Potent Reducing Effects of Vitamin D₃ on the Frequency of Apoptosis Induced by Arsenic Trioxide in NB4 Cell Line

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Background: Arsenic trioxide and 1,25-(OH)₂D₃ (vitamin D₃) 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 D₃ (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 D₃ 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 D₃ resulted in reduction of apoptosis induced by arsenic trioxide which was not dependent on the dose of vitamin D₃ (P<0.05).

Conclusion: Results indicate that arsenic trioxide is a potent inducer of apoptosis in NB4 cells and vitamin D₃ significantly decreased the sensitivity of cells to the induction of apoptosis by arsenic trioxide. These findings suggest that 1,25-(OH)₂D₃ might be involved in anti-apoptotic processes via reactive oxygen species scavenging or other mechanisms not yet known.

Keywords: Apoptosis • arsenic trioxide • NB4 cell line • vitamin D₃

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 (As₂O₃) 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 As₂O₃ 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.⁹–¹⁰ 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 As₂O₃ has hampered its acceptance as a first-choice drug for acute promyelocytic leukemia (APL).¹⁴ As₂O₃ 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 ROSs¹⁶–¹⁹ and cells containing low levels of GSH are more sensitive to
arsenic. There are reports indicating augmentation of arsenic toxicity by vitamin C in vitro. But a recent report shows that intracellular vitamin C protected cancer cells from arsenic cytotoxicity. It has also been shown that catalase suppresses arsenic-induced apoptosis. 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-di hydroxy vitamin D₃ [1,25(OH)₂D₃] 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 immunostimulatory effects. The genomic actions of 1, 25(OH)₂D₃ are modulated through its vitamin D receptor (VDR). The earliest findings were in murine and human myeloid leukemic cell lines showing that 1,25(OH)₂D₃ inhibited their proliferation and promoted their differentiation towards monocytes/macrophages. Many studies that followed have demonstrated that treatment with 1,25(OH)₂D₃ 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. 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 D₃ in combination with As₂O₃ 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 D₃ (1α, 25 D₃) and apoptosis or partial differentiation in response to As₂O₃.

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. 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. 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.

The aim of this study was to evaluate the frequency of apoptosis induced after combined treatment of As₂O₃ and vitamin D₃ 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% CO₂ humidified atmosphere in tissue culture flasks (Nunc) and passaged twice a week to maintain them in an exponential growth state.

As₂O₃ and vitamin D₃ treatment
A stock solution of As₂O₃ (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 D₃ [1,25(OH)₂D₃ (vitamin D₃)] (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 D₃ was also diluted in RPMI-1640 before use. NB4 cells, seeded at 1×10⁵ cells/mL, were treated with various doses of As₂O₃ (0.1 – 3 µM/L) and various doses of vitamin D₃ (100 – 600 nM/L) alone or in combination for 24 hours. Cells were protected...
from light to prevent inactivation of light-sensitive vitamin D₃ 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 with slight modifications, the frequency of apoptotic and non apoptotic cells in As₂O₃ and vitamin D₃ 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, 10⁵ cells were mixed with 140 μL of 0.75% low melting point (LMP) agarose (Fermentas) in phosphate buffer saline (PBS). Seventy micro liter 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 lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM tris-base, 1% N-lauryl sarcosine, 1% triton x-100, 10% dimethyl sulfoxide [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 Na₂EDTA (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. Electrophoresis 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 As₂O₃ 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 As₂O₃ treatment. Magnification 200×](image-url)
As$_2$O$_3$, 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$_2$O$_3$ (1, 1.5 and 2 μM/L) led to a pronounced decrease in the frequency of As$_2$O$_3$ induced apoptosis. This effect was more pronounced for As$_2$O$_3$ 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$_2$O$_3$ exerts remarkable biological effects on several cellular functions, including induction of apoptosis.$^{38}$ As the results in Table 1 and Figure 2 show, As$_2$O$_3$ 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$_2$O$_3$ was about 55 fold (Figure 2). This observation shows that As$_2$O$_3$ is a potent inducer of apoptosis in NB4 cells. Our observations are consistent with a recent report showing that As$_2$O$_3$ induced apoptosis in T24 human bladder carcinoma cells in a dose dependent manner$^{39}$ and in NB4 cells.$^{40}$ The mechanisms by which As$_2$O$_3$ 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$_2$O$_2$ levels in mitochondria, is involved in As$_2$O$_3$ induced apoptosis through down-regulation of peroxiredoxin, a mitochondria-specific H$_2$O$_2$ 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 2](image1.png)

