Arsenic Trioxide Induces Erythroid Differentiation and Apoptosis of K562 Human Leukemia Cells through the Down-Regulation of Bcl-2

Yong-Kyu You, M.D., Hee-Jeong Cheong, M.S., Jong-Ho Won, M.D., Sook-Ja Kim, M.S., Sang-Byung Bae, M.D., Chan-Kyu Kim, M.D., Nam-Su Lee, M.D., Kyu-Taeg Lee, M.D., Sung-Kyu Park, M.D., Dae-Sik Hong, M.D. and Hee-Sook Park, M.D.

Division of Hematology-Oncology and Institute for Clinical Molecular Biology Research, Soon Chun Hyang University College of Medicine, Seoul, Korea

Background: Arsenic trioxide (As$_2$O$_3$) has been identified as an effective drug for the treatment of acute promyelocytic leukemia (APL). However, the role of As$_2$O$_3$ during the erythroid differentiation of human leukemic cells remains unknown. In this study, we investigated the in vitro effects of As$_2$O$_3$ on the erythroid differentiation of the K562 cell line and also on the expression and regulation of the apoptotic modulators of this process.

Methods: The K562 cells were cultured in the presence of 0.1, 0.5 and 1.0 $\mu$M As$_2$O$_3$ or they were cultured in the presence of 1.0 and 10 $\mu$M all trans retinoic acid (ATRA). The expression of glycophorin A before and after treatment with As$_2$O$_3$ or with ATRA in the K562 cells was assessed by flow cytometry and western blotting. The expressions of Bcl-2 and caspase-3 were determined by western blotting.

Results: The viability of the K562 cells was not decreased after treating with 0.1 and 0.5 $\mu$M of As$_2$O$_3$, but the viability was significantly reduced at a dose of 1.0 $\mu$M. Caspase 3 activation was not observed at 0.1 and 0.5 $\mu$M of As$_2$O$_3$ until 12 days, but Caspase 3 was activated by 1.0 $\mu$M of As$_2$O$_3$ from day 3. The expression of glycophorin A was increased in dose dependent manner by As$_2$O$_3$ treatment, but this was not changed in the ATRA treated K562 cells. The expression of Bcl-2 was increased by 0.1 and 0.5 $\mu$M of As$_2$O$_3$, but it was abruptly reduced by 1.0 $\mu$M of As$_2$O$_3$.

Conclusion: These results suggest that As$_2$O$_3$ induces the erythroid differentiation of K562 cells and that 1.0 $\mu$M of As$_2$O$_3$ induces apoptosis through the down-regulation of Bcl-2. (Korean J Hematol 2005; 40:93-100.)

Key Words: Arsenic trioxide, Differentiation, Apoptosis, ATRA, K562
INTRODUCTION

Although arsenic compounds are known poisons, they have been used in traditional oriental medicine for centuries. Recently arsenic compounds, such as arsenic trioxide \((\text{As}_2\text{O}_3)\) and arsenic disulfide, have proven to be effective in the treatment of relapsed acute promyelocytic leukemia (APL). Moreover, toxicities were found to be acceptable, neither bone marrow depression nor other severe clinical side effects were observed. The mechanisms of action were shown to exert dose dependent-dual effects in APL cells, i.e., by triggering apoptosis and inducing partial differentiation.\(^1\)\(^,\)\(^3\) These effects were associated with the modulation and degradation of the \(t(15:17)\)-specific fusion protein PML-RAR\(\alpha.\)\(^4\)\(^,\)\(^5\)

However, recent reports suggest that the apoptotic effect of \(\text{As}_2\text{O}_3\) is not specific for APL cells, and that it can be observed in non-APL leukemia and lymphoma cell lines\(^6\)\(^-\)\(^9\) as well as in other tumor cell lines.\(^10\)\(^,\)\(^11\) \(\text{As}_2\text{O}_3\) can induce the activation of caspases,\(^12\)\(^,\)\(^13\) the downregulation of Bcl-2,\(^14\)\(^,\)\(^15\) the modulation of p53,\(^16\) as well as the uncoupling of the mitochondrial potential.\(^1\)\(^,\)\(^17\)

The induction of tumor cell differentiation represents an attractive strategy for the treatment of a wide range of malignancies. Differentiation of HL-60 promyelocytic leukemia cells towards neutrophils or monocytes have been shown to induce apoptotic cell death. This differentiation-induced apoptosis was found to be coupled by the downregulation of Bcl-2.\(^18\)\(^,\)\(^19\) Because little is known about \(\text{As}_2\text{O}_3\) in tumor models of erythroid differentiation, we investigated the in vitro effect of \(\text{As}_2\text{O}_3\) on the erythroid differentiation of K562 cell line using glycoporphin A, a marker for erythroid differentiation and upon the expressions and regulations of the Bcl-2 and caspase 3.

