Original Articles

ARSENIC TRIOXIDE INDUCES ONCOSIS IN K562 CELL LINE VIA CD95/CD95L PATHWAY

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Abstract : Recently it has been reported that arsenic trioxide (As_2O_3) is very effective in the treatment of acute promyelocytic leukemia (APL) by inducing apoptosis, but the molecular mechanism of its action on other leukemia remains unclear. In the present study, we tested the effect of As_2O_3 at low concentration 0.25–2.0 μM on K562, a chronic myelogenous leukemia cell line. As_2O_3 inhibited the cell growth of K562 in a similar way to APL cell line NB4. Typical oncotic cell death, such as cytoplasmic swelling and swelling of organelles, was observed by morphological study and cell cycle was arrested at G2+M phases. During the treatment of As_2O_3, the CD95 and CD95 ligand (CD95L) expressions were upregulated, and caspase 8 and caspase 3 were activated, but bcl-2 expression was not changed. Treatment of the cells with anti-CD95 monoclonal antibody or ZVAD-fmk capable of blocking the CD95 signaling pathway ameliorated As_2O_3-induced oncosis. These results suggest that the induction of oncosis by As_2O_3 involves CD95/CD95L pathway in K562 and As_2O_3 may provide a novel therapy for leukemia other than APL.

Key words : arsenic trioxide, oncosis, K562, CD95/CD95L pathway, bcl-2

INTRODUCTION

Low concentration (0.5–2.0 μM) of arsenic trioxide (As_2O_3) induces apoptosis in acute promyelocytic leukemia (APL) cell line NB4. The molecular mechanism of its action is associated with rapid degradation of PML/RARα fusion protein and downregulation of bcl-2 expression[1]. Recent in vitro studies have shown that low concentration of As_2O_3 (1–2 μM) also induces apoptosis in other leukemia[2-5] and tumor cell lines[6-9]. However, the mechanism of tumor cell killing by As_2O_3 is still unclear. Some evidence has shown that antiproliferative effects of As_2O_3 are through an independent manner of PML/RARα fusion protein and do not require downregulation of bcl-2 expression[3,10].

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Cell death is not only an essential phenomenon in normal development, but also plays a crucial role in cancer chemotherapy[11]. The end state of cellular toxicity falls into at least two distinct cell death pathways: apoptosis and oncosis, both of which progress into irreversible necrosis. Apoptosis is characterized by cellular shrinkage, chromatin condensation, and apoptotic bodies. On the other hand, oncosis is described as cellular swelling, organelle swelling, blebbing, and increased membrane permeability. Necrosis can occur after both forms of cell death[12].

In this study, we addressed the anticancer effect of As_2O_3 on K562, a chronic myelogenous leukemia cell line, and investigated the molecular mechanism of its action.

MATERIAL AND METHODS

1. Cell lines

K562, a chronic myelogenous leukemia cell line from blastic crisis, was purchased from the Japanese Cancer Research Resources Bank. We also used two human leukemia/lymphoma cell lines: HL-60, acute myeloid leukemia (the French-American-British classification, M2) cell line and RL, t(14;18) B-cell lymphoma cell line overexpressing bcl-2 as control. They were cultured in RPMI1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mmol/L L-glutamine, and 10% fetal bovine serum in a humidified atmosphere of 95% air/5% CO_2 at 37℃.

2. Reagents

As_2O_3 (Wako, Japan) was dissolved in distilled water as a stock solution of 0.01 M and diluted to working concentrations by phosphate-buffered saline (PBS) before use.

3. Assessment of cell growth inhibition

Cell growth and viability were measured with the MTT (3- [4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) test[13]. After cells were cultured with or without different concentrations (0.25-2.0 μM) of As_2O_3 for 5 days and washed twice with cold PBS, 20 μl of MTT solution was added to 200 μl of the cell suspensions in a 96 multi-well dish , and incubated at 37℃ for 2 hours. The dish was centrifuged at 400 g for 5 minutes, the medium was discarded, and 200 μl of DMSO (dimethyl sulfoxide) was added to each well to dissolve MTT formazans. Finally, optical densities (OD) at 550-650 nm were measured with an ELISA scan (SLT-Labinstruments, Austria).

