Different Responses to Arsenic Trioxide between NB4 and UF-1, Acute Promyelocytic Leukemia Cell Lines

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Acute promyelocytic leukemia (APL) is a myeloid leukemia caused by over-expression of fusion protein, PML/RAR(α), which was the result of chromosomal translocation and induces the blockage of differentiation of affected promyelocytes. Pharmacological dose of retinoic acid induces the activation of and subsequent degradation of PML/RAR(α) fusion protein, and then APL cells undergo through the normal differentiation pathway. Arsenic trioxide has proved effective in causing remission of acute promyelocytic leukemia by inducing apoptosis of this tumor cells, whereas the heterogeneity of cellular susceptibility to this cytotoxic agent limited its usage on more types of tumors in clinic.
This work showed that arsenic trioxide could induce apoptosis of a panel of acute promyelocytic leukemic cell lines, all-trans-retinoic acid (ATRA) sensitive NB4 cells and ATRA resistant UF-1 cell. They were investigated with regard to the correlation between the inherent or intrinsic cellular level of GSH and the apoptotic susceptibility of the cells to arsenic trioxide. We manifested, in two cell types, the inherently existed difference in intracellular GSH level reactive to the arsenic trioxide, and a positive correlation between the GSH level and their apoptotic sensitivity to arsenic trioxide. And it showed that arsenic trioxide could differentiate promyelocytic cancer cells to the cells possessed of dendritic cell surface markers.
Unravelling the cause of the different susceptibility between leukemic cells and proving that promyelocyte could be differentiated to dendritic cells by arsenic trioxide will help not only to understand the mechanism underlying the complete remission of acute promyelocytic leukemia induced by arsenic trioxide, but also to expand its clinical usage.

Key words – Acute promyelocytic leukemia, dendritic cell surface markers, arsenic trioxide

Introduction

Acute promyelocytic leukemia (APL) is a myeloid leukemia causing the result of chromosomal translocation and the subsequent expression of a novel fusion protein, PML/RAR(α), which induces a block to differentiation in affected promyelocytes[9,11,13]. APL cell lines, NB4 and UF-1 cells undergo differentiation when exposed to all-trans-retinoic acid (ATRA). Pharmacologic dose of retinoic acid induces the activation and subsequent degradation of PML/RAR(α), allowing granulocytic differentiation to proceed[15]. Seventy to ninety percent of patients treated with retinoic acid (RA) in combination with chemotherapy achieve complete remission, but most patients relapse and develop RA-resistant leukemia.

Several differences are contrast NB4 from UF-1 cells. The NB4 cells were established from bone marrow cells of a patient with APL in relapse. The cells were initially cultured on a bone marrow stromal layer of cells. The opposite, UF-1 cells were established from a patient who was clinically resistant to ATRA: these cells were initially cultured without any supporting hematopoietic growth factors or stromal cells. The NB4 cells have a myeloblastic morphology without the typical APL granules, whereas the UF-1 cells are hypergranulocytic promyelocytes that are consistent with APL[1,12]. More recently, there were reports in China describing beneficial effects of an arsenic trioxide in APL clinical trial. Arsenic trioxide induces remission in most patients, including those who have developed resistance to RA. Its affects are mediated in part by inducing degradation of PML/RARα and consequent differentiation of APL cells[17].

The induction of apoptosis by arsenic trioxide has been linked to the accumulation of free radicals and subsequent induction of oxidative stress. In keeping with this observation, between levels of the intracellular antioxidant,

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reduced glutathione (GSH) and arsenic trioxide sensitivity has been reported[6,7,10]. Arsenic trioxide has also been shown to cause activation of the mitogen-activated protein kinase, c-jun N-terminal kinase (JNK), extracellular signal regulated kinase (ERK), p38. Activation of MAP kinase by arsenic trioxide was shown to contribute to cellular apoptosis[3,14]. Therefore, we carried out experiments using treatments with lower doses of arsenic trioxide to examine the effects of arsenic trioxide on MAP kinase signaling. Leukemic cells can differentiate to dendritic cell (DC) like cells in culture under the influence of cytokines known to be important in normal DC development. Functional DCs can be produced from blasts in patients affected by acute myeloid leukemia[5].