MATERIALS AND METHODS

1. Cell culture

The K562 human leukemia cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in RPMI 1640 (GIBCO-BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO-BRL) and 1% penicillin-streptomycin. To induce differentiation, cells were cultured in the presence of 0.1 \(\mu\text{M}\), 0.5 \(\mu\text{M}\), and 1 \(\mu\text{M}\) \(\text{As}_2\text{O}_3\) (Sigma, St. Louis, MO, USA), or 1 \(\mu\text{M}\) and 10 \(\mu\text{M}\) all trans retinoic acid (ATRA). Fresh medium containing the differentiation inducers was added to the cultures every 72 hours. Cell viability was assessed at 3, 6, 9, and 12 days after culture by the trypan blue exclusion assay.

2. Reagents

\(\text{As}_2\text{O}_3\) and ATRA were purchased from the Sigma Chemical Co. (St. Louise, MO, USA). Monoclonal mouse antibody for glycoporphin A was obtained from Biogenesis Ltd (Poole, UK) and PE-conjugated anti-human glycoporphin A was obtained from BD Pharmingen (Mountain view, CA, USA). Monoclonal mouse anti-human bcl-2 and polyclonal rabbit anti-caspase 3 were obtained from BD Pharmingen.

3. Flow Cytometric Analysis

Flow cytometry was performed on a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA). The expression of glycoporphin A before and after treatment with \(\text{As}_2\text{O}_3\) or ATRA in K562 cells was assessed by PE-conjugated anti-human glycoporphin A. PE-conjugated anti-mouse IgG1 was used as a negative control and 10,000 events were counted for each analysis.

4. Western Blot Analysis

Expression of glycoporphin A, Bcl-2, and caspase-3 were determined by western blot analysis. Cells were lysed in ice-cold PBS containing 1% Nonidet
Fig. 1. Viability of K562 cells after treatment with As$_2$O$_3$ or ATRA. (A) The percentage of viable K562 cells after treatment with 1 $\mu$M of As$_2$O$_3$ began to decrease significantly 9 days after treatment. (B) The viabilities of K562 cells were 97%, 91%, and 74.5% by day 12 of treatment with 0.1 $\mu$M, 0.5 $\mu$M, and 1 $\mu$M of As$_2$O$_3$, respectively (*$P < 0.05$, †$P < 0.05$). The viability of K562 cells was not changed by adding 1 $\mu$M and 10 $\mu$M of ATRA until day 12.

P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2.0 $\mu$g/mL aprotinin and protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) by passing them through a 21 gauge needle. Lysed cells were centrifuged at 12,000 rpm to remove cellular debris, and the protein concentrations of the extracts were determined by colorimetric bicinchoninic acid analysis (Pierce, Rockford, IL, USA). 30 $\mu$g of proteins were separated by 10% SDS-PAGE, and the proteins were electrophoretically transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 8% non-fat dry milk in TTBS (50 $\mu$M Tris-Hcl (pH 7.5), 150 $\mu$M NaCl, 0.1% (v/v) Tween 20) for 1 hour at room temperature and incubated in primary antibody diluted to 1 : 1,000 in TTBS/8% non-fat dry milk for 4 hours at room temperature. The membrane was then washed three times with TTBS for 15 minutes and subsequently incubated in HRP-conjugated goat anti-rabbit IgG (DAKO, Glostrup, Denmark) or HRP-conjugated goat anti-mouse IgG (Bio-Rad, Hercules, CA, USA) diluted to 1 : 3,000 in TTBS/8% non-fat dry milk for 1 hour at room temperature. The membrane was then washed three times, as described above and developed using the ECL detection system (Amersham, Arlington Heights, IL, USA).

5. Statistical Analysis

All of the assays were conducted in triplicate. The results are expressed as means±SD. Statistical analysis for the viability assay was determined by students t-test. Values of $P$ less than 0.05 were considered statistically significant.