4. Morphological assessment

After treatment with or without 1.0 μM of As_2O_3 for 3 days, 2×10^4 cells were spun onto slide glasses by Cytospin (Shandon, England, 800 rpm, 8 minutes) and then stained with Giemsa solution. Morphologic changes of the cells were observed under a light microscope.

5. Cell cycle analysis

Cell cycle was analyzed by flow cytometry (FCM, Becton Dickinson). As described previously[14], 10^6 cells were incubated with or without 1.0 μM of As_2O_3 for 4 days and washed

twice with PBS and centrifuged (200 g). The cell pellets were resuspended in 1.5 ml of a hypotonic fluorochome solution [propidium iodide (PI) 50 μg/ml in 0.1% sodium citrate plus 0.1% Triton X-100 (Sigma)] in the dark overnight and examined by FCM.

6. Detection of CD95, CD95L and bcl-2 expression

The changes of CD95, CD95L and bcl-2 expressions by As_2O_3 were assessed with FCM. After treatment with or without 1.0-2.0 μM As_2O_3 for 1-4 days, 10^5 cells were washed twice with cold PBS and incubated with 5.0 μl of FITC-conjugated anti-human CD95 monoclonal antibody (MoAb) (MBL, Japan) or FITC-conjugated anti-human CD95L MoAb (Fujisawa, Japan) at 4℃ in the dark for 30 minutes. For the detection of bcl-2 protein expression[15], HL-60 and RL were used as positive controls. 5×10^6 cells were resuspended in 2ml of 2% paraformaldehyde. After incubation for 10 minutes on ice, 100μl of 1% Triton X100 was added. Then the cells were washed twice with cold PBS and incubated with 5μl of FITC-conjugated anti-bcl-2 MoAb (Fujisawa, Japan). After washing three times with cold PBS, the cells were analyzed by FCM. FITC-conjugated anti-mouse IgG 1(MBL, Japan) was used as a negative control to determine background fluorescence.

7. Measurement of caspase 8 and caspase 3 activities

The activities of caspase 8 and caspase 3 were determined with two fluorometric protease assay kits (MBL, Japan). After cells were cultured with or without 1.0 μM of As_2O_3 for 4 days, 10^6 cells were resuspended in a 96 multi-well dish and cultured with 50 μl of the chilled cell buffer on ice for 10 minutes. Then 50 μl of the 1 mM IETD-AFC (Lle-Glo-Thr-Asp-7-Amino-4 trifluoromethylcoumarin) substrate for caspase 8 or the 1 mM DEVD-AFC (Asp-Glo-Val-Asp-7-Amino-4 trifluoromethylcoumarin) substrate for caspase 3 was added to each well and incubated at 37℃ for 2 hours. Finally, samples were examined by the fluorometer with a 385 nm excitation filter and a 538 nm emission filter.

8. Blocking of the CD95/CD95L pathway by anti-CD95 MoAb and ZVAD-fmk

Anti-CD95 MoAb (MBL, Japan) or caspase family inhibitor ZVAD-fmk (MBL, Japan) was used to block the CD95/CD95L pathway. Briefly, a total of 4×10^5 K562 cells were cultured in the presence of 1.0 μg/ml of anti-CD95 MoAb or 50 μM ZVAD-fmk for 3 hours at 37℃. Then, they were treated with 1.0 μM of As_2O_3 for 2 days. Cell growth and viability were determined by MTT test. The effect of As_2O_3 on growth inhibition could be assessed as follows: inhibition of cell proliferation(%)=(1- mean OD value of treated cells/mean OD value of control cells) $\times$100.

