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Arsenic Trioxide Selectively Induces Apoptosis Within the Leukemic Cells of APL Patients with t (15;16) Translocation

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Abstract: Acute Promyelocytic Leukemia is a sub type of acute myelogenous leukemia that occurs in about 10-15% of patients with AML. Arsenic trioxide (ATO) as a single agent can induce remission in relapsed and newly diagnosed patients. Among all the arsenic putative functions responsible for these clinical responses, induction of differentiation and apoptosis with low and high doses are the most prominent mechanisms respectively. To evaluate the *in vivo* apoptotic pattern in leukemic cells of APL patients a single-laser, triple-color flowcytometric method was used, to detect leukemic apoptotic cells in a heterogeneous population of bone marrow samples with Annexin V and 7AAD. A substantial apoptosis which was selectively induced in Promyelocytic cells was detected during the early and middle stages of treatment.

Key words: Acute Promyelocytic Leukemia, arsenic trioxide, apoptosis, Fas/Apo1, flowcytometry

INTRODUCTION

Acute Promyelocytic Leukemia (APL), which comprises about 10-15% of cases of acute leukemia in adults involves a reciprocal chromosomal translocation associated with promyelocytic leukemia (PML) and retinoic acid receptor α (RAR α) genes on chromosomes 15 and 17, respectively (Warrell *et al*., 1993; Grignani *et al*., 1993). Approximately 20-30% of these patients, who are treated with the current standard ATRA and anthracyclins-based chemotherapy regimen, may suffer a severe clinical presentation known as "retinoic acid syndrome" and/or relapse in a few years (Fenaux *et al*., 2000).

Arsenic trioxide (ATO) as a single agent can induce remission in newly diagnosed and relapsed patients with little adverse effects (Antman, 2001; Kitamura *et al*., 1997; Guo-Qiang *et al*., 1997).

Reports of the safety and efficacy of ATO in clinical trials from different centers around the world prompted investigators to elucidate the mechanism of action underlying these clinical responses. Arsenic trioxide apparently affects numerous intracellular signal transduction pathways and causes many alterations in cellular functions among which the most prominant mechanisms are induction of differentiation and apoptosis with low and high does of ATO respectively (Chen *et al*., 1996; Zhen-Yi and Chen 2000; Miller, 2002; Miller *et al*., 2002).

Numerous studies have indicated that Arsenic induces apoptosis in different cell types in vitro but there are some other studies that attribute it at least in part to a cell culture artifact (Zhu *et al*., 2001).

Another challenging issue is the selective action of this drug. Some researchers claim that while sparing normal cells, Arsenic induce apoptosis only within the leukemic cells. In this study in order to elucidate the two mentioned issues we examined the apoptosis induced within the Leukemic cells of APL patients during Arsenic therapy by using a tricolor flow cytometric method in the promyelocytic population.

MATERIALS AND METHODS

Patient's samples: Three male patients diagnosed with de novo APL, based on morphological characteristics of the French-American-British classification and confirmed with cytogenetic analysis by detection of PML/RARá chimeric gene using reverse transcription-polymerase chain reaction (data not shown), were informed consent before receiving 10 mg/day ATO solution (0.1% prepared by Dr.Hossaini, faculty of Pharmacy, Tehran University of

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Medical Sciences), as a single agent for treatment. Bone Marrow specimens (BM) which were treated with EDTA, prepared in three time intervals up to 30 days during therapy and stained with Wright-Giemsa for morphological study. Mononuclear cells (MNC's) were obtained from the Bone Marrow specimen by density gradient centrifugation using Ficoll-Paque (density:1.077) (Pharmacia fine chemicals) and used for the analysis.

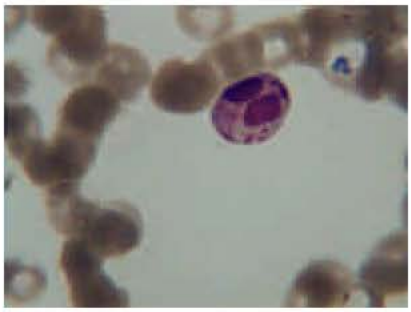
Apoptosis assay: Cell morphology was examined by Wright Giemsa staining. Apoptotic cells that show nuclear shrinkage, blebs and apoptotic bodies were clearly identified in both B.M samples and Ficoll separated MNC's.