Promyelocytes express CD33, which is an antigen usually found on immature myeloid and leukemic cells, whereas CD11b is found on mature myeloid cell. Based on the evidence, we conducted an experimental study on APL cell lines NB4, UF-1 and found that arsenic trioxide induced the cell apoptosis. The expression of CD83 was evaluated by flow cytometry. CD83 was expressed in arsenic trioxide-treated NB4 leukemic cells. These results suggested that the differentiation of NB4 cell by arsenic trioxide causes the cells to express DC markers and showed the possibility that arsenic trioxide treated NB4 cells are able to present tumor antigens to T cells.

Materials and Methods

Reagents

Arsenic trioxide and BM-cycline were obtained from Sigma, (St. Louis. MO, USA). Antibodies to human CD11b/Mac1-PE, CD33-FITC were purchased from BD BioScience (Flanklin Lakes, NJ, USA) and Biolegend for FACS analysis.

MAP kinase antibodies from SantaCruz Biotechnology (Santa Cruz, CA, USA) for Western blot. Glutathione detection kit was acquired from CALBIOCHEM (San Diego, CA, USA). RPMI-1640 medium and fetal bovine serum (FBS) were obtained from GIBCO-BRL (Grand Island, NY, USA).

Treatment with arsenic trioxide and cell culture

NB4 APL cell line was maintained in RPMI-1640 medium supplemented with 10% FBS and 100 U/ml Penicillin Streptomycin in six well culture plate. Also, UF-1 APL cell line was cultured in RPMI-1640 medium containing with 15% FBS and 100 U/ml Penicillin Streptomycin in six well culture plate. All cells were grown in a humidified chamber at 37℃ with 5% CO_2 environment. For western blot, two cells in culture were treated with 4~16 μM of arsenic trioxide for 20 min - 24 h. For Glutathione detection assay and DNA fragmentation assay, cells in culture were treated with 20 μM arsenic trioxide for 1~48 h.

Assays for cellular proliferation

Cellular proliferation was measured by cell viability and nonradioactive cell proliferation assay system, MTT assay. MTT solution was dissolved in 1× PBS (5 mg/ml). Stock solution was stored at 4℃ wrapping with foil. This stock solution was applicable for 3 weeks. Working solution for MTT assay was 0.5 mg/ml.

Cells were collected by centrifugation at 1200 rpm for 5 min. Supernatant was removed and each pellet was resuspended carefully in 1ml of ice cold PBS. Cells (4×10^4) were treated with arsenic trioxide for 24 h - 72 h in 96 well plate. After incubation, cells were centrifuged at 1200rpm for 15min. One hundred microliter of supernatant was removed, One hundred microliter of MTT solution was added to each well. Cells were incubated at 37℃ for 4hours in dark. Cells were centrifuged at 1200 rpm for 15 minutes. Supernatant was removed carefully, 60 μl of the resulting supernatant were used for the assay, and then 100 μl of DMSO was added to each well and the absorbance at 550nm was measured.

Glutathione detection assay

Cells (5×10^6) were collected by centrifugation at 1500rpm for 5 min. Supernatant was removed, and each pellet was resuspended carefully in 1ml of ice cold PBS. Cells were contrifuged at 1500 rpm for 5min again, supernatant was removed, the pellets was resuspended with 500 μl of 5% metaphosphoric acid. Cells were homogenized by pipetting. The homogenates were transferred to 1.5 ml E-tubes, and centrifuged at 15,000 rpm for 10 minutes at 4℃. Twenty to three hundred microliter of resulting supernatant were applicated to the assay. Initial volume was 300 μl. Final volume was adjusted to buffer 3 (200 mM potassium phosphate, pH 7.8 [25℃], containing 0.2 mM diethylene triamine pentaacetic acid and 0.025% LUBROL). Fifty microliter of solution R1 was added and mixed thoroughly. Fifty microliter of solution R2 was added and mixed thoroughly.

