Original Article

Potent Reducing Effects of Vitamin D_3 on the Frequency of Apoptosis Induced by Arsenic Trioxide in NB4 Cell Line

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Background: **Arsenic trioxide and 1,25-$(OH)_2D_3$ (vitamin D_3) are used for the treatment of lymphocytic leukemia. However, the effects of combined treatment of these drugs are controversial. In this study, the combined effects of these drugs on the induction of apoptosis in NB4 cells were investigated using the neutral comet assay.**

Methods: **NB4 cells were treated with various doses of arsenic trioxide (0.1 – 3 µM) and vitamin D_3 (100 – 600 nM) alone or in combination. Twenty-four hours after treatment, neutral comet assay was performed and apoptotic cells were scored under a fluorescent microscope following staining with ethidium bromide.**

Results: **Results show that all doses of arsenic trioxide used in this study induced apoptosis in NB4 cells. The frequency of induced apoptosis was dose dependent and significantly higher than the controls (P<0.05 – 0.01). In contrast, vitamin D_3 at concentrations of 100 – 600 nM produced no significant effect on apoptosis induction compared to the controls. Treatment of NB4 cells with a combination of arsenic trioxide and vitamin D_3 resulted in reduction of apoptosis induced by arsenic trioxide which was not dependent on the dose of vitamin D_3 (P<0.05).**

Conclusion: **Results indicate that arsenic trioxide is a potent inducer of apoptosis in NB4 cells and vitamin D_3 significantly decreased the sensitivity of cells to the induction of apoptosis by arsenic trioxide. These findings suggest that 1,25-$(OH)_2D_3$ might be involved in anti-apoptotic processes via reactive oxygen species scavenging or other mechanisms not yet known.**

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Keywords: Apoptosis • arsenic trioxide • NB4 cell line • vitamin D_3

Introduction

Arsenic, a semimetal commonly found in soil, water and air, is an environmental toxin but it has also been used as a therapeutic agent for more than 2400 years. Recently, considerable interest has developed around arsenic trioxide (As_2O_3) for its anticancer properties. The therapeutic effects of arsenic are dependent on its ability to induce cell cycle arrest and induction of apoptosis. Studies carried out on a large number of leukemic cell lines, including erythroleukemia cell lines, have also shown that this compound mainly exerts an apoptotic effect.[1–4] These findings were also observed in several megakaryocytic cell lines (Meg01 and M07e); the growth of which are clearly inhibited by As_2O_3 due to induction of apoptosis.[5,6] Some of the apoptotic effects of arsenic are attributed to its ability to down-regulate B-cell lymphoma 2 (Bcl-2),[7,8] and the activation of caspases, which ultimately lead to cell death.[8–10] Several studies have also shown that arsenic is a potent inducer of reactive oxygen species (ROSs) that contribute significantly to cell killing.[11–13] However, because of these properties, the reported chronic toxicities and carcinogenicity of AS_2O_3 has hampered its acceptance as a first-choice drug for acute promyelocytic leukemia (APL).[14] As_2O_3 induces not only apoptosis but partial differentiation in APL cells *in vitro* and *in vivo*.[14–16]

Antioxidant molecules such as glutathione (GSH) reduce the cytotoxic effects of arsenic largely by quenching ROSs[16–19] and cells containing low levels of GSH are more sensitive to

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arsenic.[16] There are reports indicating augmentation of arsenic toxicity by vitamin C *in vitro*.[16,20–23] But a recent report shows that intracellular vitamin C protected cancer cells from arsenic cytotoxicity.[24] It has also been shown that catalase suppresses arsenic-induced apoptosis.[25] These observations suggest that down-regulating antioxidant molecules, or the biochemical pathways that generate them, could be therapeutically useful in altering the cytotoxicity of arsenic.

