Arsenic Trioxide Selectively Induces Apoptosis within the Leukemic Cells of APL Patients with t(15;17) Translocation Possibly through the Fas Pathway

Ardjmand. A.R.[1], Alimoghaddam. K.[2], Zaker F.[3], Ghavamzadeh A.[2], Jahani.M.[2]

[1]*Correspondance: Hematology, Oncology & BMT research center, Shariati Hospital, Kargar Ave. 14114, Tehran, Iran; Email: arard-jmand@yahoo.com*
[2]*Hematology Oncology & B.M.T Research Center, Tehran University of Medical Science.*
[3]*Cellular & Molecular research center, Iran University of medical science*

Abstract
Background: Acute Promyelocytic Leukemia is a sub-type of acute myelogenous leukemia that occurs in about 10-15% of patients with AML. Approximately 20%-30% of these patients, who are treated with the current standard All Trans Retinoic Acid (ATRA) and Anthracyclin-based chemotherapy regimen, suffer relapse in less than a year. Arsenic trioxide (ATO) as a single agent can induce complete remission even in refractory and relapsed patients with few adverse effects. The investigators efforts regarding elucidation of the mechanisms of action underlying these clinical responses has shown that Arsenic apparently affects numerous intracellular signal transduction pathways and causes many alterations in cellular function, among which the most prominent ones are the induction of differentiation & apoptosis with low & high doses of arsenic, respectively.
Purposes: *In vivo* apoptosis on these patients has not been evaluated yet and despite previous *In vitro* studies, which mostly reveal Fas/Apo1 is not expressed during ATO treatment, its *in vivo* expression has not been evaluated yet.
Materials & methodes: In order to study the apoptotic pattern in leukemic cells of APL patients, we conducted a single-laser, triple-color flowcytometric experiment, to detect leukemic apoptotic cells in a heterogeneous population of bone marrow samples with the Annexin V & 7AAD technique. The Fas expression was also evaluated in promyelocyte population cells in a dual color panel.
Results & Conclusion: A substantial Apoptosis was selectively detected in Promyelocytic cells during the early and middle stages of treatment and the concurrent Fas expression indicates its involvent in Apoptosis induced by Arsenic Trioxide.

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, is characterized by prominent malignant promyelocytes in the bone marrow. The pathogenesis of APL involves a reciprocal chromosomal translocation associated with promyelocytic leukemia (PML) and a retinoic acid receptor α (RAR α) genes on chromosomes 15 and 17, respetively.[1, 2] 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.[3]

Arsenic trioxide (ATO) as a single agent can induce complete remission, even in refractory and relapsed patients with little adverse effects.[4, 5, 6]

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 the induction of differentiation and apoptosis with low and high doses of ATO, respectively.[7, 8, 9, 10]

Despite thorough investigation currently being done on different aspects of arsenic, the mechanisms under which ATO exerts its effect has not been elucidated yet. Mitochondrial $\Delta\psi_m$ collapse following arsenic treatment,[11, 12] which could be due to the activation of Caspase 8,[13, 14] is suggestive of the involvement of its upstream death receptor Fas/Apo1 (CD95). Participation of this classical death receptor is still controversial and, in contrast to some evidence against its involvement, the apoptosis induced via this system has recently been reported.[15, 16]

Here to study the apoptosis induced within the leukemic cells of APL patients during arsenic therapy, we conducted a tricolor flow cytometric experiment by using the Annexin V and 7-AAD technique and a dual color panel for the evaluation of the *Fas* expression in the promyelocytic population.

Patients, Materials, and Methods

Three male patients, 34, 40 and 55 years of age, were diagnosed with de novo APL, based on morphological characteristics of the French-American-British classification, and confirmed with cytogenetic analysis and detection of PML/RARα chimeric genes, using a reverse transcription-polymerase chain reaction (data not shown).

Informed consent was obtained from all patients before receiving 10 mg/day AS2O3 solution, 0.1% (prepared by Dr.Hossaini, faculty of Pharmacy, Tehran University of Medical Sciences), as a single agent for remission induction in a standard treatment protocol. Bone marrow specimens (BM) were treated with EDTA and prepared at three 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 described below.

Apoptosis assay

Cell morphology was examined by Wright Giemsa staining. Apoptotic cells that showed nuclear shrinkage, blebs and apoptotic bodies were clearly identified in both BM samples and Ficoll separated MNC's.