RESULT

1. Viability of K562 cells after treatment with As$_2$O$_3$ or ATRA

In the first set of experiments, we investigated the viability of K562 cells after treatment with
As$_2$O$_3$ or ATRA by using the trypan blue exclusion assay (Fig. 1). The percentage of viable K562 cells after treatment with 1.0 $\mu$M of As$_2$O$_3$ began to decrease significantly 9 days after treatment. The viabilities of the K562 cells were 97%, 91%, and 74.5% by day 12 of treatment with 0.1 $\mu$M, 0.5 $\mu$M, and 1.0 $\mu$M of As$_2$O$_3$, respectively ($P < 0.05$). The viability of K562 cells was not changed by adding 1.0 $\mu$M and 10 $\mu$M of ATRA until day 12.

2. Induction of apoptosis by As$_2$O$_3$

To determine whether the cytotoxic effect induced by As$_2$O$_3$ is induced by apoptosis, we examined the expression of caspase-3, which plays an essential role in the induction of apoptosis, during the treatment of K562 cells with As$_2$O$_3$ or ATRA by a western blot analysis (Fig. 2). After
Fig. 4. Expression of Bcl-2 in K562 cells after treatment with As$_2$O$_3$ or ATRA. The expression of Bcl-2 was increased in a time and dose dependent fashion in K562 cells treated with 0.1 $\mu$M or 0.5 $\mu$M of As$_2$O$_3$ but abruptly decreased in K562 cells from 9 days after incubation with 1.0 $\mu$M of As$_2$O$_3$. Bcl-2 expression was not changed in K562 cells incubated with ATRA.

3. Erythroid differentiation of K562 cells

We next assessed the expression of glycophorin A in K562 cells after treatment with As$_2$O$_3$ or ATRA by flow cytometry and western blot. Flow cytometric analysis showed that glycophorin A expression was increased in a dose dependent fashion in K562 cells after 3 days of incubation with As$_2$O$_3$, but was unchanged in K562 cells incubated with ATRA (Fig. 3A). Western blot analysis showed that treatment with As$_2$O$_3$ induced the upregulation of glycophorin A in a dose dependent fashion, whereas glycophorin A remained unaltered when treated with ATRA (Fig. 3B).

4. Expressions of Bcl-2

Although, ATRA resistant K562 cells can undergo erythroid differentiation in the presence of 0.1 $\mu$M and 0.5 $\mu$M of As$_2$O$_3$, apoptosis was observed only at 1.0 $\mu$M of As$_2$O$_3$. To investigate whether abnormal expressions of apoptotic modulators are involved during erythroid differentiation and apoptosis, we examined the expressions of Bcl-2 in K562 cells after treatment with As$_2$O$_3$ or ATRA. The expression of Bcl-2 was increased in a time and dose dependent fashion in K562 cells treated with 0.1 $\mu$M or 0.5 $\mu$M of As$_2$O$_3$ but abruptly decreased in K562 cells treated with 1.0 $\mu$M of As$_2$O$_3$. Bcl-2 expression was unchanged in K562 cells incubated with ATRA (Fig. 4).