The biologically active form of vitamin D, 1,25-dihydroxy vitamin D_3 [$1,25(OH)_2D_3$] possesses *in vitro* multiple anti-cancer activities including growth arrest, induction of apoptosis and differentiation of a variety of different types of malignant cells as well as several immuno-stimulatory effects. The genomic actions of 1, $25(OH)_2D_3$ are modulated through its vitamin D receptor (VDR).[26] The earliest findings were in murine and human myeloid leukemic cell lines showing that $1,25(OH)_2D_3$ inhibited their proliferation and promoted their differentiation towards monocytes/macrophages.[27] Many studies that followed have demonstrated that treatment with $1,25(OH)_2D_3$ resulted in growth arrest, induction of monocytic differentiation and apoptosis in a variety of acute myeloid leukemia (AML) cell lines including HL-60, U937, NB4 and THP-1.[27–29] Cell death by apoptosis is a natural regulatory process in the body but in cancer, cells often fail to undergo apoptosis leading to malignant outgrowth. Vitamin D compounds can induce apoptosis in a number of different cancer cell types by several distinct pathways. Based on these reports we used various doses of vitamin D_3 in combination with As_2O_3 to study apoptosis formation in an NB4 cell line. NB4 cells, as a model of acute promyelocytic leukemia have been shown to undergo monocytic differentiation in response to 1α, 25 dihydroxy vitamin D_3 (1α, 25 D_3) and apoptosis or partial differentiation in response to AS_2O_3.[14,30]

Apoptosis is an energy-dependent process of self-induced cell death, characterized by nuclear condensation, cell shrinkage, membrane blebbing and the degradation of DNA into discrete fragments comprised of multiples of 180 – 200 base pairs. Severe fragmentation of cellular DNA happens during apoptosis and can be readily measured by single cell gel electrophoresis, also known as the comet assay.[30] There are different methods available for measuring apoptosis. Some are based on morphology, others on biochemical such as DNA laddering, TUNEL assay (transferase-mediated dUTP-biotin nick end labeling of DNA fragments), *in situ* end labeling (ISEL), comet assay and flow cytometric methods. The value of comet assay in measuring apoptosis in comparison with electron microscopy and flow cytometry using annexin V and propidium iodide is shown by other investigators.[31,32] These studies confirm the consistency of the comet assay for detection of apoptosis in single cells and provide evidence for its applicability as an additional method to detect apoptosis. The comet assay has been used to measure apoptosis in several other studies describing apoptotic cells as structures with diffuse fan-like tails and small heads, whereas normal cells form larger, more defined heads with minimal DNA diffusion.[31–36]

The aim of this study was to evaluate the frequency of apoptosis induced after combined treatment of As_2O_3 and vitamin D_3 in an NB4 cell line.

Materials and Methods

Cell line

NB4 cells were obtained from the National Cell Bank of Iran (NCBI, Pasteur Institute, Tehran, Iran). The cells were maintained in RPMI-1640 medium (Sigma) supplemented with penicillin (100 U/ml), streptomycin (100 µg/mL), L-glutamine (2 mM/L, Sigma) and 10% fetal calf serum (Gibco-BRL). Cells were kept at 37°C in a 5% CO_2 humidified atmosphere in tissue culture flasks (Nunc) and passaged twice a week to maintain them in an exponential growth state.

As_2O_3 and vitamin D_3 treatment

A stock solution of AS_2O_3 (Sigma Aldrich, Product #255483; mol wt = 197.841) was prepared in PBS (1 mmol/L) and working concentrations were made by serial dilution of the stock solution in RPMI-1640 before use.

1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$ (vitamin D_3)] (ACROS Organics, ID 14094; mol wt= 384.838, USA) was dissolved in absolute ethanol (0.1 mol/L), light protected and stored at -20°C. A stock solution of vitamin D_3 was also diluted in RPMI-1640 before use. NB4 cells, seeded at 1×10^5 cells/mL, were treated with various doses of AS_2O_3 (0.1 **–** 3 µM/L) and various doses of vitamin D_3 (100 – 600 nM/L) alone or in combination for 24 hours. Cells were protected

from light to prevent inactivation of light-sensitive vitamin D_3 after treatment.

Viability test

Viability of cells was determined by using trypan blue staining before and after treatment. Samples of cells were obtained, mixed with an equal volume of 0.4% trypan blue, and then counted on a hemocytometer slide (improved Neubauer) under a light microscope (Zeiss, Germany) with 10x objective lens to determine the number of viable cells. Only samples with greater than 95% viability were considered for treatment with drugs and the comet assay analysis.