To evaluate the apoptosis pattern in the malignant population, we used a similar method, as previously described.[17] Briefly, MNC Ficoll separated cells were stained with fluresein isothiocyanate (FITC)-conjugate 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 350g, and the cell pellet was resuspended in Ca^{2+} binding buffer (HEPES 2.6gr, NaCL 8.18gr, $CaCL_2$ 0.28gr, DW 50ml, PH= 7.3-7.4). Then, a phycoerythrine conjugated Annexin V (IQ product) was added according to the recommendation of the manufacturer and incubated for 20 min on ice in the dark. Finally, for nuclei counterstaining, cells were resuspeded in 1µg/ml 7-AAD (ICN) solution for 10 min and immediately run on FACScalibur™ flowcytometer (Beckton Dickenson). Multivariate data was analyzed with CELLquest™ software (Beckton Dickenson). For the discrimination of early from late apoptosis, leukemic apoptotic cells (dual population of FL1 vs FL2 histogram) were gated out and displayed in a FL2 vs. FL3 histogram.

The immunophenotypic study of CD95/Fas

Fas (CD95) expression within the leukemic cells of the heterogeneous population of BM samples were detected by direct immunofluorecence dual-color staining via incubating cells, simultaneously with the use of flureseein isothiocyanate (FITC)-conjugated and phycoerythrine (PE)-conjugated monoclonal antibodies: CD33/CD95 (DAKO). Negative controls, as irrelevant monoclonal immunoglobulin of the same isotype, were analyzed concurrently.

Results

Morphological evaluation *in vivo* during ATO therapy:

During ATO therapy, blasts and promyelocytes gradually decreased, while cells with a morphologic maturation of Myelocyte, Metamyelocyte and Band cells increased. During the preparation 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).

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

Evaluation of apoptosis in the context of promyelocytic cells.

For the detection of Leukemic cells undergoing apoptosis, cells that had been stained both with CD33-FITC and Annexin V-PE were gated out and displayed in Annexin V-PE vs. 7-AAD histogram to discriminate between the early and late stages of apoptosis. Since the apoptosis was studied *In vivo* and no necrosis normally occurs in the body, even in the case of chemotherapy, and the duration of apoptosis is generally very short, (even shorter than the duration of mitosis), the Annexin V^+ populations with lower 7-AAD RFI (Annexin$^+$7-AAD^{low}) and higher 7-AAD RFI (Annexin$^+$7-AAD^{Hi}) were considered early and late apoptosis, respectively.

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

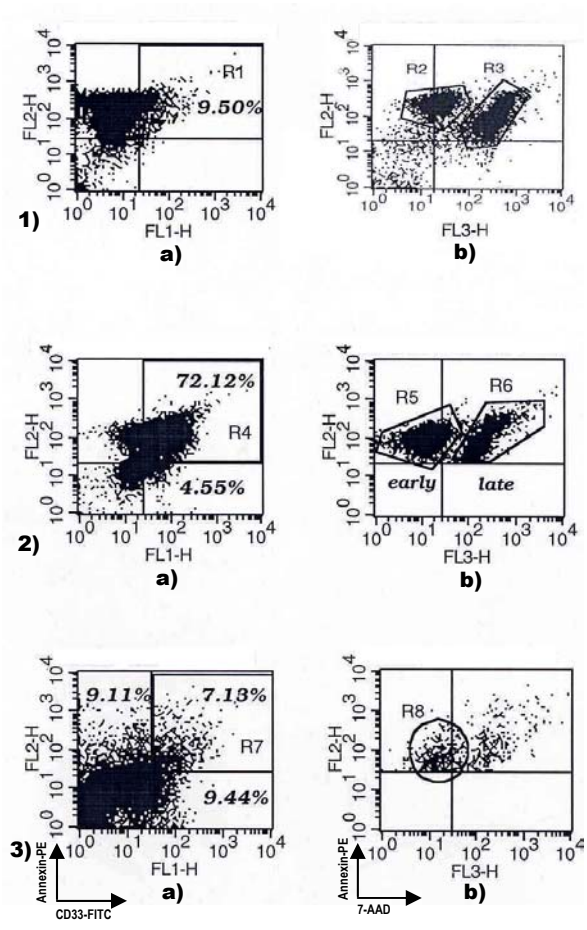

Fig. 2. Lukemic cells undergoing apoptosis are gated out (a) and displayed in Anexin vs. 7-AAD cytogram (b) for discrimination of apoptosis from necrosis in early (1), middle (2) and late (3) stages of treatment.