9. Statistical analysis

Results were expressed as mean$\pm$standard deviation (S.D.). Statistical significance ($p<0.05$) was assessed using Student's *t* test.

RESULTS

1. Effects of As_2O_3 on cell growth inhibition

Effects of As_2O_3 on the growth inhibition of K562 cells are shown in Fig. 1. Although the inhibitory effects were weak at lower concentrations (0.25–0.5 μM), As_2O_3 inhibited proliferation of K562 cells in dose- and time-dependent manners. The inhibition of cell proliferation by As_2O_3 was similar to that reported on NB4[1].

2. Morphological changes and cell cycle arrest

After treatment with 1.0 μM As_2O_3 for 3 days, typical oncotic changes, such as cytoplasmic swelling and swelling of organelles, were observed in K562 cells. On the other hand, typical apoptotic changes, such as chromatin condensation, cellular shrinkage and apoptotic bodies were observed in HL-60 and RL (Fig. 2A). To investigate whether the inhibition of cell growth is due to cell cycle arrest, we examined cell cycle by FCM. As shown in Fig 2B, cell cycle arrested at G2+M phases in K562 compared with apoptotic changes of cell cycle arrested at subG1 in HL-60 and RL.

3. Changes in CD95/CD95L and bcl-2 expression

To understand the mechanisms responsible for As_2O_3-induced oncosis, changes of the expression of CD95 and CD95L in K562 cells were examined by FCM. Both CD95 and CD95L proteins were originally expressed and upregulated by As_2O_3 for one day (Fig. 3). Incubation for more than 2 days did not further increase in the expression of these proteins (data not shown). Next, we examined whether bcl-2 expression was regulated by As_2O_3 by using HL-

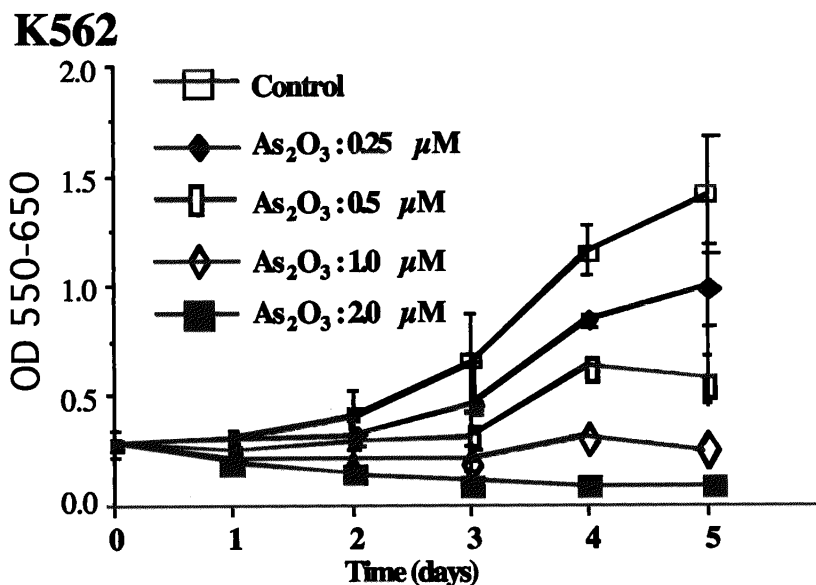

Fig. 1. As_2O_3 induced cell growth inhibition in K562 cells with various concentrations for 5 days. Viability of cells was measured by MTT assay. The data are shown as mean±SD from three independent experiments.