The sample was incubated at 25℃ for 10 min in the dark. The final absorbance at 400nm was measured.

DNA fragmentation assay

Cells (1×10^7) were collected by centrifugation at 1500 rpm for 5 min. Supernatant was discarded, and each pellet was resuspend in 3 ml of ice cold PBS (0.1X), and transferred 1 ml to E-tubes. tubes were centrifuged at 1500 rpm for 3 min at 4℃. Supernatant was removed, and 500 ㎕ lysis buffer solution I was added to each tube, and pipetted roughly. Fifty hundred microliter of lysis buffer solution II was added to each tube, pipetted strongly and 50 ㎕ Proteinase K was added to each tube. All tubes were incubated at 48℃ water bath for over night.

Next day, 233 ㎕ of ice cold NaCl was added to each tube. Samples were incubated at 0℃ ice box for 20 min. And then, tubes were centrifuged at 18,000rpm for 20 min at 4℃. Seven hundred supernatant was obtained, same volume of isopropanol was added to each tube. Tubes were mixed carefully. After stored at -20℃ for 2 h or over night and tubes were centrifuged at 20,000 rpm for 20 min, supernatant was discarded, pellets were dried for 30 min. One hundred microliter of TE buffer supplemented with 2 ㎕ RNase(0.2 mg/ml) was added to the tubes, and incubated at 37℃ for 2 h. The final absorbance at 550 nm was measured. The samples were electrophoresed with 10% agarose gel at 50 V for 2 h.

Western blot analysis

Cells (5×10^6) were washed with cold PBS, then cell pellets were resuspended in 300 ㎕ lysis buffer {For 10ml lysis buffer; 1mM Sodium orthovanadate 50 ㎕, 10 ug/ml each aprotinin 10 ㎕, leupeptin 10 ㎕, and pepstatin A 100 ㎕, 1 mM PMSF(phenylmethylsulfonyl fluoride) 100 ㎕, 1mM Sodum Fluoride 50 ㎕, 10 mM Iodoacetamide 100 ㎕ , 1 M Tris-cl pH 7.5 500 ㎕, NaCl 0.09 g, 0.5 M EDTA 20 ㎕, Na-deoxycholate to 0.25%, 1% NP-40, Sodium molybdate 0.24 mg} at 4℃ for 30min. Extracts were then centrifuged at 14,000 캐m in a microfuge at 4℃, and supernatants were transferred to fresh tubes. Protein concentration was measured by BCA analysis at 400 nm. To detect JNK1, p38 and ERK1, SDS-PAGE was performed. Proteins were transferred to PVDF (Amersham) at Semi-dry transfer kit (Bio-rad). Membrane were blocked with 4% skim milk in TBST (NaCl, KCl, Tris-base, 0.05% Tween-20) for over night at 4℃. The membrane was hybridized for 4 h at room temperature with antibody against JNK1 (Santa Cruz) (1:2000 dilution). Following three times washing with TBST for 1h, blots were incubated with a goat antirabbit antibody for 2hr at RT. Bands were visualized by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) at LAS 3000.

Annexin-V analysis

Cells (2×10^6) were washed twice in cold PBS, the pellets were resuspended in cold binding buffer. And then, 10 ㎕ of annexin V-FITC was added to each tube. Each tubes were voltexed gently and incubated for 15 minutes on ice, protected from the light. Without washing, 380 ㎕ of cold binding buffer was added to each tube. 10 ㎕ of PI was added to each tube and control sample was prepared without antibody staining. Samples were analyzed by flow cytometry.

Detecion of CD83 by flow cytometric assay

Cells (2×10^6) were collected by centrifugation at 1500 rpm for 5 min and cells were washed twice, transferred to 1.5 ml tubes. After centrifuged at 1500 rpm for 5 min at 4°C, supernatant was discarded. One hundred microliter of cold PBS was added to each tube, and then 2 ㎕ of CD83-PE staining antibody was added to each tube. All tubes were incubated for 45min at ice. After centrifuged at 20,000 rpm for 5 min at 4°C, supernatant was discarded, and then cells were resuspended in 1 ml of cold PBS, washed again in 1 ml of cold PBS and resuspended in 1 ml of cold PBS, transfered to polypropylene FACS tube for flow cytometry analysis. Cells were kept on ice prior to analysis.