**Figure 2.** Percentage of apoptotic cells in NB4 cell line treated with various doses of As$_2$O$_3$ (μM/L). Error bars indicate standard error of mean values obtained from 5 independent experiments

![Figure 3](image2.png)

**Figure 3.** Frequency of As$_2$O$_3$ 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
that ROS-induced DNA damage is involved in apoptotic death.\textsuperscript{39,42} Results shown in Table 1 and Figure 2 may indicate that As\textsubscript{2}O\textsubscript{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\textsuperscript{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\textsuperscript{45,46} Consistent with this notion is the observation that high levels of GSH are associated with cellular resistance to arsenic\textsuperscript{13,16,47–50} and decreasing intracellular GSH concentrations cause increased sensitivity to As\textsubscript{2}O\textsubscript{3}.\textsuperscript{16}

Addition of vitamin D\textsubscript{3} in the cellular environment of NB4 cells, led to a considerable decrease in the frequency of As\textsubscript{2}O\textsubscript{3} induced apoptosis (Table 1, Figure 3). Our observation is consistent with the recent report by Bao et al.\textsuperscript{51} who have shown potent anti-apoptotic effects of vitamin D\textsubscript{3} on non-malignant prostate cells from oxidative stress induced cell death by elimination of ROS induced cellular injury\textsuperscript{51} and the report by Zhang and Zanello\textsuperscript{52} who have shown that

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Treatment} & \textbf{No. of experiments} & \textbf{No. of cells analyzed} & \textbf{Total no. of normal cells} & \textbf{Total no. of apoptotic cells} \\
\hline
\textbf{Control} & 10 & 5000 & 4973 & 27 \\
\hline
\textbf{Control vitamin D\textsubscript{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 \\
\hline
\textbf{As\textsubscript{2}O\textsubscript{3} treatment (μM)} & & & & \\
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 & 5 & 2357 & 1520 & 837 \\
\hline
\textbf{As\textsubscript{2}O\textsubscript{3} (1)* + vitamin D\textsubscript{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 \\
\hline
\textbf{As\textsubscript{2}O\textsubscript{3} (1.5)* + vitamin D\textsubscript{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 \\
\hline
\textbf{As\textsubscript{2}O\textsubscript{3} (2)* + vitamin D\textsubscript{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 \\
\hline
\end{tabular}
\caption{Total frequency of normal and apoptotic cells observed in each study group treated either with As\textsubscript{2}O\textsubscript{3} or vitamin D\textsubscript{3} alone or in combination}
\end{table}

\textsuperscript{*Concentration of As\textsubscript{2}O\textsubscript{3} in μM/L; **concentration of vitamin D\textsubscript{3} in nM/L.}
physiological doses of vitamin D₃ could protect osteoblasts against apoptosis. Also the anti-apoptotic effect of vitamin D₃ has been previously shown in various models of neural damage.\textsuperscript{53} 1,25 D₃ protects against various stress stimuli including H₂O₂.\textsuperscript{41} It has been shown that 1,25 D₃ also acts as an antioxidant in leukemic cells\textsuperscript{54} and a clear efficiency on the apoptotic machinery in β-cells.\textsuperscript{55} In the present study, a significant decrease in the frequency of apoptosis in the various treatment groups clearly gives evidence that vitamin D₃ reduces sensitivity of NB4 cells to As₂O₃ and has an inhibitory role when used in combination (Figure 3).

In conclusion, our observations as well as other reports indicate that As₂O₃ is a potent inducer of apoptosis and that 1,25-(OH)₂D₃ is a potent anti-apoptotic agent by means of ROS scavenging or other mechanisms not yet fully understood.

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