DISCUSSION

As$_2$O$_3$ at a dose of 1~2 $\mu$M was shown to have therapeutic effect against APL cells, even in patients resistant against ATRA or conventional chemotherapy, with minimal toxicity by induction of apoptosis of APL cells.$^{3,10,20,21}$ The success of As$_2$O$_3$ in inducing high rates of complete remission in patients who have relapsed with this aggressive, acute disease provides an impetus for uncovering its specific mechanisms of action that underlie these dramatic clinical responses. Although there has been considerable focus on PML-RAR$\alpha$ and PML in terms of response to arsenic, recent investigation has yielded considerable data on the mechanisms by which arsenic acts on other cellular pathways. Arsenic acts on cells through a variety of mechanisms, influencing numerous signal transduction pathways, and this results in a vast range of cellular effects that include apoptosis induction, growth inhibition, promotion or inhibition of differentiation, and angiogenesis inhibition.$^{22}$ These cellular effects suggest a rationale for the evaluation of As$_2$O$_3$ as a possible therapy in other hematologic malignancies. Therefore, we investigated the in vitro effect of As$_2$O$_3$ on the K562 erythroid leukemia cell line.
In this study, we examined the in vitro effect of As\(_2\)O\(_3\) on the erythroid differentiation of K562 cells and upon the expressions and regulations of the apoptotic modulators of this process. We used low doses of As\(_2\)O\(_3\) (0.1\(\sim\)1 \(\mu\)M) because 1\(\sim\)2 \(\mu\)M of the plasma concentration of As\(_2\)O\(_3\) is thought to be a safe range in human.\(^{29}\) K562 cells undergo erythroid differentiation after treatment with As\(_2\)O\(_3\). We found that the erythroid differentiation of K562 cells was observed from 0.1 \(\mu\)M of As\(_2\)O\(_3\) but significant loss of cell viability by activation of an apoptotic process was only observed at 1.0 \(\mu\)M of As\(_2\)O\(_3\). Activation of cleaved caspase-3 was also observed in K562 cells treated with 1.0 \(\mu\)M of As\(_2\)O\(_3\). Caspase-3 was not activated in K562 cells treated with 0.1 \(\mu\)M and 0.5 \(\mu\)M of As\(_2\)O\(_3\). Moreover, the apoptosis of K562 cells was associated with the diminished expression of Bcl-2 protein. The expression of Bcl-2 increased in a time and dose dependent fashion in K562 cells treated with 0.1 \(\mu\)M and 0.5 \(\mu\)M of As\(_2\)O\(_3\) but abruptly decreased in K562 cells treated with 1.0 \(\mu\)M of As\(_2\)O\(_3\). As\(_2\)O\(_3\) has been shown to exert dose-dependent dual effects in APL cells, i.e., by triggering apoptosis and inducing partial differentiation.\(^{1-3,23}\) Low dose As\(_2\)O\(_3\) (0.1\(\sim\)0.5 \(\mu\)M)-induced differentiation might be mediated directly or indirectly by RARa-related signaling pathways in APL cells. On the other hand, high dose As\(_2\)O\(_3\) (1.0 \(\mu\)M)-induced apoptosis involves collapse of the mitochondrial transmembrane potential, triggers the release of pro-apoptotic factor from mitochondria to cytoplasm, which is followed by caspase activation and the degradation of specific substrates.\(^1\) Although As\(_2\)O\(_3\) induced differentiation has been selectively observed in APL cells previously, in this study, we demonstrate the As\(_2\)O\(_3\) induced erythroid differentiation of K562 erythroid leukemic cells. 0.1\(\sim\)0.5 \(\mu\)M of As\(_2\)O\(_3\) induced glycophorin A expression without a loss of cell viability in K562 cells. These results suggest that low dose As\(_2\)O\(_3\) induces erythroid differentiation in K562 cells, and that the activation of Bcl-2 was found to inhibit apoptosis. Benito et al.\(^{18}\) demonstrated the erythroid differentiation of K562 and HEL cells after treatment with retinoic acid, hemin, or TGF-\(\beta\). The erythroid differentiation of both HEL and K562 cells led to progressive loss of cell viability by activating an apoptotic process. In our study, the apoptosis of K562 cells was associated with the diminished expression of Bcl-2 protein. The expression of Bcl-2 increased in a time and dose dependent fashion in K562 cells treated with 0.1 \(\mu\)M and 0.5 \(\mu\)M of As\(_2\)O\(_3\) but abruptly decreased in K562 cells treated with 1.0 \(\mu\)M of As\(_2\)O\(_3\). These results indicate that apoptosis and erythroid differentiation proceed simultaneously, but that they can be uncoupled by the expression of Bcl-2. This data is in agreement with that reported for HL-60 myeloid leukemic cells, in that the overexpression of Bcl-2 cells did not affect their maturation, but prevented the apoptosis induced by myeloid differentiation.\(^{19,24,25}\) Thus, the dose dependent dual effects in K562 cells treated with As\(_2\)O\(_3\) can be explained by a decreased Bcl-2 expression. These results suggest that 0.1\(\sim\)0.5 \(\mu\)M of As\(_2\)O\(_3\) induced erythroid differentiation in K562 cells and up-regulate Bcl-2 inhibited apoptosis. On the other hand, 1.0 \(\mu\)M of As\(_2\)O\(_3\) potentially down-regulates Bcl-2 gene expression. There are some arguments for the change of Bcl-2 expression in K562 cells treated with As\(_2\)O\(_3\). Zhu et al.\(^{26}\) reported Bcl-2 expression did not change in K562 cells after incubation with 1.0 \(\mu\)M of As\(_2\)O\(_3\) for 2 days but other investigators showed down-regulation of the Bcl-2 after incubation for 4 days with 2.0 \(\mu\)M of As\(_2\)O\(_3\).\(^{27}\) In our data, we found definite down-regulation of the Bcl-2 expression from 9 days after incubation with 1.0 \(\mu\)M of As\(_2\)O\(_3\).