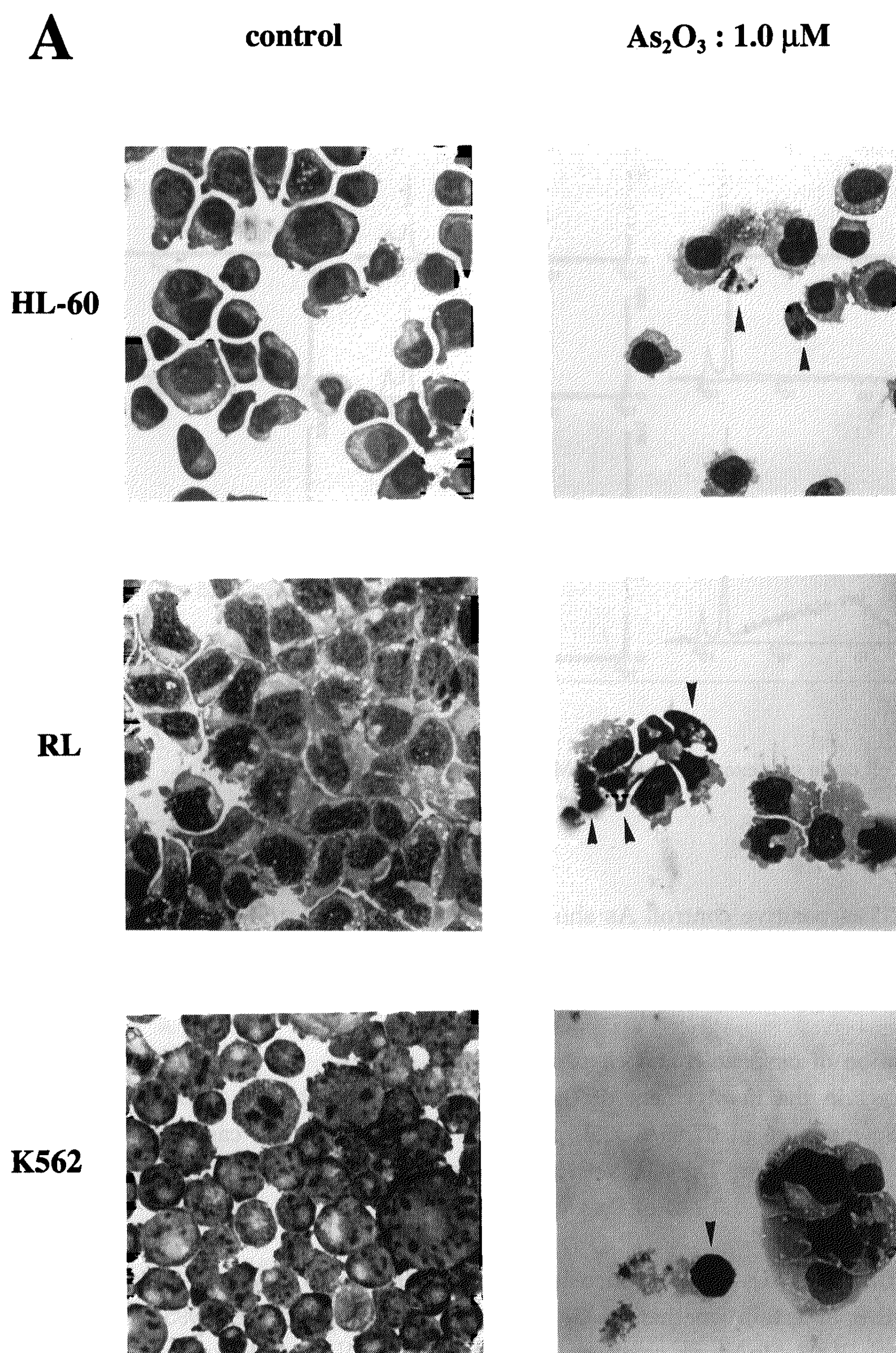

Fig. 2A. Changes of morphology and cell cycle in HL-60, RL and K562 by As_2O_3. Apoptotic changes of morphology (arrow) and cell cycle in HL-60 and RL were shown as positive control. Oncotic changes of morphology (arrow) and cell cycle in K562 were also shown. Morphologic changes were photographed after treatment with 1 μM As_2O_3 for 3 days.

