

Arsenic trioxide induce apoptosis independent of TNFR-I and CD30 pathways in Acute promyelocytic leukemia patient with t(15;17) translocation.

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Abstract

Arsenic trioxide (ATO) has been reported to induce apoptosis in Leukemic cells of Acute Promyelocytic Leukemia (APL) patients through different pathways. However, the exact mechanism of ATO-induced apoptosis is not yet clear. Co stimulation of death receptors CD30 and tumor necrosis factor receptor type one (TNFR-I) is one of the postulated mechanisms. In the present study we aimed to evaluate their involvement in fresh Promyelocytic cells separated from bone marrow of APL patients. Immunomagnetic separated cells were treated up to 48 hr at clinically tolerable concentrations of ATO (0.5-2.0 $\mu\text{mol/l}$) and expression of TNFR-I and CD30 were evaluated within the apoptotic and live populations using a sensitive triple color flow cytometric method for measuring apoptosis in combination with dual color immunofluorescence.

Our results suggest that the expression of TNFR-I and CD30 might not be related to ATO-induced apoptotic cell death.

Key words: Acute Promyelocytic Leukemia, Arsenic trioxide, apoptosis, CD30, TNFR-I

Introduction:

Acute Promyelocytic Leukemia (APL), which comprises about 10%-15% of cases of acute leukemia in adults, is characterized with prominent malignant Promyelocytes in bone marrow. The pathogenesis of APL involves a reciprocal chromosomal translocation associated with promyelocytic leukemia (PML) and retinoic acid receptor α (RAR α) genes on chromosomes 15 and 17 respectively.¹⁻²

Arsenic trioxide (ATO) as an effective treatment with little adverse effects³⁻⁴ apparently affecting numerous intracellular signal transduction pathways and causing many alterations in cellular functions among which the most prominent mechanisms are induction of differentiation and apoptosis with low and high doses of ATO respectively.⁵⁻⁶

Induction of apoptosis occurs through multiple pathways. Some principal signals pathways of apoptosis have been identified. One pathway involves activation of death receptors such as CD95, tumor necrosis factor (TNF) receptor and TNF-related apoptosis-inducing ligand (TRAIL), followed by recruitment of the Fas-associated protein with death domain and activation of caspase 8. The other pathway is triggered by disruption of the mitochondrial membrane by many insults and results in the loss of mitochondrial transmembrane potential ($\Delta\psi_m$)

and release of mitochondrial proteins such as cytochrome c and apoptosis-inducing factor into cytosol followed by activation of caspase 9. Caspase 8 and 9 activated by either pathway transduce signals which activate caspase 3 and subsequently cleave poly (ADP-ribose) polymerase (PARP).

Since activation of Caspase 8, which induces mitochondrial $\Delta\psi_m$ collapses⁷⁻⁸ following arsenic treatment⁹⁻¹⁰ it is absolutely required for TNF mediated apoptosis¹¹. This time we decided to check the TNF receptor type I (TNFR-I) involvement in ATO apoptosis induction. But due to the diverse biological effects of TNF, evaluation of its co-stimulatory family member, CD30, also seemed to be necessary.¹²

Here, to study the expression of TNFR-I and CD30 within the apoptotic and live Leukemic cells of de novo APL patients, we employed a tricolor flow cytometric method for measuring apoptosis in combination with dual color immunofluorescence.