By using the technique of neutral comet assay[33,35–36] with slight modifications, the frequency of apoptotic and non apoptotic cells in As_2O_3 and vitamin D_3, treated alone or in combination, as well as control untreated NB4 cells were determined. Briefly, the samples were centrifuged for 5 min at 2500 rpm, and the supernatant was removed, 10^5 cells were mixed with 140 μL of 0.75% low melting point (LMP) agarose (Fermentas) in phosphate buffer saline (PBS). Seventy micro litter of the resulting suspension was layered on top of each window of microscope slides precoated with a supporting layer of 1% normal melting point (NMP) agarose (Fermentas) in distilled water, then covered with coverslips and kept in 4°C for about 5 min in order to solidify the gel. The cover-slips were removed and the slides were then soaked in freshly prepared lysing solution (2.5 M NaCl, 0.1 M EDTA, 10 mM tris-base, 1% N-lauryl sarcosine, 1% triton x-100, 10% dimethyl sulphoxide [DMSO]) for about 30 minutes at 4°C in the dark to remove DNA-bound proteins so as not to interfere with migration in an electric field. All materials used for preparation of the lysis solution were from Merck, Germany. The final pH of the lysis solution was adjusted to about 10.

After lysing, the slides were washed three times in electrophoresis buffer consisting of 90 mM tris base, 90 mM boric acid and 2.5 mM Na_2EDTA (Merck, Germany), at a pH of 8.3 – 8.4. Slides were then transferred onto a submarine horizontal electrophoresis chamber containing a fresh electrophoresis buffer. Electrophresis was performed at 20 volts (0.8 V/cm) and 8 mA for 15 min. The slides were then washed with distilled water for 5 min in order to anneal the DNA and temperature. The air dried slides were stained with then fixed in ethanol for 5 min at room ethidium bromide solution (20 μg/mL) and covered with cover-slips before analysis. Cells were analysed using a fluorescent microscope (Nikon) equipped with an excitation filter (510 – 550 nm) and barrier filter (590 nm), at 200× magnification, for the presence of apoptotic and non apoptotic cells. A typical photomicrograph of normal and apoptotic cells is shown in Figure 1. A total number of 500 cells were randomly analysed for each slide. For each sample, at one run, at least 1000 cells were analysed. (For one run of each sample, at least 1000 cells were analysed.)

Statistical analysis

Data were statistically analysed with Mann-Whitney non-parametric and ANOVA tests using SPSS (version 12) software. *P*-value of less than 0.05 was considered as significant.

Results

Results are summarized in Table 1 and shown in Figures 2 and 3. As seen, the frequency of apoptosis is very low in control untreated NB4 cells (Table 1 and Figure 2). Treatment of NB4 cells with As_2O_3 led to an increase in the frequency of apoptosis in a dose dependent manner at the dose range used in this study (0.1 – 3 μM/L). Statistical analysis shows a significant difference for all doses used compared to controls ($P<0.05$ for the dose of 0.1 μM/ L and $P<0.01$ μM/L for other concentrations). The maximum number of apoptosis was achieved with the use of 3 μM/L

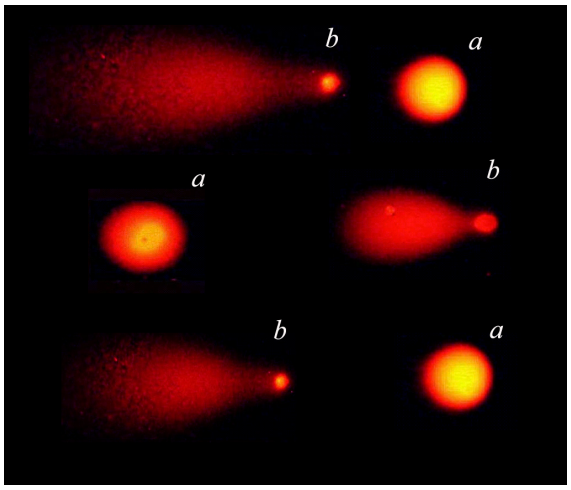

Figure 1. A typical photomicrograph of normal **(a)** and apoptotic **(b)** NB4 cells following As_2O_3 treatment. Magnification 200×