The selective induction of apoptosis was shown by getting back the $CD33^+$-Annexin V^+ population in the cytogram of the same sample. The apoptosis induced within the leukemic and not the lymphoid gate clearly show the selective effect of arsenic. (Fig.3)

Involvement of *Fas*/CD95 in apoptosis induction.

From the three, two of the patients showed a correlation between the direct positive the Pro

Fig. 3. Gating back the leukemic apoptotic cells (Gray populations-R1) and non-leukemic non-apoptotic cells (Bright Gray -R2) (a) and displaying them back in its cytogram (b), we show the selective apoptosis which is induced only within the blastic cells.

myelocytic cells expressing *Fas*, and the apoptosis rate of the leukemic cells. (Fig.4)

Discussion

Numerous studies have indicated that arsenic induces apoptosis in different cell types *in vitro,* but there are some other studies attributing this, at least in part, to a cell culture artifact.[18] Nevertheless, although some methods have been developed for the detection of apoptosis *in vivo,* none of them are applicable to humans.[19-20]

In this study, we demonstrated that arsenic induces apoptosis selectively within the leukemic

cells of APL patients, mostly during the early phase of treatment and that the Fas/Apo1 pathway is at least partially involved.

The observation of apoptotic cells in BM and PB samples and its detection by Annexin V and 7-AAD as a sensitive method, indicates the finite role of arsenic in apoptosis. The substantial apoptosis rate in the early stages of treatment within the leukemic cells, especially when they are increased in number, appropriately confirmed the previous results.[21] The Promye locyte GPX (Glutathione Peroxides) and low levels of catalase, which results in the reduction of reduced glutathione (GSH) levels,[10] and the high rate of mitosis in these cells as a prerequisite for arsenic putative cell cycle specific arrest,[22] are highly suggestive of its selectivity.

Fig. 4. Evaluation of apoptosis and expression of *Fas* in Promyelocyte of three APL patients during the first and second weeks of treatment. While the expression of *Fas* was not correlated with apoptosis rate in patient 1, *this death receptor* down regulated concurrently with decrement of apoptosis in the other 2 cases.

The CD95/CD95L pathway has been described as a key signal pathway to regulate cell death. CD95 and CD95L activates a signal cascade via the *Fas*-associated death domain (FADD) protein to cleave procaspase 8 to caspase 8. Then caspase 8 cleaves procaspase 3 to caspase 3 as the final step of cell death.[23, 24]

Although activation of caspase 8 following ATO treatment proposes the involvement of its upstream death receptor *Fas*, caspase 8 signals are not necessarily evoked by *Fas,* and there are some other death receptors associated ligand or stimuli-like TRAILs and anticancer drugs or radiation which can activate caspase 8. The diverse, clinical results accompanied with the different patterns of the *Fas* expression within the leukemic cells of APL patients indicates the *Fas* pathway involvement, but the mRNA expression of this receptor and functional assessment of its downstream molecule FADD, as well as the involvement of other related genes through the Micro-array technique, is highly recommended for confirmation.

In this study, we showed that arsenic selectively induces apoptosis within the leukemic cells of APL patients, but due to the diverse physiopathologic, pharmacokinetic and pharmacogenetic status of individuals, and possibly the heterogeneous nature of the disease, the involvement of *Fas* was depicted differently and, thus, must be further studied.

Acknowledgments

A grant from the Tehran University of Medical Sciences (Tehran-Iran) supported this work and we thank Miss Hayat for flow cytometry technical support.