Results

Proliferation studies by arsenic trioxide

To assess the effects of arsenic trioxide, the MTT assay was used. NB4 and UF-1 cells were treated for 24 h to 72 h with various concentrations of arsenic trioxide (Fig. 1, 2 and 3). Arsenic trioxide inhibited the cellular proliferation of NB4 and UF-1 cells in a dose and time - dependent manner. Proliferation of NB4 cells was more extensively inhibited by arsenic trioxide than that of UF-1 cells.

Detection of glutathione on arsenic trioxide treated cell

Concentration of reduced glutathione of NB4 cells was

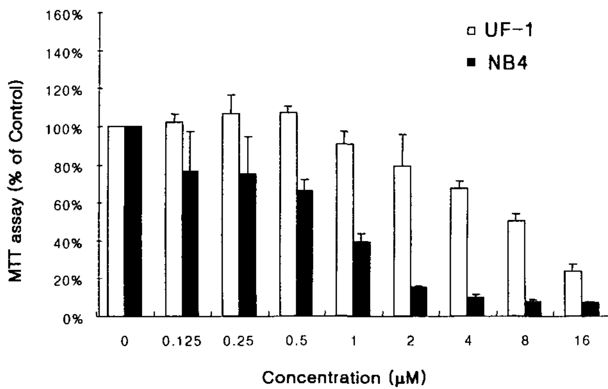

Fig. 1. Effects of arsenic trioxide on the cell proliferation of NB4 and UF-1 cells treated for 24h. in culture cells. Cells (4×10^6) were cultured in the presence of various concentrations of arsenic trioxide for 24 h, and MTT incorporation was measured. Each data (mean ± S.D.) point out of 5 individual experiments.

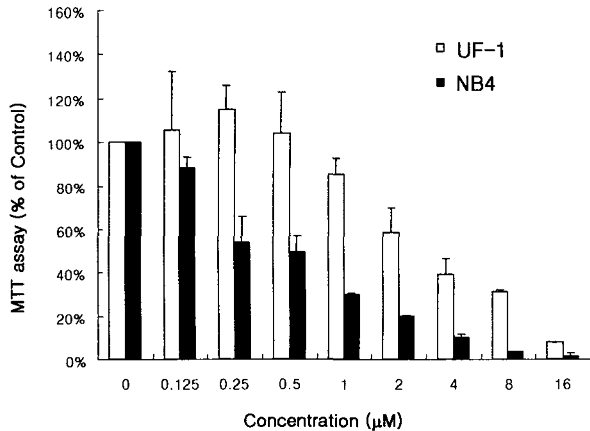

Fig. 2. ·Effects of arsenic trioxide on the cell proliferation of NB4 and UF-1 cells in culture cells treated for 48h. Cells (4×10^6) were cultured in the presence of various concentrations of arsenic trioxide for 48 h, and MTT incorporation was measured. Each data (mean ± S.D.) point out of 5 individual experiments

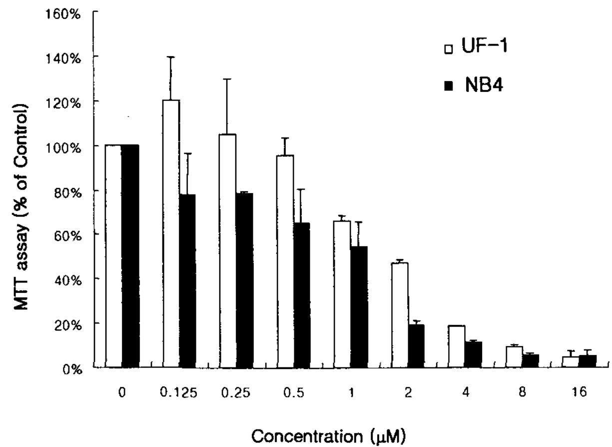

Fig. 3. Effects of arsenic trioxide on the cell proliferation of NB4, and UF-1 cells in culture cells treated for 72 h. Cells (4×10^6) were cultured in the presence of various concentrations of arsenic trioxide for 72 h, and MTT incorporation was measured. Each data (mean ± S.D.) point out of 5 individual experiments