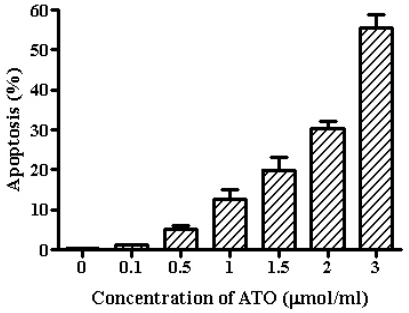

Figure 2. Percentage of apoptotic cells in NB4 cell line treated with various doses of As_2O_3 (µM/L). Error bars indicate standard error of mean values obtained from 5 independent experiments

As_2O_3, about 60%.

As shown in Table 1, various doses of vitamin D_3 used in this study (100 – 600 nM/L) did not increase the frequency of apoptosis in treated cells. There was no statistically significant difference between vitamin D_3 treated samples and the control (P>0.05). However, the combination of vitamin D_3 with various doses of As_2O_3 (1, 1.5 and 2 µM/L) led to a pronounced decrease in the frequency of As_2O_3 induced apoptosis. This effect was more pronounced for As_2O_3 at a concentration of 1 µM/L compared to concentrations of 1.5 and 2 (Figure 3). Also, no dose effect was seen for the various doses of vitamin D_3 used in this study (P>0.05) (Figure 3).

Discussion

Apoptosis, a genetically programmed event, is a common mode of cell death in a variety of normal tissues characterized by chromosomal DNA fragmentation.[33,37] As_2O_3 exerts remarkable biological effects on several cellular functions, including induction of apoptosis.[38] As the results in Table 1 and Figure 2 show, As_2O_3 at various concentrations (0.1 – 3 µM/L) used in this study induced apoptosis in NB4 cells in a dose dependent manner. The frequency of apoptosis almost doubled at a concentration of only 0.1 µM/L compared to the control. The increase by using 3 µM/L As_2O_3 was about 55 fold (Figure 2). This observation shows that As_2O_3 is a potent inducer of apoptosis in NB4 cells. Our observations are consistent with a recent report showing that As_2O_3 induced apoptosis in T24 human bladder carcinoma cells in a dose dependent manner[39] and in NB4 cells.[40] The mechanisms by which As_2O_3 induces cell death are not completely elucidated; however, several reports indicate that the generation of ROSs is a significant component of its cytotoxic action.[11–13,41] Production of ROSs by any means in the cellular environment may lead to induction of DNA damage with alterations in the expression of a large number of genes, leading to an alteration in the cell cycle progression and ultimately to cell death. Vivas-Mejia et al.[41] have shown that inhibition of glutathione peroxidase, an enzyme that regulates H_2O_2 levels in mitochondria, is involved in As_2O_3 induced apoptosis through down-regulation of peroxiredoxin, a mitochondria-specific H_2O_2 scavenger. This and many other reports support the involvement of ROSs in induction of apoptosis. A variety of damage, including single and double strand breaks (DSB), is induced in cellular DNA by ROSs. It has been previously shown that DSB triggers apoptosis and that the frequency of DSB correlates with the yield of apoptosis. These observations support the view

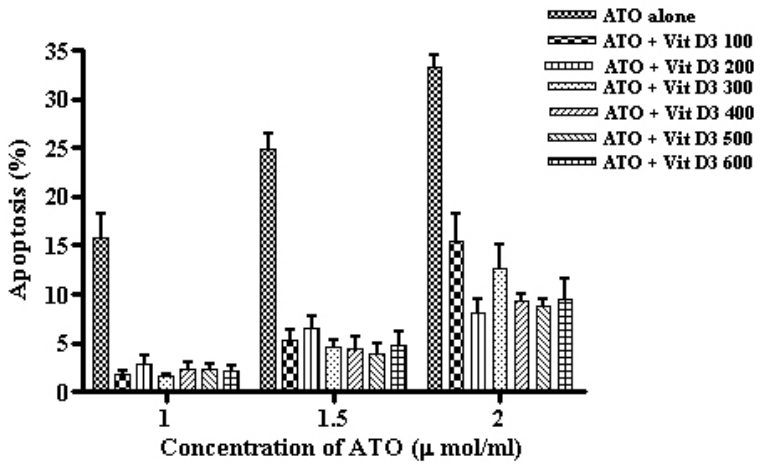

Figure 3. Frequency of As_2O_3 induced apoptosis in the presence of various doses of vitamin D_3 (nM/L). Error bars indicate standard error of mean values obtained from 5 independent experiments

