MOLECULAR MECHANISM OF ARSENIC TRIOXIDE-INDUCED APOPTOSIS IN HL-60 AND RL CELL LINES

JIEBIN ZHU[1] and SHINOBU NAKAMURA[2]

1) *Cellular Transplantation Biology, Kanazawa University Graduate School of Medical Science*

2) *Department of General Medicine and Clinical Investigation, Nara Medical University*

Received October 17, 2003

Abstract : Recently, it has been reported that arsenic trioxide (As_2O_3) is an effective anticancer agent for acute promyelocytic leukemia (APL). In the present study, we examined the anticancer effects of As_2O_3 at low concentration (0.25~2.0μM) on two human leukemia/lymphoma cell lines, HL-60 and RL, in vitro. We found that As_2O_3 inhibited the growth of HL-60 and RL similar to the reported APL cell line, NB4. Typical apoptosis was observed in morphological study and DNA fragmentation assay, as well as a cell cycle arrest at subG1. To address the mechanism of As_2O_3-induced apoptosis, we also examined the effect of As_2O_3 on the CD95/CD95L pathway and bcl-2 protein expression. The results showed that the CD95/CD95L expressions were upregulated; meanwhile, caspase 8 and caspase 3 were activated. However, the bcl-2 protein expression was downregulated. Using anti-CD95 monoclonal antibody to block the CD95 pathway, As_2O_3-induced apoptosis was ameliorated. These data suggest that in HL-60 and RL cell lines the CD95/CD95L pathway and downregulation of bcl-2 protein expression are involved in As_2O_3-induced apoptosis.

Key words : arsenic trioxide, HL-60, RL, apoptosis, CD95/CD95L pathway

INTRODUCTION

Arsenic trioxide (As_2O_3) has been reported to be very effective in the treatment of acute promyelocytic leukemia (APL), even in patients relapsed after all-trans retinoic acid (ATRA)-induced and chemotherapy-maintained complete remission and without severe myelosuppression[1]. Recent studies showed that As_2O_3 induced apoptosis in some cancer cell lines, such as: chronic B-lymphocytic leukemia[2], acute myeloid leukemia[3,4], T-cell leukemia[5], megakaryocytic leukemia[6], multiple myeloma[7,8], lymphoma[9], neuroblastoma[10], human small cell lung cancer[11], human ovarian carcinoma[12], and human breast cancer[13]. The molecular mechanism of As_2O_3 treatment is controversial. The modulation of PML/RARα fusion protein and downregulation of bcl-2 expression in NB4, APL cell line with t(15;17) translocation, induced by As_2O_3 was reported[1]. But recent studies showed that the antiproliferative effect of As_2O_3 may be independent on changes of PML/RARα fusion protein[3,4,14], and bcl-2 expression. These reports suggested that it might be via modulation of the glutathione redox system in NB4 cells[9] and via microtubule inhibition in myeloid leukemia cells, K562[4].

Current evidence suggests that the mechanism of some anticancer agents involves the activation of the CD95/CD95L pathway[15-18]. In the present study, we investigated anticancer molecular mechanism of As_2O_3 on two leukemia/lymphoma cell lines, HL-60 and RL, and

provide evidence that As_2O_3 induces apoptosis through the CD95/CD95L pathway and downregulation of bcl-2 protein expression.

MATERIALS AND METHODS

1. Cell lines

Two human leukemia/lymphoma cell lines were used. HL-60, acute myeloid leukemia (the French-American-British classification, M2) cell line, was purchased from the Japanese Cancer Research Resources Bank, and t(14;18) B-cell lymphoma RL cell line overexpressing bcl-2 was obtained from the American Type Culture Collection (Rockville, MD, USA). They were cultured in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100μg/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. Reagent

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

3. Cell growth and viability assay

Cell growth and viability were determined by MTT (3-[4,5-dimethylthiazol-2-yl] -2,5-diphenyltetrazolium bromide) test[19]. Briefly, the cells (2×10^5 /ml) were harvested with or without different concentration (0.25-2.0 μM) of As_2O_3 for 1-5 days, respectively. At assay time, the cells were washed twice with cold PBS and 200μl of the cell suspension were cultured in 96 multi-well dish, then 20 μl of MTT solution were added to each well and kept at 37℃ for 2 hours. After 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. Then optical densities were measured by ELISA scan at 550-650 nm (SLT-Labinstruments, Austria).

