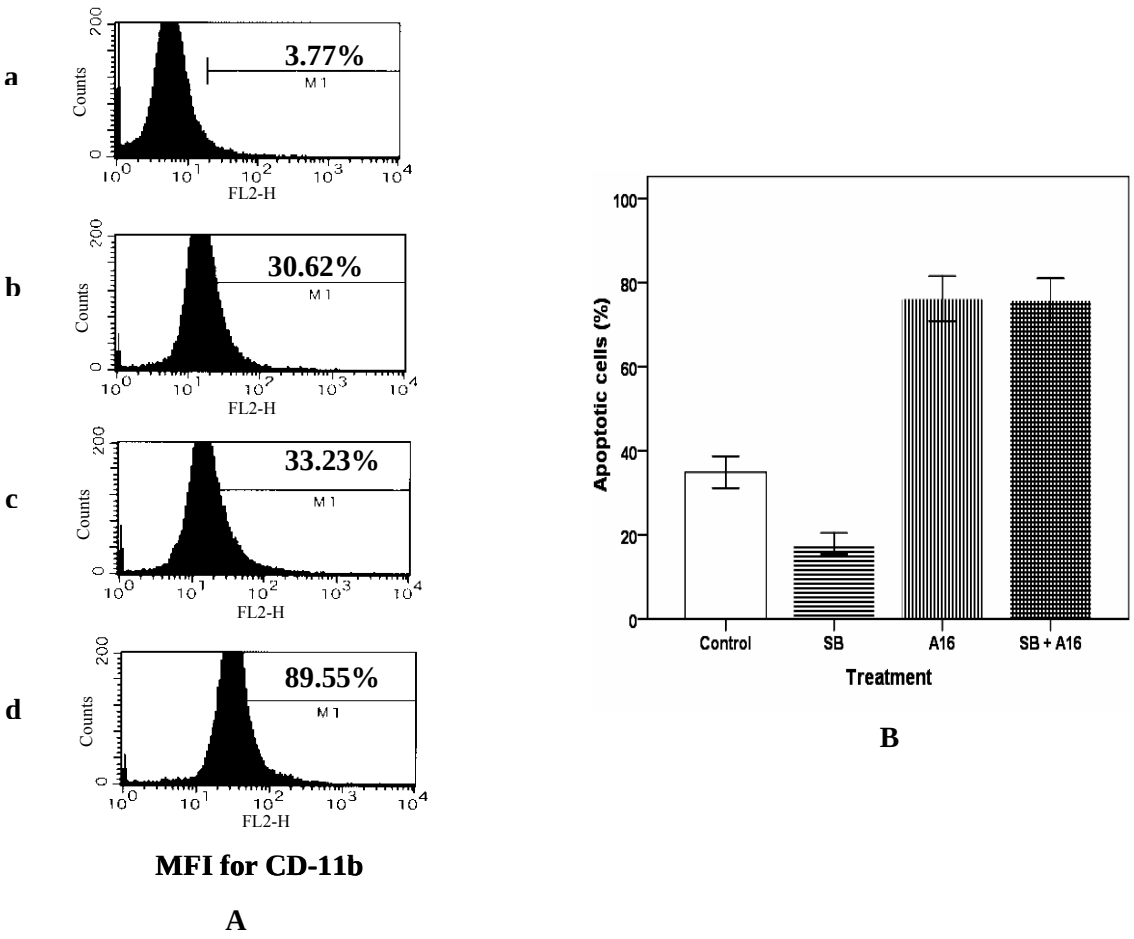


Fig. 9. The modifying effects of p38 MAPK inhibitor (SB203580) on (A) As_2S_2-induced differentiation and (B) apoptosis in HL-60 cells. (A) After treatment with 8 μM As_2S_2 and 10 μM SB203580 alone or in combination for 72 h, the cells were stained for CD11b and analyzed with flow cytometer. a; Control, b; 10 μM SB203580 (SB), c; 8 μM As_2S_2 (A8), d; SB + A8. (B) After treatment with 16 μM As_2S_2 (A16) and 10 μM SB203580 (SB) alone or in combination for 72 h, apoptotic cells were identified by Annexin V using flow cytometer. Data are the mean ± SD of 3 independent experiments. Means were compared by 1-way ANOVA.

leading to severe dysfunction and cell death [23]. The amounts of cellular reactive oxygen species generated by 8 μM As_2S_2 was significantly lower than that generated by 16 μM As_2S_2 (Fig. 4). Whereas, when a high level of oxidative stress was additionally provided by H_2O_2, As_2S_2-induced differentiation was attenuated and apoptosis was enhanced in HL-60 cells (Fig. 5). These results suggested that a moderate oxidative stress promotes cell proliferation and differentiation, while more severe oxidative stress induces apoptosis and decreases viability in HL-60 cells.