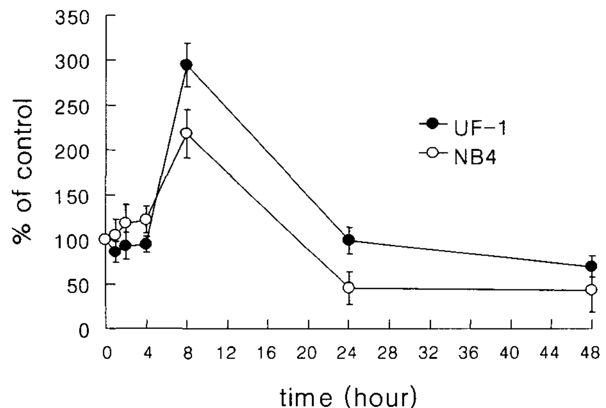

Fig. 4. Reduced glutathione level of APL cells lines (NB4 and UF-1) injured by arsenic trioxide. Cells in culture (1×10^7) were treated for 1-48 h with 20 μM of arsenic trioxide. Each data point represents the means of 3 individual experiments.

increased rapidly from 4 h to 8 h (Fig. 4). The peak concentration of reduced glutathione was detected at 8 h. Concentration of reduced glutathione of UF-1 cell is decreased initially at 1 h and 2 h. However at 4 h, like NB4 cell, concentration of reduced glutathione of UF-1 cells was increased rapidly from 4 h to 8 h. After 8 h, concentration of reduced glutathione of both cells were decreased gradually.

Elucidation of apoptosis on NB4 and UF-1 by DNA fragmentation assay

We have achieved DNA electrophoresis with extraction of chromosomal DNA of NB4 and UF-1. The ladder of NB4 was detected appeared from 8 h (Fig. 5). On the other hand, DNA fragmentation of UF-1 was shown from 24 h (Fig. 6). The difference between NB4 and UF-1 is very obvious. Although cell number of two cells lines was same as 1×10^7, DNA concentration of cell extract of NB4 cells was higher than that of UF-1 (data not shown).

ERK1 inhibition on arsenic trioxide treated cell

MAP kinase signaling pathways have been implicated in survival and death, Apoptosis, signaling in response to numerous stimuli in a variety of cell types. In our experiment,

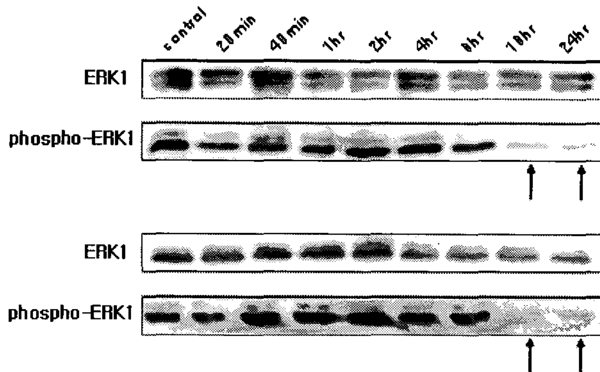

Fig. 5. Effect of arsenic trioxide on expression of ERK1 in NB4 cells was assessed by western blot. NB4 cells were treated with 4-16 μM arsenic trioxide for up to 24 h. Presence of phospho-ERK1 was detected with mouse monoclonal antibody and anti-mouse IgG-horseradish peroxidase/ECL detection. Arrows indicate the decreased expression of phospho-ERK1 from NB4 cells after 16 h treated with arsenic trioxide.