4. Morphological observation by Giemsa stain

After treatment with or without 1.0 μM of As_2O_3 for 3 days, 100 μl of cell suspension were spun onto slides by Cytospin (Shandon, 800 rpm, 8 minutes) and stained with Giemsa solution. The morphologic changes of cells were observed and photographed with light microscopy.

5. Cell cycle analysis and quantification of apoptotic cells

Cell cycle was analyzed by flow cytometry (FCM, Becton Dickinson). As described previously[20], a total of 10^6 cells with or without 1.0 μM of As_2O_3 treatment for different days were washed twice with PBS and centrifuged (200×g), then the cell pellet was resuspended in 1.5 ml 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. Apoptosis was quantified by measuring subG1 fraction.

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

CD95 and CD95L expressions were assessed by FCM. After treatment with or without

1.0–2.0 μM As_2O_3 for 1 day, 10^5 of cells were washed twice with cold PBS and incubated with 5.0 μl of FITC-conjugated anti-mouse IgG1 (MBL, Japan), anti-human CD95 monoclonal antibody (MoAb) (MBL, Japan) or anti-human CD95L MoAb (Fujisawa, Japan) at 4℃ in the dark for 30 minutes. Then the cells were washed three times with cold PBS and measured by FCM.

For the detection of bcl-2 protein expression[21], after treatment with or without 1.0 μM As_2O_3 for 2 days, pellet of 5×10^6 cells was resuspended in 2 ml of 2% paraformaldehyde. After incubation for 10 minutes on ice, 100 μl of 1% Triton-X100 was added. Ten minutes later, cells were washed twice with cold PBS and incubated with 5 μl of FITC-conjugated anti-mouse IgG1 (MBL, Japan) or anti-bcl-2 MoAb (Fujisawa, Japan) for 30 minutes. After washing three times, the cells were analyzed by FCM.

7. Caspase 8 and caspase 3 activity assay

Caspase 8 and caspase 3 activities were determined by fluorometric protease assay kits (MBL, Japan). Briefly, after cells were incubated with or without 1.0 μM of As_2O_3 for 2 days, pellet of 10^6 cells were resuspended in 50 μl of chilled cell lysis buffer and incubated on ice for 10 minutes. 50 μl of the 1 mM IETD-AFC (Ile-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 the cultured cells and incubated at 37℃ for 2 hours. Then samples were examined by the fluorometer with a 385 nm excitation filter and a 538 nm emission filter.

8. Blocking of the CD 95 pathway by anti-CD95 IgG MoAb

Anti-CD95 IgG MoAb (MBL, Japan) was used to block CD95 pathway. Briefly, a total of 4×10^5 cells with or without 1.0 μg/ml of anti-CD95 MoAb were incubated for 3 hours at 37℃. Then the cells were treated with or without 1.0 μM of As_2O_3 for 4 days. Finally the apoptotic cells were determined by PI staining.

9. Statistical analysis

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

RESULTS

1. Effect of As_2O_3 on cell growth inhibition

Cell proliferation was assessed by MTT assay. Time-dependent and dose-dependent cell growth inhibitions at the concentration of As_2O_3 (0.25–2.0 μM) are shown in Fig.1. As_2O_3 at 1.0–2.0 μM significantly inhibited the growth of both cell lines throughout course (day1–5). These results showed that As_2O_3 was a potent inhibitor of the proliferation of leukemia/lymphoma cells.