Material and Method:

Isolation of Promyelocytes and Arsenic treatment:

Bone marrow (BM) samples were collected from 5 de novo APL patients based on FAB classification and cytogenetic studies for t(15;17) translocation. MNC's were isolated

from heparinized BM samples after centrifugation at $600\times g$ for 20 min over Ficoll-Hypaque (1.077). (Pharmacia, Uppsala, Sweden) Freshly obtained MNCs' were then further purified for promyelocyte as previously described¹³ and according to Dynal bead negative selection system's instruction. Briefly, a total number of 4×10^6 of obtained MNC's were incubated with anti human HLA-DR and Anti-human CD3 antibodies (Serotec, London, U.K) for 30 min on ice. Then cells were washed for three times in RPMI 1% FBS (Gibco, Gaithersburg, MD) and incubated with Daynabeads pan mouse IgG for the next 30 min on ice and continuous gentle shaking. Finally by pipetting the fluid from the bottom of the tube, HLA-DR negative Promyelocytes were obtained and cultured at 10^5 cells/well in a 24-well culture plate in the presence of 0.5, 1.5 and 2 $\mu\text{mol/l}$ Arsenic trioxide (Sigma, St. Louis, MO) in RPMI 1640 with 10% Fetal Calf Serum (FCS) in a humidified, 5% CO_2 atmosphere at 37°C .

TNFR-1 (CD120a) and CD30 expression within the context of apoptotic and live cells.

To measure apoptosis in combination with cell surface immunophenotyping, we used DNA dye 7-AAD.¹⁴

After 36 and 48 hr of treatment, cultured Promyelocytes were harvested and washed twice in cold PBS, 1% FCS and 10^5 of cells were incubated for 10 min in 2% mouse serum before staining with anti-human CD120a-FITC and anti-human CD30-PE MoAb (Serotec, London, U.K) or FITC and PE isotype control (Dako, Denmark) respectively. For discrimination of apoptosis from secondary necrosis, cells were then incubated with 7-AAD (20 $\mu\text{g}/\text{l}$) in PBS for 20 min at 4°C , washed in PBS, and immediately submitted for flowcytometric analysis. A minimum of 10,000 events was acquired on each sample. For the analysis of apoptotic cells, gates were set on cells staining dimly with 7-AAD; and cells staining brightly with 7-AAD were discriminated as dead cells and were not considered within the analysis.

Statistical data analysis:

Control and ATO treated cells were compared for differences in expression of CD120a and CD30 in apoptotic and live cells after 36 and 48 hr of culture.

Statistical comparisons were performed using the Paired-t test. Significance was considered at P values of < 0.05 .

Results:

Expression of CD120a and CD30.

To study the mechanism responsible for arsenic induced cell death, changes in expression of CD120a and CD30 on Promyelocytes of APL patients were examined after 36 and 48 hr of treatment. In this regard, first living, apoptotic and dead cells were discriminated by virtue of their size changes and 7-AAD DNA dye uptake. (Fig.1)

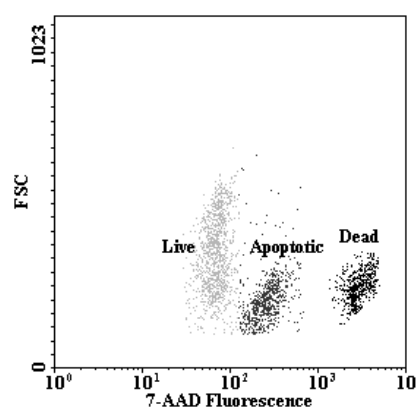


Fig.1 Discrimination of Live, Apoptotic and Dead cells by using Forward light scatter (FSC) and 7-AAD DNA dye uptake. Despite necrosis, cells under going apoptosis show a cellular condensation and hence a decrement in FSC signal, on the other hand due to membrane permeability disruption and inability of cells in excluding 7-AAD, dead cells uptake more DNA dye and show brighter 7-AAD fluorescence. Accordingly green, red and black populations are live, apoptotic and dead cells respectively.