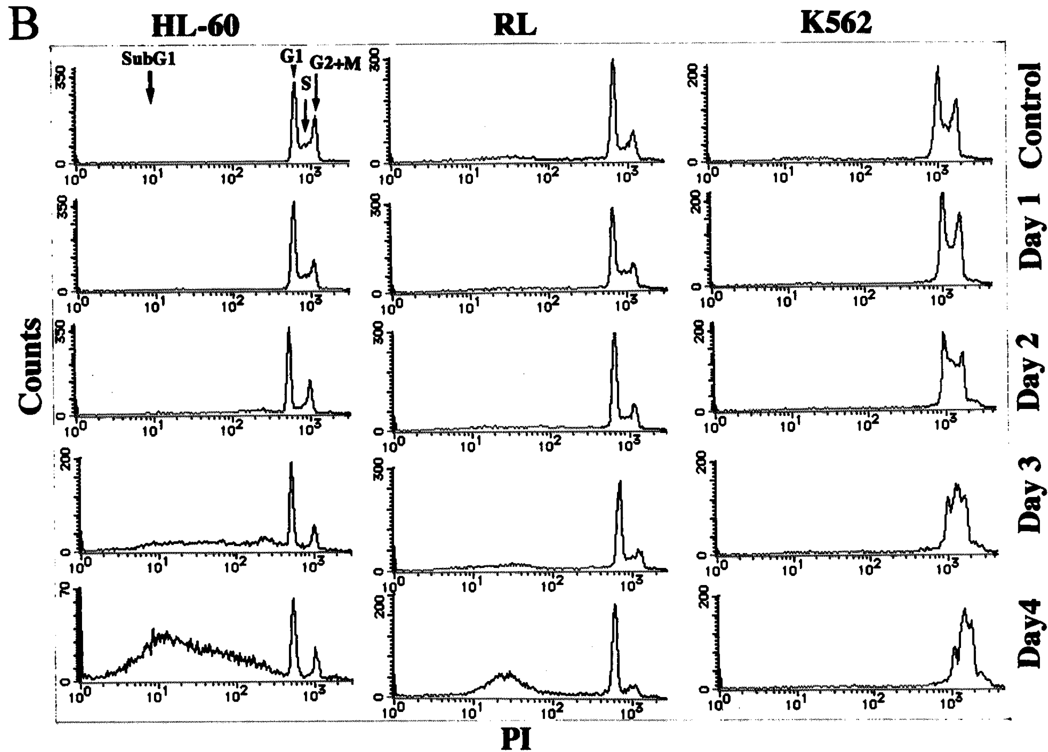

Fig. 2B. Cell cycle was examined after 1 μM As_2O_3 treatment for 4 days by FCM.

60 and RL as positive control. As shown in Fig. 4, bcl-2 was downregulated significantly in HL-60 and RL cells, which is similar to that in NB4[1], but no detectable change in K562 was observed.

4. Activation of caspase 8 and caspase 3

To ascertain the involvement of the CD95/CD95L pathway in As_2O_3-induced oncosis, we studied downstream of CD95/CD95L, activities of caspase 8 and caspase 3 during treatment of As_2O_3. As shown in Table 1, activities of caspase 8 and caspase 3 increased significantly after incubation with 1.0 μM of As_2O_3.

5. Inhibition of As_2O_3-induced oncosis by anti-CD95 MoAb or ZVAD-fmk

To further ascertain whether As_2O_3 induces oncosis only via the CD95/CD95L pathway, we added anti-CD95 MoAb or caspase family inhibitor ZVAD-fmk to block the CD95/CD95L pathway. As shown in Table 2, the addition of anti-CD95 MoAb or ZVAD-fmk significantly suppressed cell death of K562 induced by As_2O_3, but not completely.