2. Morphological changes and cell cycle arrest

After the treatment of As_2O_3 (1.0 μM) for 3 days, typical apoptotic changes, such as chromatin condensation, cellular shrinkage and apoptotic bodies were observed (Fig. 2A). To investigate whether the inhibition of cell growth is due to cell cycle arrest, we examined the

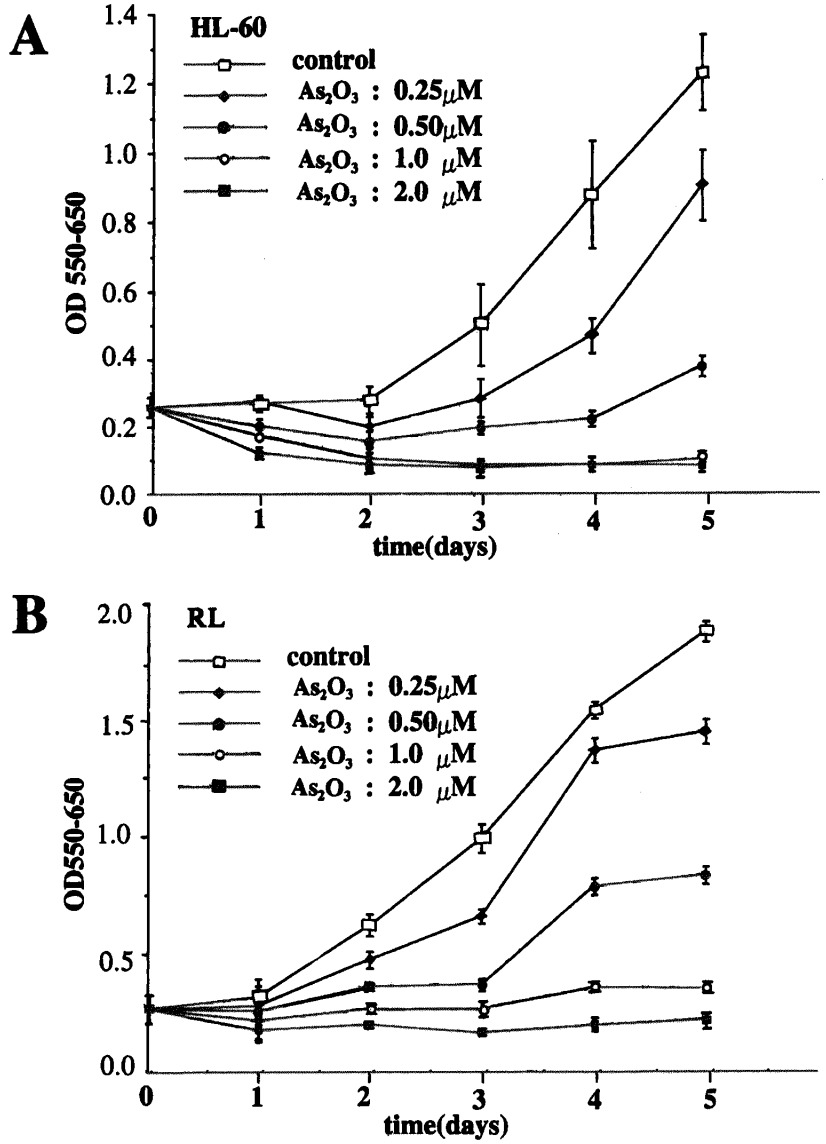

Fig. 1. As_2O_3 induces cell growth inhibition in HL-60 (A) and RL (B) with various concentrations for 5 days. Viability of cells was measured by MTT assay. The data are shown as mean±SD from three independent experiments.

distribution of cellular DNA contents by FCM. FCM analysis showed a distinct subG1 peak from cells treated with 1.0 μM As_2O_3 for 4 days (Fig. 2B).

3. Upregulation of CD95/CD95L expression

To understand mechanisms responsible for As_2O_3-induced apoptosis, the expressions of CD95 and CD95L were measured during the treatment of As_2O_3 on HL-60 and RL cells. Both CD95 and CD95L proteins originally expressed on two cell lines were upregulated by As_2O_3