Table 1. Total frequency of normal and apoptotic cells observed in each study group treated either with As_2O_3 or vitamin D_3 alone or in combination

Treatment	No. of experiments	No. of cells analyzed	Total no. of normal cells	Total no. of apoptotic cells
Control	10	5000	4973	27
Control vitamin D_3 (nM)				
100	10	5000	4965	35
200	10	4950	4912	38
300	10	5000	4961	39
400	9	4500	4467	33
500	9	4500	4463	37
600	9	4500	4467	33
As_2O_3 treatment (μM)				
	5			
0.1	5	2547	2516	31
0.5	5	2523	2402	121
1	5	2454	2177	277
1.5	5	2537	2120	417
2	5	2447	1870	571
3		2357	1520	837
As_2O_3 (1)* + vitamin D_3**				
100	5	2535	2488	47
200	5	2518	2458	60
300	5	2326	2280	46
400	5	2500	2443	57
500	5	2432	2380	54
600	5	2403	2357	46
As_2O_3 (1.5)* + vitamin D_3**				
100	5	2466	2352	114
200	5	2457	2329	128
300	5	2348	2256	92
400	5	2736	2637	99
500	4	2111	2038	73
600	4	2048	1965	83
As_2O_3 (2)* + vitamin D_3**				
100	5	2651	2398	253
200	5	2573	2401	172
300	5	2494	2286	208
400	5	2352	2180	172
500	5	2570	2404	166
600	5	2338	2185	153

*Concentration of As_2O_3 in μM/L; **concentration of vitamin D_3 in nM/L

that ROS-induced DNA damage is involved in apoptotic death.[39,42] Results shown in Table 1 and Figure 2 may indicate that As_2O_3 induced apoptosis might be due to ROSs generation leading to a variety of DNA damage involved in the apoptotic process.

ROSs, utilized as essential intermediate messenger molecules in the cell death signaling cascade[43,44] might be the reason that antioxidants are capable of inhibiting apoptosis. Therefore, antioxidants and free radical scavengers that partition into the aqueous phase of the cytosol can delay or inhibit apoptosis.[45,46] Consistent with this notion is the observation that high levels of GSH are associated with cellular resistance to arsenic[13,16,47–50] and decreasing intracellular GSH concentrations cause increased sensitivity to As_2O_3.[16]

Addition of vitamin D_3 in the cellular environment of NB4 cells, led to a considerable decrease in the frequency of As_2O_3 induced apoptosis (Table 1, Figure 3). Our observation is consistent with the recent report by Bao et al.[51] who have shown potent anti-apoptotic effects of vitamin D_3 on non-malignant prostate cells from oxidative stress induced cell death by elimination of ROS induced cellular injury[51] and the report by Zhang and Zanello[52] who have shown that

physiological doses of vitamin D_3 could protect osteoblasts against apoptosis. Also the anti-apoptotic effect of vitamin D_3 has been previously shown in various models of neural damage.[53] 1,25 D_3 protects against various stress stimuli including H_2O_2.[41] It has been shown that 1,25 D_3 also acts as an antioxidant in leukemic cells[54] and a clear efficiency on the apoptotic machinery in β-cells.[55] In the present study, a significant decrease in the frequency of apoptosis in the various treatment groups clearly gives evidence that vitamin D_3 reduces sensitivity of NB4 cells to As_2O_3 and has an inhibitory role when used in combination (Figure 3).

In conclusion, our observations as well as other reports indicate that As_2O_3 is a potent inducer of apoptosis and that 1,25-$(OH)_2D_3$ is a potent anti-apoptotic agent by means of ROS scavenging or other mechanisms not yet fully understood.

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