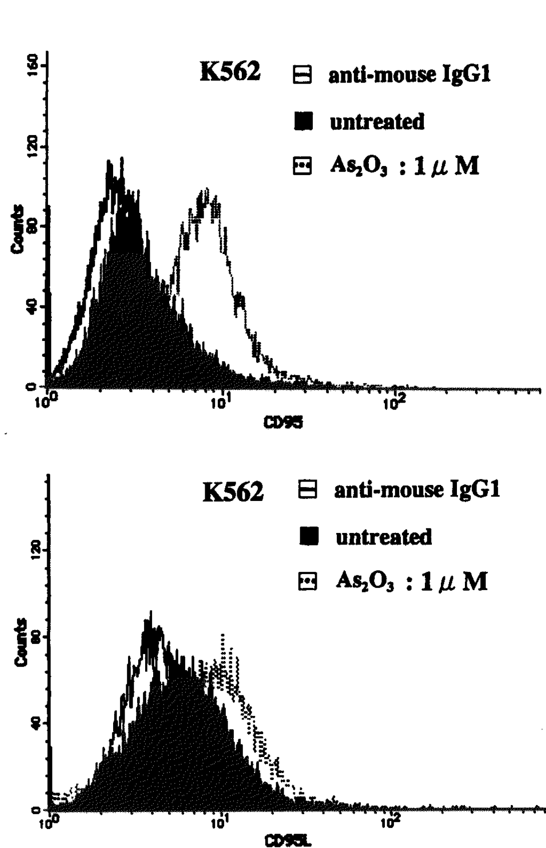

Fig. 3. Upregulation of CD95/CD95L expression by As_2O_3. After K562 cultured with or without 1μM As_2O_3 for one day, CD95/CD95L were analyzed by FCM. Top: CD95, Bottom: CD95L.

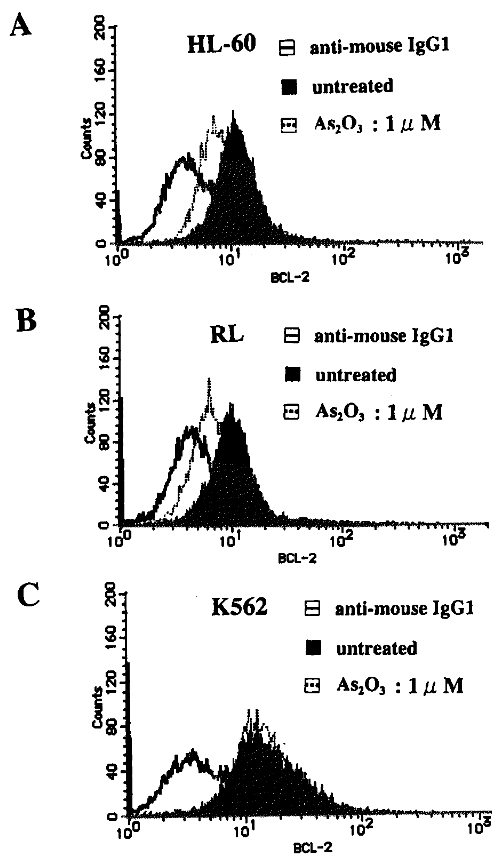

Fig. 4. Downregulation of bcl-2 expression by As_2O_3. HL-60 and RL were employed as positive control for detection of bcl-2 expression. After incubation with or without 1μM As_2O_3 for 2 day, bcl-2 expression was analyzed by FCM. (A) : HL-60, (B) : RL, (C) : K562.