Glutathione is the main non-protein antioxidant in cells, and provides electrons for enzymes such as glutathione peroxidase, which reduce H_2O_2 to H_2O. Glutathione is also known to protect cells from toxic insult by detoxifying toxic metabolites of drugs and reactive oxygen species [24, 25]. A previous work revealed that intracellular glutathione levels were regulated in a complex fashion during the maturation of HL-60 cells [26], in which a small transient increase in glutathione levels was observed during cell differentiation induction triggered by 1α,25-dihydroxyvitamine D_3 or dimethyl sulfoxide. They further demonstrated that transient glutathione depletion as a result of using low doses of glutathione-depleting agents enhanced the differentiation

process [26]. In the present study, when HL-60 cells were treated with As_2S_2 at concentrations of 8 and 16 μM, cellular glutathione amount transiently increased in response to reactive oxygen species generation (Fig. 6A). Furthermore, as the cellular reactive oxygen species rose to the maximum level, cellular glutathione amount simultaneously depleted to the minimum (Fig. 6A). The degrees of reactive oxygen species generation associated with glutathione reduction in cells treated with low concentration (8 μM) of As_2S_2, which induced cell differentiation, were lower than those in cells treated with high concentration (16 μM) of As_2S_2, which induced apoptosis. Oxidative stimuli are known to increase expression of the rate limiting enzyme in glutathione synthesis and hence elevate glutathione levels [27]. The initial increase in the amount of reactive oxygen species may result in a transiently high level of glutathione. However, severe oxidative stress caused by the addition of H_2O_2 or the treatment with high concentration of As_2S_2 (16 μM) exceeded the elimination capacity of antioxidants such as glutathione, which ultimately attenuated differentiation and induced apoptosis in HL-60 cells (Fig. 3 and Fig. 5).

Many studies have reported mitochondrial membrane potential-dependent apoptosis induced by arsenic [14, 17, 18]. In the present work, loss of mitochondrial membrane potential was also observed in the apoptosis induced by high concentration (16 μM) of As_2S_2 in HL-60 cells. On the other hand, much less has been revealed about the role of mitochondrial membrane potential in cell differentiation. Recently, the decrease of mitochondrial membrane potential was reported in As_4S_4-induced differentiation of HL-60 cells [28], in which it was shown that the decreased mitochondrial membrane potential in 6 μM As_4S_4-induced cell differentiation was mild and did not lead to apoptosis. In the present study, loss of mitochondrial membrane potential was observed in cells treated with 8 μM As_2S_2, a concentration which induces cell differentiation. Whereas, severe and irreversible loss of mitochondrial membrane potential was observed in cells treated with 16 μM As_2S_2, a concentration which induces cell apoptosis. The mitochondria can be viewed as a central regulator of the decision between cell survival and death [29]. The Bcl-2 family proteins play important roles in the regulation of apoptosis by targeting the mitochondria to exert their proapoptotic or antiapoptotic effects. There were evidences that the antiapoptotic function of Bcl-2 is dependent on its phosphorylation status rather than its expression level [17, 30]. A detailed analysis of relationship between the loss of mitochondrial membrane potential and the expression levels of Bcl-2 family proteins is currently underway.

p38 MAPK acts as a sensor of moderate oxidative stress, and is activated in a reactive oxygen species-dependent manner [19]. It was considered that both the extent and duration of activation of p38 MAPK contribute to the determination of cell fate, such as survival, differentiation and apoptosis [31]. Interestingly, p38 MAPK has previously been shown to mediate both proapoptotic/growth inhibitory [32] and antiapoptotic/progrowth [33] signals in different systems, apparently depending on the stimulus and cell type involved. We showed that p38 MAPK inhibitor (SB203580) enhanced the CD11b expression level (Fig. 9A). A recent paper has demonstrated that inhibition of p38 MAPK enhances differentiation induced by As_2O_3 in NB4 cells, suggesting that p38 MAPK signaling cascade is activated in a negative regulatory feedback manner to attenuate the anti-leukemic effects of As_2O_3 [14]. Our present study demonstrated that the activation of p38 MAPK was observed, followed by the accumulation of intracellular reactive oxygen species. Interestingly, expression levels of p38 MAPK decreased after treatment with 8 μM but increased with 16 μM treatment of As_2S_2 for 1 h (Fig. 7A), suggesting that much stronger stimuli was produced by 16 μM of As_2S_2 during the short period of time. Although an increase of apoptotic cells was accompanied by the activation of p38 MAPK (Fig. 8C), almost no alteration in the number of apoptotic cells was observed after treatment with As_2S_2 in combination with p38 MAPK inhibitor (Fig. 9B). Taking these observations into account, we suggest that the activation of p38 MAPK resulting from oxidative stress is implicated in the negative regulation of cell differentiation, rather than apoptosis induction, in HL-60 cells.

CONCLUSIONS

Our results demonstrated that As_2S_2 induces both differentiation and apoptosis depending on its dose in HL-60 cells. The results also suggest that moderate level of cellular reactive oxygen species is implicated in 8 μM As_2S_2-induced differentiation, while severe oxidative stress induced by 16 μM As_2S_2 leads to cell apoptosis. The generation of reactive oxygen species is suggested to be a contributing factor to the transient increase in glutathione, and it further involves in differentiation and apoptosis induction. We also suggest that activation of p38 MAPK was implicated in As_2S_2-induced differentiation rather than apoptosis induction in the HL-60 cells. Our present observations concerning biphasic actions of As_2S_2 related to dose imply the rationale and future directions of As_2S_2 as a potential anticancer drug candidate.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest to disclose.

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