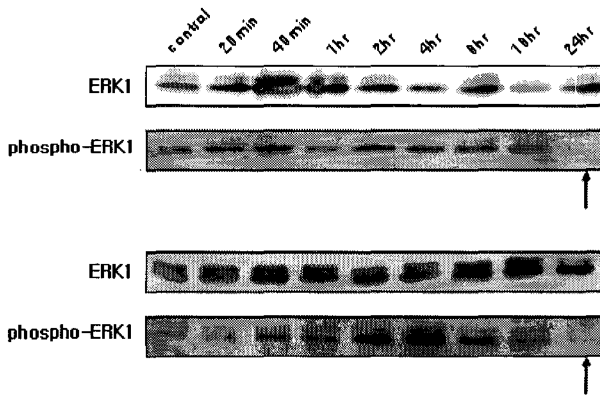

Fig. 6. Effect of arsenic trioxide on expression of ERK1 in UF-1 cells. UF-1 cells were treated with 4-16 μM arsenic trioxide for up to 24 h. Presence of phospho-ERK1 was detected with mouse monoclonal antibody and anti-mouse IgG-horseradish peroxidase/ECL detection. Arrows indicate the decreased expression of phospho-ERK1 from UF-1 cells after 24 h treated with arsenic trioxide.

we attempted to clarify the phosphorylation of ERK in arsenic trioxide treated cells. The result of western blot, ERK1 was phosphorylated at 16-24 h in NB4 cell.

Annexin V-FITC staining in NB4 cell treated with arsenic trioxide.

NB4 cells were cultures for up to 6 days with 4-16 μM arsenic trioxide for up to 6 days. NB4 cells were harvested after 1, 3, 6 days of treatment, and annexin V-FITC staining to detect live, apoptotic and necrotic NB4 cells were

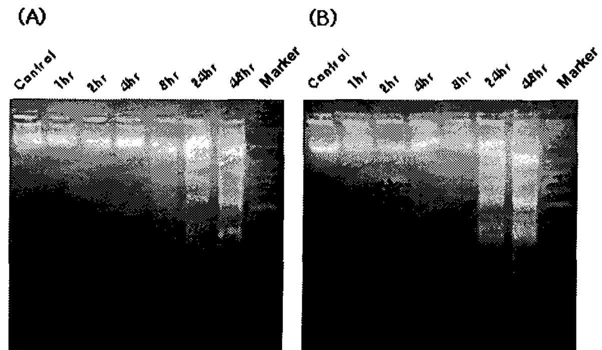

Fig. 7. Induction of apoptosis by arsenic trioxide in NB4 and UF-1 cells. DNA fragmentation assay to detect apoptosis were performed with extracts from NB4 and UF-1 cells treated with 20 μM arsenic trioxide for up to 48 h. (A) NB4 cell ; DNA fragmentation was detected after 8 h treated with arsenic trioxide. (B) UF-1 ; DNA fragmentation was detected after 24 h treated with arsenic trioxide.

performed by flow cytometry. As shown Fig. 8, the percentage of late apoptotic or necrotic cells prominently increased after 3 days treated with 16 μM arsenic trioxide. Until 3 days after treated with arsenic trioxide, the percentage of live cells showed no significant difference in all tested concentration.

Expression of DC markers on arsenic trioxide treated NB4 cells.

NB4 cells were treated with 4-16 μM arsenic trioxide for up to 6 days to induce differentiation. NB4 cells were harvested after 1, 3, 6 days of treatment, and the expression

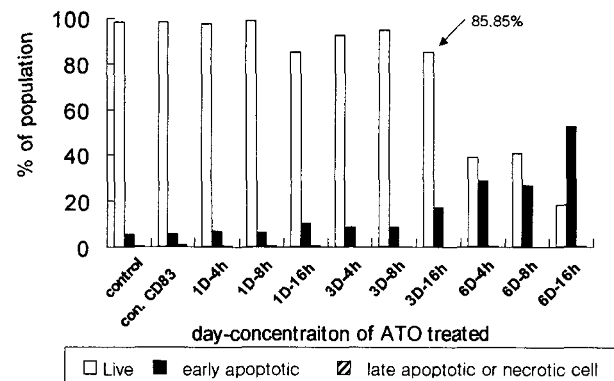

Fig. 8. Annexin V-FITC staining in NB4 cell treated with arsenic trioxide. NB4 cells were cultured with 4-16 μM arsenic trioxide for up to 6 days. Annexin V-FITC staining to detect live, apoptotic and necrotic NB4 cells were performed as shown in figure. This result is a representative of five independent experiments.