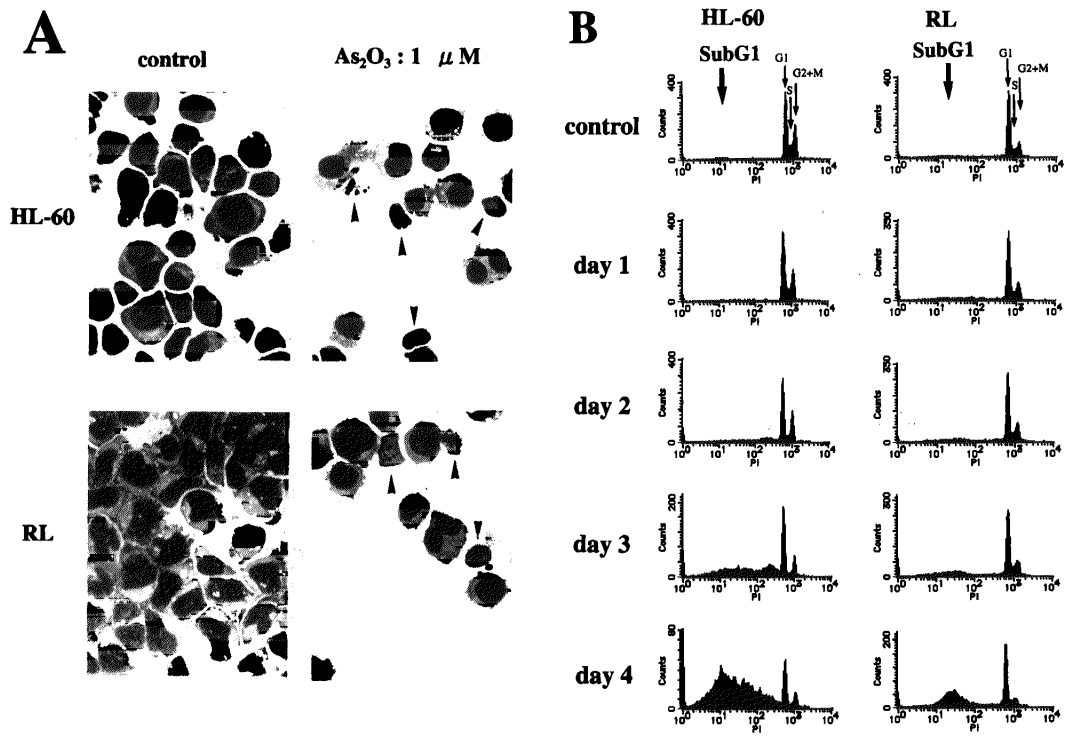

Fig. 2. Apoptotic changes of cell morphology and cell cycle by As_2O_3.
(A) Apoptotic changes showed after treatment with 1μM As_2O_3 for 3 days in HL-60 and RL by original magnification (×400). (B) Cell cycle arrested at subG1 after 1μM As_2O_3 treatment for 4 days in HL-60 and RL by FCM.

Table 1. Activation of caspases 8 and 3 by As_2O_3.

	Caspase 8		Caspase 3	
	Control	As_2O_3	Control	As_2O_3
HL-60	201.7± 58	386.5± 83.2 *	229.9± 31.8	391.8± 62.2 *
RL	165.3± 55	257.1± 75.1 *	112.5± 9.6	202.3± 50.9 *

After HL-60 and RL incubated with or without 1μM As_2O_3 for 2 days, caspases 8 and 3 were activated. The data are shown as mean±SD from three independent experiments. *($P<0.05$) were considered to represent a significant difference between treatment and control.

treatment for one day (Fig. 3).

4. Activation of caspase 8 and caspase 3

We studied whether As_2O_3 affects the downstream of CD95/CD95L, including caspase 8 and caspase 3. Fluorometric protease assay showed that the functions of caspase 8 and caspase 3 increased significantly in HL-60 and RL incubated with 1.0 μM As_2O_3 for 2 days (Table 1).