Principally, cells undergoing apoptosis activate a series of molecular and biochemical events which lead to their total physical disintegration. One of the early events is cell dehydration, cytoplasm and nuclear chromatin condensation. Cell size and shape are characterized by light scattered at both forward and right angle of the laser beam in flowcytometry systems. Although a transient increase in right angle scatter can be seen during apoptosis in some cell systems, in later stages of apoptosis, the intensity of light scatter at both forward and right angle directions is decreased.¹⁵ Cell membrane permeability change is another event that features distinguishing dead from

live cells and can be detected by the ability of intact membrane in excluding charged cationic dyes such as trypan blue, propidium iodide (PI), ethidium bromide or 7-aminoactinomycin D (7-AAD). During apoptosis the plasma membrane transport function becomes transiently defective prior to total loss of the ability to exclude these charged fluorochromes. At this stage of apoptosis, the rate of uptake of these nuclear dyes increases compared to live cells.

So the more cells progress in apoptosis, the more DNA dye they uptake.

As illustrated by color gating, compared to live cells (green), apoptotic populations (red) are significantly ($p < 0.05$) over expressing the CD120a molecule albeit irrelevant to arsenic. But in the case of CD30, since its isotype control also shows a higher affinity to apoptotic cells we believe that there is no over expression of this molecule. (Fig.2 and 3)

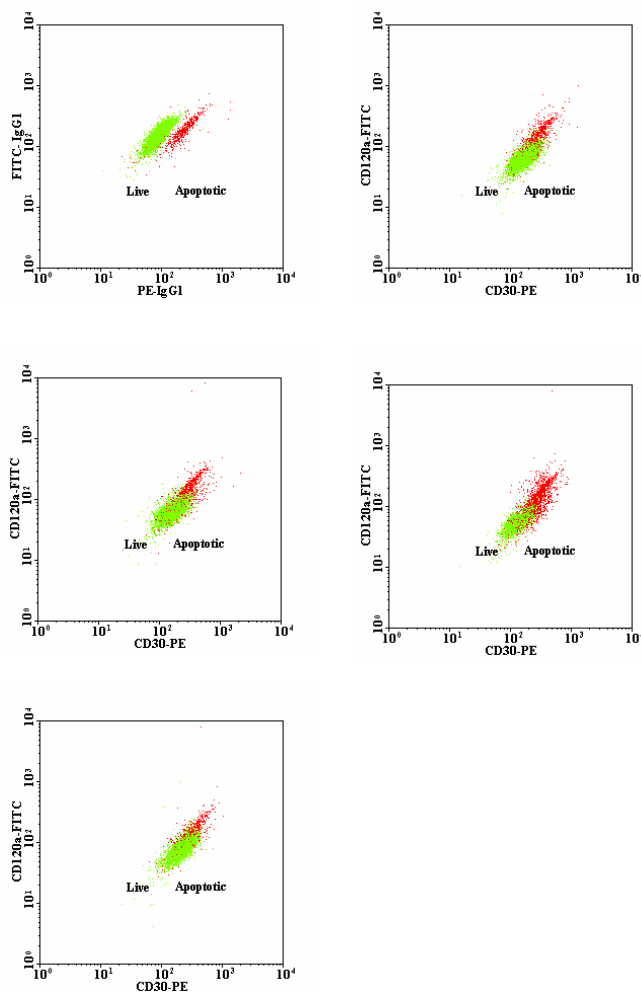


Fig.2 Expression of TNFR-I CD120a and CD30 on apoptotic (Red) and live (Green) cells of non-arsenic and arsenic treated cells: A. nonspecific binding of apoptotic and live cells to nonspecific Abs IgG1-FITC and IgG1-PE represents higher affinity of apoptotic cells to IgG1-PE. B. non-arsenic treated promyelocytes as control. C-E, 0.5, 1.5, 2.0 μ Mol arsenic treated cells after 48 h

Discussion:

One of the important mechanisms of apoptosis is regulated by death receptors associated death domain signaling intermediates, which contain receptor activation induced binding death do-

main proteins, such as tumor necrosis factor (TNF) receptor-associated death domain protein (TRADD) and Fas-associated death domain protein (FADD) and further activation of caspase cascades that initiate apoptotic death.¹⁶