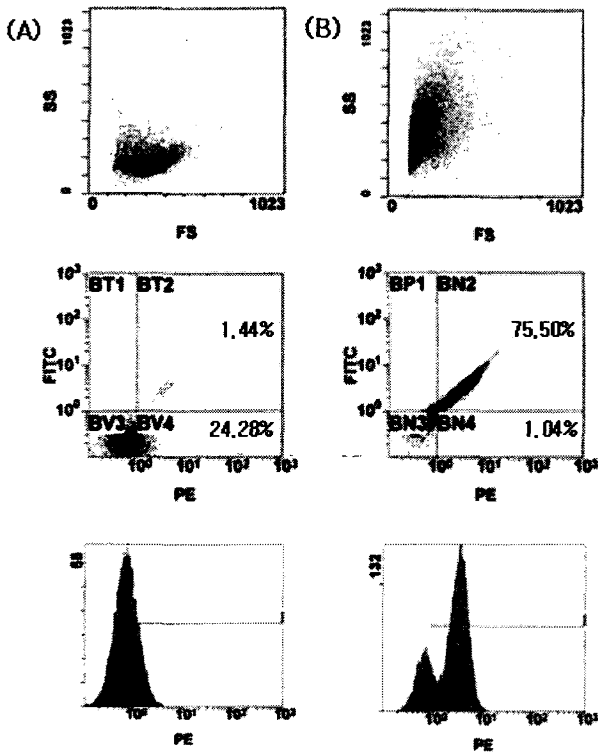

Fig. 9. Expression of CD83 in NB4 cell treated with arsenic trioxide. NB4 cells were cultures for 3 days treated with 16 μM arsenic trioxide. The cells were then harvested and the surface expression of CD83 were measured as shown. Panel A's are the result of flow cytometry analysis which were not treated with arsenic trioxide and those of panel B's were treated with arsenic trioxide. Upper two figure show that granularity within the NB4 cells increased when treated with arsenic trioxide. Middle and lower figures show that the percentage of $CD83^+$-expressing cells prominently increased after 3 days when treated with 16 μM arsenic trioxide. These results are representative of three independent experiments.

of DC marker, CD83, was determined by flow cytometry. As shown Fig. 9, the percentage of $CD83^+$-expressing cells prominently increased after 3 days when treated with 16 μM arsenic trioxide (78%). After 6 days treated with 4, 8, 16 μM of arsenic trioxide, the percentage of $CD83^+$ cells were 71%, 69%, 91%, respectively.

Discussion

Arsenic trioxide has shown substantial efficacy in treating both newly diagnosed and relapsed patients with acute promyelocytic leukemia (APL). As a single agent, it induces complete remissions, causing few side effects. These successes have prompted investigations to elucidate the mechanisms of action underlying these clinical responses. Arsenic trioxide induce degradation of the PML/RAR(α) chimeric fusion protein. The vast majority of cases of APL are characterized by the t(15;17) translocation. This translocation generates a fusion between the PML gene and the RAR(α) gene, which encodes a transcription factor[8]. The resulting PML/RAR(α) fusion protein blocks the expression of genes required for normal myeloid differentiation. Sequence analysis of the PML gene has indicated the presence of a cysteine-rich region that may be a principal candidate for interaction with trivalent arsenic. The endogenous PML protein in normal cells has been shown to be localized to a novel macromolecular structure in the nuclear body. Expression of the PML/RAR(α) fusion protein in leukemic cells disrupts the nuclear bodies, and the PML protein in dispersed into smaller fragments of these structure[1].