To evaluate the apoptosis pattern in the malignant population, we used a similar method as described earlier by Herault *et al*. (1999). Briefly, MNC Ficoll separated cells were stained with fluoroscein isothiocyanate (FITC)-conjugated antibody and its relative isotype negative control: CD33-FITC/FITC IgG1 Isotype control (DAKO). The supernatant was removed after two washes with PBS by 5 min centrifugation at 350 g. and the cell pellets were resuspended in Ca^{2+} binding buffer and stained with phycoerythrin (PE) conjugated Annexin V (IQ product) according to recommendation of manufacturer and incubated for 20 min on ice and in the dark. Finally, for nuclei counter staining, cells were resuspended in 1 μg mL^{-1} 7-AAD (ICN) solution for 10 min and immediately analysed by FACScalibur™ (Beckton Dickenson). Multivariate data was analyzed with CELLquest™ software (Beckton Dickenson). For discrimination of early from late apoptosis, leukemic apoptotic cells (dual pos population of FL1 vs FL2 histogram) were gated out and displayed in FL2 vs. FL3 histogram.

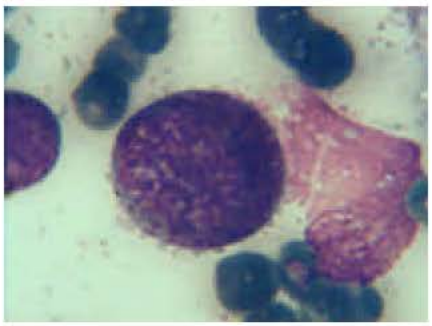
RESULTS

Morphological evaluation *in vivo* during ATO therapy: During ATO therapy, blasts and promyelocytes gradually decreased, while cells with morphologic maturation of Myelocyte, Metamyelocyte and Band cells increased. On glass slides, a very small fraction of BM cells both from whole WBC and from MNC Ficoll separated cells, showed morphological apoptosis in all patients (Fig. 1).

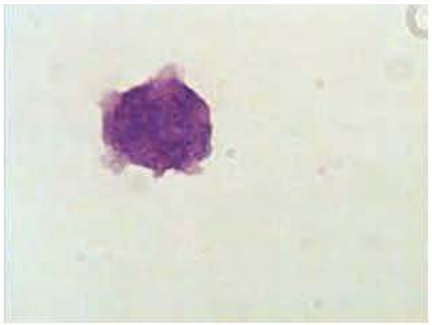
Evaluation of apoptosis in the context of promyelocytic cells: For detection of leukemic cells undergoing apoptosis, cells that stained with both CD33-FITC and Annexin V-PE were gated out and displayed in Annexin V-PE vs. 7-AAD histogram to discriminate early from late stages of apoptosis. Since apoptosis was studied *in vivo* and no necrosis normally occurs in the body, even in the case of chemotherapy and duration of apoptosis is



(a)



(b)



(c)

Fig. 1: Cells exhibiting morphological apoptosis in (a) Peripheral Blood (b) Bone Marrow and (c) Ficoll separated MNC's

generally too short, even shorter than duration of mitosis, the Annexin V^+ populations with lower 7-AAD RFI ($Annexin^+7\text{-}AAD^{low}$) and with higher 7-AAD RFI ($Annexin^+7\text{-}AAD^{Hi}$) was considered as early and late apoptosis respectively.

Considering both early and late apoptosis, the present study detected a considerable apoptosis induced selectively in leukemic cells during early and middle stages of treatment (Fig. 2).

The selective induction of apoptosis was shown by gating back the $CD33^+$-Annexin V^+ population in