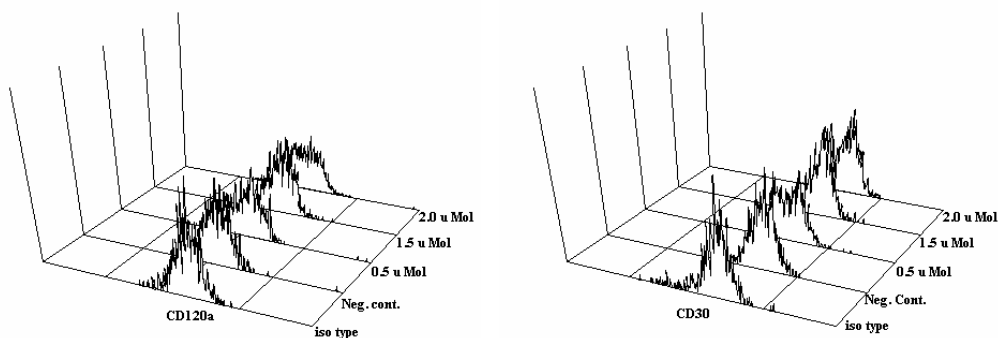


Fig.3 Expression of TNFR-I (CD120a) and CD30 on apoptotic cells of different doses of arsenic after 48 hr. treatment. Events were normalized and no over expression were detected on both receptors.

A major apoptotic signal in MNCs is mediated by TNF- α and its receptor. TNF- α which was shown to be produced as a proinflammatory cytokine from the inorganic arsenicals treated peritoneal macrophage¹⁷ is a pleiotropic cytokine that mediates a wide variety of biological responses, including the effects on the growth and differentiation of normal and malignant hematopoietic cells, and also the induction of tumor cell death. Two types of TNF receptors have been identified for the TNF, namely TNFR-I and TNFR-II which may mediate the activation of independent downstream signaling pathway¹⁸ TNFR-II functions primarily to bind ligand rapidly, passing soluble TNF trimers to TNFR-I¹⁹. Signaling by TNF receptor causes the association of an adaptor protein TRADD with the intercellular death domain of the TNFR-I molecule.²⁰ TRADD mediates the subsequent recruitment of an adapter protein FADD to form a death inducing signaling complex, which initiates apoptosis through activation of caspase cascades. Signaling through TNFR-I is, however, more complex than CD95. Indeed, in addition to recruiting adaptor molecules that bind and activate caspases, TNFR-I recruits proteins that engage various signal transduction pathways, some of which either abrogates or potentiates the apoptotic response. For example, in some cells exposed to TNF, NF κ B is induced through the activation of TNF receptor associated factor II (TRAF II), and the cells survive (NF κ B protects some cells from TNF induced programmed cell death). However, ligation of CD30 induces TRAF II degradation, which then disconnects the TNF receptor and NF κ B activation. Consequently, when cells are simultaneously stimulated through the

TNFR-I and CD30 molecules, the cells will undergo apoptosis.

Expression of CD30 in several types of malignancies including Hodgkin's disease, anaplastic large-cell lymphoma, immunoblastic lymphoma, multiple myeloma, adult T-cell leukemia lymphoma and also its TNF family member CD30L in a variety of hematopoietic cells²¹ prompt us to evaluate the above co-stimulatory pathway in arsenic apoptosis induction.

In the present study, CD30 expression was not observed while significant ($p < 0.05$) over expression of TNFR-I on apoptotic populations of arsenic treated and non-treated Promyelocytes after 36 and 48 hr indicated that arsenic does not affect TNFR-I expression.

Since the PML/RAR- α fusion protein of leukemic promyelocytes, which is responsible for resistance induction to TNF- α mediated apoptosis²² was shown to be degraded by arsenic⁶ and that TNF- α dose not necessarily induce or enhance apoptosis through its ligand binding pathway,²³ hypothetically, arsenic may restore the abrogated sensitivity of these cells to TNF- α rather than induce their over expression.

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