Table 1. Changes in activities of caspase 8 and caspase 3 after As_2O_3 treatment in K562

Caspase	Control	Day 1	Day 2	Day 3	Day 4
Caspase 8	136.6± 25.3	217.4± 31.1 *	271.5± 8.4 *	267.3 ± 25.3 *	254.7± 21.1 *
Caspase 3	182.5± 36.5	267.7± 34.1 *	298.8± 55.4 *	286.1± 54.2 *	197.1± 28.7

Activation of caspases 8 and 3 in K562 cells by As_2O_3. After incubation with or without 1μM As_2O_3 for 4 days, activities of caspases 8 and 3 were detected with fluorometric protease assay. The data are shown as mean±SD from three independent experiments. There was a significant difference between control and treated groups (*: $P<0.05$).

Table 2. Effects of anti-CD95 MoAb and ZVAD-fmk on the oncosis induced by As_2O_3 in K562

	As_2O_3	Anti-CD95MoAb	ZVAD-fmk
Day 1	**17.4± 8.1**	**8.1± 8.3 ***	**1.0± 1.73 ***
Day 2	**18.4± 7.4**	**7.6± 7.5 ***	**11.4± 2.7 ***

Anti-CD95 MoAb and ZVAD-fmk suppressed cell death of K562 induced by As_2O_3. After pretreatment with anti-CD95 MoAb or ZVAD-fmk, K562 cells were cultured with 1.0 μM As_2O_3 for 2 days. Cell growth and viability were detemined by MTT test. The effect of As_2O_3 on growth inhibition was assessed as follow: inhibition of cell proliferation (%) = (1-mean OD value of treated cells/mean OD value of control cells)×100. The date are shown as mean±SD from three independent experiments.
*: significant difference ($P<0.05$)

DISCUSSION

The molecular mechanism of As_2O_3-induced cell death is very complex, depending on dose, cell type, and cellular environment[16]. In the present study, we studied the action of in vivo plasma level concentrations, 0.25-2.0 μM As_2O_3 on K562[17]. It was shown that the effects of antiproliferation were similar to that in an APL cell line, NB4[1]. Furthermore, typical oncosis was confirmed by cellular swelling, swelling of organelles, blebbing of cytoplasm, no significant changes in nucleus, and cell cycle arrested at G2+M phases. These results confirmed that the cell growth inhibition in K562 is due to oncosis.

The CD95/CD95L pathway involves expression of transmembrane receptor CD95 and CD95L. CD95 activates the Fas-associated death domain (FADD) protein to cleave pro-caspase 8 to caspase 8, then caspase 8 cleaves pro-caspase 3 to caspase 3 which is the final step of cell death[18-20]. It was reported that CD95/CD95L plays a novel role in anticancer drugs[21-23]. In this study, the expression of CD95 and CD95L on K562 were upregulated by As_2O_3 in 1 day. Furthermore, As_2O_3 treatment of K562 for 2 days activated both caspase 8 and caspase 3. These findings suggest that the CD95/CD95L pathway plays a major role in the As_2O_3-induced oncosis in K562. To further determine whether As_2O_3 induces oncosis only via the CD95/CD95L pathway, anti-CD95 MoAb or ZVAD-fmk was used to block the CD95/CD95L pathway. As_2O_3-induced oncosis was suppressed partially. This finding provides evidence that the molecular mechanism of As_2O_3-induced oncosis may be not only via CD95/CD95L pathway but also via other pathways.

CD95-independent pathway including mitochondrial death pathway has been also described as a central role in As_2O_3-induced cell death, throughout the release of cytochrome c[24], activating downstream caspases[25], and downregulation of bcl-2 expression[1,6,10]. In our previous study[26], bcl-2 expression was downregulated by As_2O_3 in positive control, HL-60 and RL. However, no detectable change of bcl-2 expression was found in K562. This discrepancy may be due to the possibility that bcl-2 directly or indirectly determines cell death fate: apoptosis or oncosis. The relationship between bcl-2 expression and cell morphological changes should be further studied.

We conclude that As_2O_3 induces growth inhibition, cell cycle arrest and eventually oncosis via the CD95/CD95L pathway in K562 cells. These results suggest that As_2O_3 may be useful in the treatment of chronic myelogenous leukemia other than APL.

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