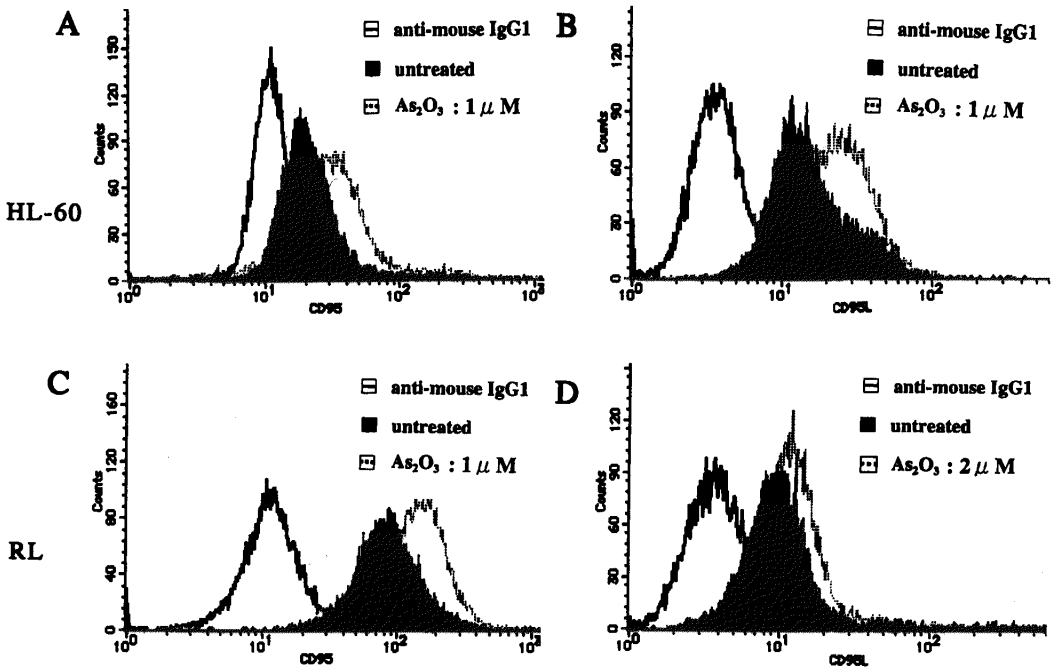

Fig. 3. Upregulation of CD95/CD95L expression by As_2O_3. (A, B) HL-60 cultured with or without 1μM As_2O_3 for 24 hours. (C, D) RL cultured with or without 1μM As_2O_3 in CD95 expression and 2μM As_2O_3 in CD95L expression for 24 hours.

5. Inhibition of As_2O_3-induced apoptosis by anti-CD95 MoAb

In order to confirm whether As_2O_3 induces apoptosis via CD95/CD95L pathway only, as shown in Fig. 4, CD95/95L pathway was blocked by anti-CD95 MoAb. As_2O_3-induced apoptosis decreased significantly in number in HL-60 and RL; however, anti-CD95 MoAb did not block As_2O_3-induced apoptosis completely.

6. Downregulation of bcl-2 expression

We studied whether bcl-2 protein regulates As_2O_3-induced apoptosis in HL-60 and RL. As shown in Fig. 5, both cell lines highly expressed bcl-2 protein originally. After treatment with 1.0 μM As_2O_3 for 2 days, bcl-2 was downregulated significantly.

DISCUSSION

Recently, As_2O_3 is a very effective agent in the treatment of patients with APL[22,23], but the molecular mechanism of its action has not been well understood. Many targets of As_2O_3 have been reported in different papers, for example, PML/RARα fusion protein[1], glutathione redox system[9] and bcl-2[1-3,8,10]. In the present study, we tested anticancer effects of As_2O_3 on other leukemia cell lines, HL-60 and RL at low concentrations of 0.25-2.0 μM, which was used to keep the same concentrations with in vivo plasma level[25]. Time- and dose-dependent inhibitions of cellular proliferation were similar to that in APL cell line, NB4[1]. In addition, the morphologic