The block in myeloid differentiation by PML/RAR(α) can be released by treatment with retinoic acid (RA), providing the basis for all-trans retinoic acid (ATRA) therapy for APL. RA-induced differentiation of APL blasts results in degradation of the fusion protein and the relocation of wild-type PML from the deviant nuclear structures to its normal location in nuclear bodies[4]. In NB4 APL cells, the PML/RAR(α) fusion protein displays a micropunctate distribution in nuclei and cytoplasm, arsenic trioxide also causes degradation of the PML/RAR(α) fusion protein, although probably by a different mechanism than RA[4].

Arsenic trioxide has been proposed as an alternative to treatment with ATRA because it can induce complete remissions in both RA-sensitive and RA-resistance APL cells. The NB4 cells were established from bone marrow cells of a patient with APL in relapse. The cells were initially cultured on a bone marrow stromal layer of cells. The opposite, UF-1 cells were established from a patient who was clinically resistant to ATRA. Therefore, we have shown that differences between NB4 and UF-1 on arsenic trioxide. To determine the appropriate arsenic trioxide concentration for experiments, we have investigated cell viability by MTT assay. Treated with arsenic trioxide, cell proliferation of NB4 was decreased immediately. But cell proliferation of UF-1 cells was not inhibited initially at low concentration of arsenic trioxide below 0.5 μM. Inhibition of cell proliferation in UF-1 cells by arsenic trioxide was gradually increased time-dependent manner. Inhibition of cell proliferation in NB4 cells by arsenic trioxide over 4 μM concentration showed similar pattern compared with that of UF-1 cells when treated for 24 h to 72 h.

MAP kinase signaling pathways have been implicated in survival and death, Apoptosis, signaling in response to numerous stimuli in a variety of cell types. In our experiment, we attempted to clarify the phosphorylation of ERK in arsenic trioxide treated cells. The result of western blot, ERK1 was phosphorylated at 16-24h in NB4 cell. DNA fragment was detected at 8 h on arsenic trioxide treated NB4 cells, otherwise at 24 hr on UF-1 cells. After treated with 4-16 μM arsenic trioxide for up to 6 days, the expression of DC marker of NB4 cells, CD83, was determined by flow cytometry. A significant increase in the surface expression of CD83 of arsenic trioxide treated NB4 cells was observed at 3 days after treated with 16 μM arsenic trioxide (78%) without cytokines. After 6 days treated with 4, 8, 16 μM of arsenic trioxide, the percentage of $CD83^{+}$ cells were 71%, 69%, 91%, respectively. It has been suggested that CD83 is a specific surface marker of DC maturation. These finding shows that arsenic trioxide can differentiate promyelocytic leukemic cells to dendritic cells without cytokines such as GM-CSF. However, the mechanism by which arsenic trioxide up-regulates DC-maker during these differentiation processes remain to be identified.

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초록 : 급성 전골수성 백혈병 세포주간의 삼산화비소에 대한 반응

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급성 전골수성 백혈병은 염색체 전위의 결과로 생긴 PML/RAR(α) 융합 단백의 과발현으로 영향을 받은 전골수세포의 분화 정지로 발생하는 골수성 백혈병의 일종이다. 삼산화 비소는 세포고사를 유발하여 급성전골수성 백혈병의 관해를 유도한다는 것이 밝혀졌으나 이 약제에 대한 감수성이 다양하여 고형암에 적용하기에는 제한점이 있다. All-trans-retinoic acid (ATRA)에 감수성인 NB4 세포주와 내성인 UF-1 세포주 모두에 삼산화 비소가 세포고사를 유도하였다. 백혈병 세포주를 삼산화 비소로 처리하여, 세포내 GSH 농도가 낮아지고 세포고사의 감수성이 높아지는 상관관계를 찾았으며 전골수성 암세포를 수지상 세포 표면 표식자를 가진 세포로 분화시켰다. ATRA에 대한 감수성인 세포주와 내성인 세포주의 삼산화 비소에 대한 반응의 차이를 이해하고, 전골수 세포가 수지상 세포로 분화하는 과정을 규명한다면, 삼산화 비소에 의한 전골수성 백혈병의 완전관해의 기전을 밝힐 수 있고 또한 임상적용을 확대할 수 있을 것이다.