Our study suggests that As\(_2\)O\(_3\) can induce K562 erythroid leukemic cell differentiation and apoptosis. 0.1\(\sim\)0.5 \(\mu\)M of As\(_2\)O\(_3\) induced the erythroid differentiation of K562 cells but only 1.0 \(\mu\)M of As\(_2\)O\(_3\) induced apoptosis. Dose dependent dual effects in K562 cells treated with As\(_2\)O\(_3\) can be explained partly by a decreased Bcl-2 expression,
배경: 삼산화비소(Arsenic trioxide, As₂O₃)는 금성전
골수세백혈병의 효과적인 치료제로 알려져 있다. 그러나
아니라 As₂O₃는 금성골수성백혈병 이외의 골수성백
혈병에서도 백혈병 세포의 세포고사를 유도하여 백혈
병 세포의 증식을 억제한다. 그러나 As₂O₃를 이용한 기
존의 연구들은 주로 골수구계의 분화 및 세포고사에
관한 연구가 대부분이었으며 백혈병 세포의 적혈구계
분화에 대한 As₂O₃의 역할에 대하여는 아직 연구가 미
진한 상태이다.

방법: 본 연구에서는 As₂O₃를 이용하여 적혈구계 금
성골수성백혈병에서 기원한 K562 세포주의 문화를 유도
하였으며 이 과정에서 세포고사에 관여하는 세포고사에
관한 지속적인 연구가 필요하리라 생각한다.

결과: K562 세포의 활성도는 0.1과 0.5μM의 As₂O₃
를 투여한 경우에는 변화가 없으나 1μM을 투여한
경우에는 의미 있게 감소하였다. Caspase 3은 0.1와
0.5μM의 As₂O₃를 투여한 경우에는 변가 없었으나 1μM을 투여한
경우는 유사한 경로를 그렸다. Glycophorin A는 As₂O₃를 투여
한 경우 유리하게 발현이 증가하였으나 ATRA
를 투여한 경우에는 발현이 감소하였고, Bcl-2의 발현은 1
과 0.5μM의 As₂O₃를 투여한 경우에는 변화가 없었으나 1μM을 투여한
경우에는 발현이 감소하였다.

결론: As₂O₃는 저용량의 투여에 의하여 K562 세포
의 적혈구계 분화를 유도하였으며 1μM을 투여한 경우
에는 K562 세포의 apoptosis를 유도하였다. As₂O₃에
 의한 K562 세포의 세포고사는 Bcl-2의 발현 감소와 관계
가 있었다. 본 연구의 결과는 As₂O₃가 골수세포의 적혈
구계 분화 및 세포고사에 관여함을 보여주며 향후 이에
관한 지속적인 연구가 필요하다라 생각한다.

요 약

although the precise roles and mechanisms involved
require further investigation.

REFERENCES

ced apoptosis and differentiation are associated respec-
tively with mitochondrial transmembrane potential
collapse and retinoic acid signaling pathways in acute
cellular and molecular mechanisms of arsenic trioxide
(As₂O₃) in the treatment of acute promyelocytic leu-
kenia: As₂O₃ induces NB4 cell apoptosis with down-
regulation of Bcl-2 expression and modulation of
PML-RAR alpha/PML proteins. Blood 1996;88:1052-
61.
trioxide (As₂O₃) in the treatment of acute promyelo-
cytic leukemia (APL): I. As₂O₃ exerts dose-dependent
4. de The H, Chomienne C, Lanotte D, Degos L, Dejean
A. The t(15;17) translocation of acute promyelocytic
leukemia fuses the retinoic acid receptor alpha gene
mosomal translocation t(15;17) in acute promyelocytic
leukemia fuses RAR alpha with a novel putative
trioxide and melarsoprol induce apoptosis in plasma
cell lines and in plasma cells from myeloma patients.
of apoptosis and cell cycle arrest by arsenic trioxide
and characterization of an arsenic-sensitive monoblastic
leukemia cell line (SigM5). Brit J of Haematol 2000;
9. Rojewski MT, Baldus C, Knauf W, Thiel E, Schre-
zenmeier H. Dual effects of arsenic trioxide (As
cappa and c-Jun-N-terminal kinase on p53-indepen-
11. Zhang TC, Cao EH, Li JF, Ma W, Qin JF. Induction
of apoptosis and inhibition of human gastric cancer
MGC-803 cell growth by arsenic trioxide. Eur J