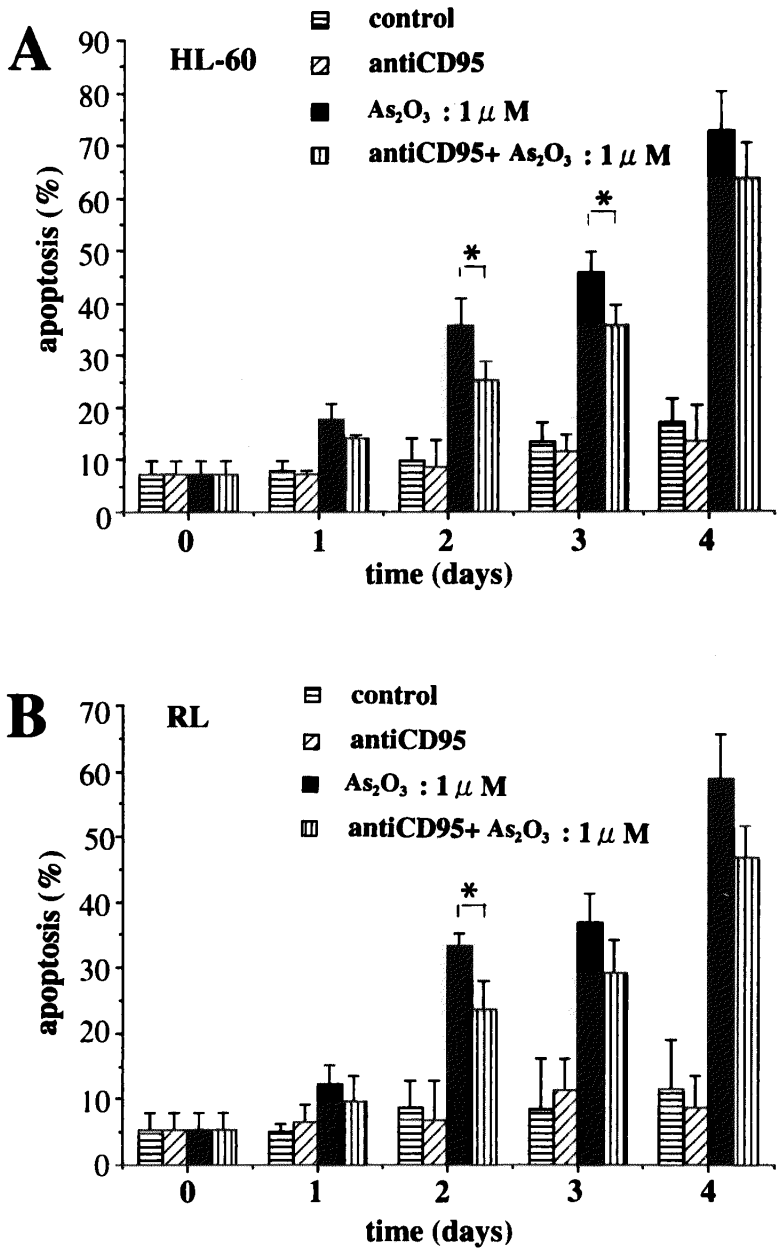

Fig. 4. Effect of 1μM As_2O_3 on blocking the CD95/CD95L pathway by anti-CD95 MoAb. Using PI staining, apoptotic cells of HL-60 and RL were determined for 4 days by FCM. The data are shown as mean ± SD from three independent experiments. *($P<0.05$) shows significant difference.

changes of apoptotic death, for example cellular shrinkage, chromatic condensation, apoptotic bodies and cell cycle arrest in subG1 were found. These findings suggest that As_2O_3 induces cell killing via apoptosis.

CD95 and CD95L activate a signal cascade via Fas-associated death domain (FADD) protein