References

1- Warrell RP Jr, The H, Wang Z-Y, et al. Acute promyelocytic leukemia. N Engl J Med. 1993;329:177-189

2- Grignani F, Ferrucci PF, Testa U, et al. The Acute promyelocytic leukemia-specific PML-RARα fusion protein inhibits differentiation and promotes survival of myeloid precursor cells. Cell 1993;74:423

3- Fenaux P, Chevert S, Gueric A et al. Long term followup confirms the benefit of all-trans retinoic acid in Acute promyelocytic leukemia. Leukemia 2000;14:1371-1377

4- Antman KH. The history of arsenic cancer therapy. Oncologist. 2001;6:1-2

5- Kitamura K, Yoshida H, Ohno R, Naoe T. Toxic effects of arsenic (As3+) and other metal ions on acute promyelocytic leukemia cells. Int J Hematol.1997;65(2): 179-185

6- Guo-Qiang Chen, Xue-Geng Shi, Wei Tang, et al. Use of Arsenic Trioxide (As2O3) in the Treatment of Acute Promyelocytic Leukemia (APL): I. As2O3 Exerts Dose-Dependent Dual Effects on APL Cells. Blood 1997;89:3345-3353

7- Chen GQ, Zhu J, Shi XG, et al. In vitro studies on cellular and molecular mechanisms of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia: As2O3 induces NB4 cell apoptosis with downregulation of Bcl-2 expression and modulation of PML-RAR alpha/PML proteins. Blood 1996;88(3):1052-1061

8- Zhen-Yi Wang and Zhu Chen, Differentiation and Apoptosis induction therapy in acute promyelocytic leukemia. The lancet oncology 2000;1:101-106

9- Miller WH Jr. Molecular targets of arsenic trioxide in malignant cells. Oncologist 2002;7,1:1-13

10- Miller WH, Schipper HM, Lee JS, et al. Mechanisms of action of arsenic trioxide. Cancer Res 2002;62(14):3893-3903
11- X Cai, Y-L Shen, Q Zhu, et al. Arsenic trioxide-induced apoptosis and differentiation are associated respectively with mitochondrial transmembrane potential collapse and retinoic acid signaling pathways in acute promyelocytic leukemia. Leukemia 2000;14:262-270
12- S-H Woo, I-C Park, M-J Park, et al.Arsenic trioxide induces apoptosis through a reactive oxygen species-dependent pathway and loss of mitochondrial membrane potential in Hela cells. Int J Oncol 2002;21:57-63
13- Li H, Zhu H, Xu Cl, et al. Cleavage of Bid by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 1998;94:491-501
14- Luo X, Budihardjo I, Zou H, et al. Bid, a Bcl2 interacting protein, mediates cytochrom C release from mitochondria in response to activation of cell surface death receptors. Cell 1998;94:481-490
15- Kitamura K, Minami Y, Yamamoto K, et al. Involvement of CD95-independent caspase 8 activation in arsenic trioxide-induced apoptosis. Leukemia 2000;14(10):1743-1750
16- Zhu J,Okumura H, Ohtake S, et al. Arsenic trioxide induces apoptosis in leukemia/lymphoma cell lines via the CD95/CD95L system. Oncol Rep 2003;10(3):705-709
17- Herault O, Colombat P, Domenech J, et al. A rapid single-laser flow cytometric method for discrimination of early apoptotic cells in a heterogenous cell population. Br J Haematol 1999;104(3):530-507
18- Zhu J, Lallemand-Breitenbach V, de The H. Pathways of retinoic acid or arsenic trioxide-induced PML/RARalpha catabolism, role of oncogene degradation in disease remission. Oncogene 2001;20(49):7257-7265
19- Van Engeland M, Nieland LJ, Ramaekers FC, et al Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry 1998;31(1):1-9.
20- Blankenberg F.G, Katsikis P, Tait J.F, et al. In vivo detection and imaging of phsphatidylserine expression during programmed cell death. Proc. Nact. Acad. Sci. USA.1998;95:6349-6354
21- Yongkui Jing, Jie Dai, Ruth M.E. Chalmers-Redman, Willam G.et al. Arsenic Trioxide Selectively Induces Acute Promyelocytic Leukemia Cell Apoptosis Via a Hydrogen Peroxide-Dependent Pathway. Blood;199994:2102-2111
22- Halicka HD, Smolewski P, Darzynkiewicz Z, et al. Arsenic trioxide arrests cells early in mitosis leading to apoptosis. Cell Cycle. 2002;1(3):201-209
23- .Ashkenazi A, Dixit VM. Death receptor: Signaling nd modulation. Sciences 1998;281:1305-1308
24- Muzio M, Chinnaiyan AM, Kischkel FC, et al. FLIC, a novel FADD-homologous ICE-CED-3-like protease, is recruited to the CD95 (Fas/Apo1) death-inducing signaling complex. Cell 1996;85:817-827