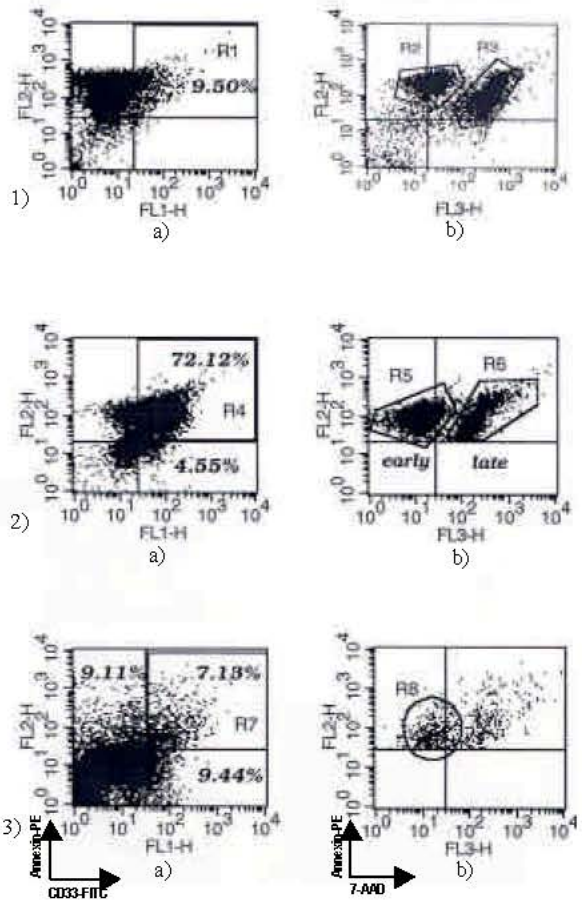


Fig. 2: Leukemic cells undergoing apoptosis are gated out and displayed in Anexin vs. 7-AAD cytogram for discrimination of apoptosis from necrosis in (a) early (b) middle and (c) late stages of treatment

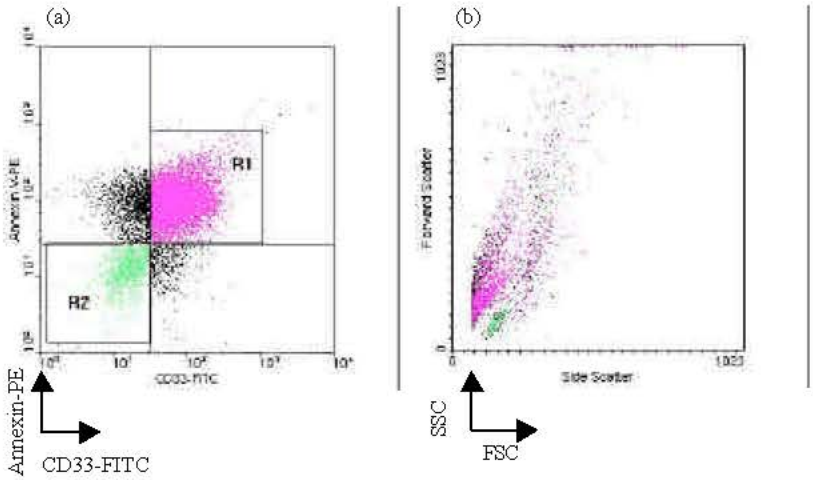


Fig. 3: Gating back the leukemic apoptotic cells (Red populations-R1) and non-leukemic non-apoptotic cells (Green population–R2) (a) in its cytogram (b) we show the selective apoptosis which is induced only within the blastic gate

Cytogram of the same sample. The apoptosis induced within the leukemic and not the lymphoid gate; clearly showed the selective effect of Arsenic (Fig. 3).

DISCUSSION

In vivo studies of apoptosis are a challenging issue especially in human cell biology research. Due to the *in vivo* dynamic changes, some scientists believe that the in vitro outcomes are not entirely extendable to their similar *in vivo* studies. Despite substantial evidence confirming the apoptosis inducing function of ATO, some still believe this to be a cell culture artifact. Some methods are used for apoptosis detection *in vivo*, but a few of them are applicable to human research. Observation of apoptotic cells in both B.M and P.B samples and their detection by Annexin V and 7-AAD as a sensitive method indicate the important role of Arsenic trioxide in *in vivo* apoptosis induction and confirms the earlier studies.

Arsenic trioxide was shown to exert its function selectively and only in the malignant promyelocytes (Yongkui *et al*., 1994). Promyelocytes have low levels of GPX (Glutathione Peroxides) and Catalase, which are responsible for the cellular antioxidant effects and therefore, they are more susceptible to free oxygen species compared to normal cells. In addition, it seems that arsenic trioxide is more effective on cells with a high rate of cell division, which is likely to account for its selective function. (Yongkui *et al*., 1994). The results of the present study show a substantial apoptosis in the early and middle stages of treatment in which promyelocytes increase and this is consistent with the earlier results.

This study shows that arsenic not only induces apoptosis *in vivo* but also exerts its function selectively within the leukemic cells of APL patients.

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