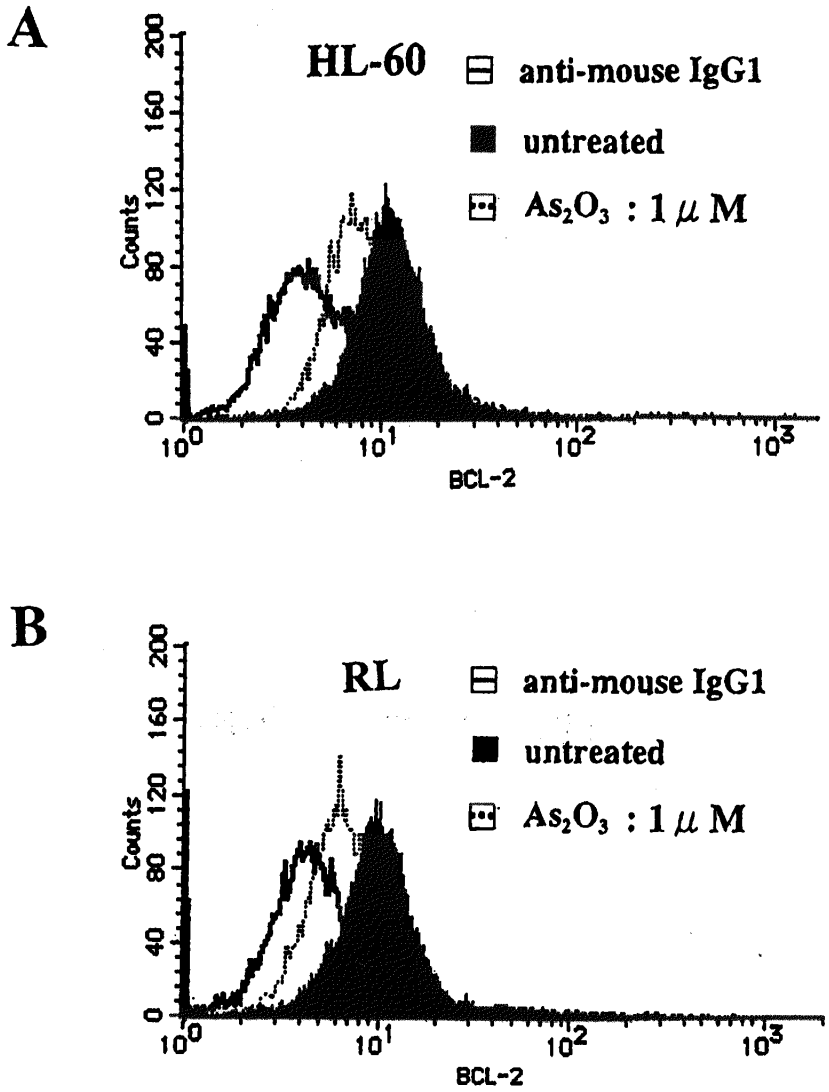

Fig. 5. Downregulation of bcl-2 protein expression by FCM. HL-60 and RL cultured with or without 1.0 μM As_2O_3 for 2 days.

to cleave procaspase 8 to caspase 8, then caspase 8 cleaves procaspase 3 to caspase 3 as final step of cell death[24-28]. Current evidence suggests that the mechanism of some anticancer agents is involved in the activation of the CD95/CD95L pathway which is a key signal pathway to regulate apoptosis[15-18]. We studied whether this pathway is involved in As_2O_3-induced apoptosis in HL-60 cells and RL cells. The CD95 and CD95L proteins originally expressed on two cell lines were upregulated by As_2O_3. These findings suggest that the CD95/CD95L pathway may be involved in the apoptosis of the cell lines induced by As_2O_3. To further support this hypothesis, we determined activities of caspase 8 and caspase 3, which are downstream of CD95/CD95L. The results showed that caspase 8 and caspase 3 were activated by As_2O_3. It is also reported that caspase 8 and casapse 3 play an important role in the process of

As_2O_3-induced apoptosis of leukemia cell lines[8,10]. These indicate that the CD95/CD95L pathway is involved in As_2O_3-induced apoptosis of HL-60 and RL. To further confirm whether cell killing by As_2O_3 was only through the CD95/CD95L pathway, we blocked the CD95 pathway by using anti-CD95 MoAb. As_2O_3-induced apoptosis was suppressed. From this result it was demonstrated that the CD95/CD95L pathway is an important molecular mechanism in As_2O_3-induced apoptosis in HL-60 and RL. However, anti-CD95 MoAb did not block As_2O_3-induced apoptosis completely. It indicates that As_2O_3-induced apoptosis is not only via the CD95/CD95L pathway but also via other pathways which remain to be determined.

We also studied the mitochondria pathway which has been reported as a CD95-independent death pathway and playing a central role in apoptotic action of As_2O_3[1-3,8,10]. It is well known that bcl-2 protein is an important factor of the mitochondria pathway. In the present study, bcl-2 expression in HL-60 and RL cells was downregulated by As_2O_3. This result suggests that downregulation of bcl-2 expression may be another important mechanism of As_2O_3-induced apoptosis in HL-60 and RL.

The molecular mechanism of As_2O_3-induced apoptosis is very complex. We conclude that As_2O_3 induces growth inhibition, cell cycle arrest and apoptosis via the CD95/CD95L pathway and downregulation of bcl-2 expression in HL-60 and RL cell lines. As_2O_3 might be useful in the treatment of leukemia and lymphoma other than APL.

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