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Zinc protects against arsenic-induced apoptosis in a neuronal cell line, measured by DEVD-caspase activity

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Abstract

Acute and chronic arsenic exposure results in toxicity in humans and causes many neurological and other manifestations. For the first time the present study reports that zinc decreases arsenic-induced apoptosis and also confirms a single report of apoptosis induced by arsenic in a neuronal cell line. Apoptosis measured by DEVD-caspase activity peaked between 10 μM and 20 μM of arsenic trioxide. Higher concentrations of arsenic up to 40 μM caused increasing cell death with diminishing DEVD-caspase activity. The beneficial effect of zinc was proportional to its concentration with a significant decrease in arsenic-induced DEVD-caspase activity at 50 μM and 75 μM zinc ($P < 0.05$). This finding may be of therapeutic benefit in people suffering from chronic exposure to arsenic from natural sources, a global problem especially relevant to millions of people on the Indian subcontinent.

Introduction

In addition to its deliberate use in acute and chronic poisoning, arsenic has therapeutic applications in western medicine. In the 18th to 20th century it was used to treat illnesses such as syphilis, eczema, leukemia and Vincent's angina (Ratnaike 2003). Safer and more effective drugs have replaced the use of arsenic. Currently arsenic trioxide is the drug of choice in the treatment of acute promyelocytic leukemia (Sun *et al.* 1992; Shen *et al.* 1997; Soignet *et al.* 1998).

The present interest in arsenic toxicity arises from the large number of people in more than 18 countries who are exposed to arsenic; the majority from natural sources in aquifers supplying drinking water (Ratnaike 2003). A major clinical feature of arsenic poisoning is toxicity to the central and peripheral nervous systems (Le Quesne 1982; Campbell & Alvarez 1989). The manifestations are cognitive impairment, encephalopathy and damage to the peripheral nervous system that leads to peripheral neuropathy (Abernathy *et al.* 1999; Hall 2002).

Arsenic is not a component of human biological systems, and is toxic to human cells with the potential to cause apoptosis. Apoptosis, or programmed cell death, is the physiological, gene-controlled process whereby individual cells in multicellular animals are deliberately eliminated to achieve homeostasis and normal tissue and organ development (Chinnaiyan & Dixit 1996). Apoptosis occurs by controlled proteolysis of cellular components and a family of enzymes known as caspases are the principal proteases involved; measurement of their activation reflects apoptotic activity.

Arsenic causes apoptosis by damaging the DNA of cells, perhaps through induction of reactive oxygen species (Pelicano *et al.* 2002; Shen *et al.* 2003). Arsenic also has a direct effect on mitochondrial membranes, leading to the release of an apoptosis-inducing factor (Larochette *et al.* 1999; Lorenzo *et al.* 1999; Susin *et al.* 2000).

There is only one report of arsenic-induced apoptosis in a neurological cell line (Akao *et al.* 1999). The present study seeks to confirm arsenic-induced apoptosis in a neuroepithelial cell line. A second part of the study explores whether zinc has a beneficial effect on arsenic-induced apoptosis in these cells, based on the fact that we have shown in this laboratory that zinc

prevents butyrate-induced apoptosis in a neuronal cell line (Ho *et al.* 2000).

Materials and methods

Materials

Major materials used included reagent grade sodium butyrate, sodium bicarbonate and NaOH (Merck), fetal bovine serum (FBS; Chase Laboratories), gentamicin (Pharmacia and Upjohn), penicillin, streptomycin and trypsin-EDTA (ICN Biomedicals), carbobenzoxy-asp-glu-val-asp-7-amino-4-trifluoro-methyl-coumarin (zDEVD-AFC; Calbiochem) and 6-well tissue culture plates (Falcon, Becton Dickenson). All other materials, including RPMI-1640 powdered medium, As_2O_3 (arsenic-III oxide), HEPES, EDTA, CHAPS, NP-40 (Igepal CA-630 nonionic detergent) and $ZnSO_4$ were reagent grade sourced from Sigma-Aldrich Co., unless otherwise specified.

Cell line

2.3D cells are an immortalised adherent cell line that can be induced by fibroblast growth factor to differentiate into astrocytes and neurons (Bartlett *et al.* 1988). The morphology and antigenic phenotype of the cloned cell line is characteristic of normal neuroepithelium (Bartlett *et al.* 1988).

Cell cultures

2.3D cells were grown in culture flasks in a humidified atmosphere containing 5% CO_2 in RPMI-1640 tissue culture medium, pH 7.4 supplemented with 25mM HEPES, 24mM sodium bicarbonate, 100 IU/ml penicillin, 100 μg/ml streptomycin, 160 μg/ml gentamicin and 10% heat-inactivated FBS (called *medium* for convenience). Cells were harvested with trypsin-EDTA before cell confluence occurred. A count of viable cells was done using a hemocytometer and 0.15% trypan blue (Searle Diagnostic). A starting cell concentration of 10^5 cells/ml was obtained by adding an appropriate volume of medium. Two millilitres of this suspension was pipetted into each well of 6-well plates.

The culture plates were incubated until the cells were between 25% and 50% confluence in 5% CO_2 at 37 °C. Plates showing uneven growth of cell layers were discarded. The wells were replenished with fresh medium containing arsenic and/or zinc to final volumes of 2 ml. The cultures were incubated for 20 h after the addition of arsenic and/or zinc to triplicate wells.

Arsenic trioxide

Each experiment used fresh dilutions made in RPMI-1640 of As_2O_3 from a stock solution in 1 N NaOH. The pH was adjusted to 7.4 with HCl (APS Finechem, Aust.) and sterilised by microporous membrane filtration (Millipore) before adding FBS to 10%. Dilutions of As_2O_3 between 5 μM and 40 μM were made. In regard to apoptosis, a standard positive control was sodium butyrate dissolved in RPMI-1640 and membrane sterilised (Ho *et al.* 2000).

Zinc sulfate

To determine the beneficial effects of zinc, a series of concentrations of $ZnSO_4$ from 12.5 μM to 75 μM were made in medium from a 25 mM aqueous stock. An initial dilution of 1:10 in H_2O avoided precipitation of salts.

Controls

Sodium butyrate at concentrations from 1 to 12 mM were positive controls to induce apoptosis. Negative controls were medium without added arsenic or zinc. All culture wells were set up in triplicate.

Buffers

Lysis buffer was prepared fresh, using milli-Q water containing 0.5% NP-40, 0.05 M Tris and 0.05 M EDTA, pH adjusted to 7.4. Caspase buffer was prepared with 50 mM HEPES, 1% sucrose and 0.1% CHAPS in milli-Q water, pH adjusted to 7.4 with 5M NaOH, then used for up to two weeks. Fresh dithiothreitol and zDEVD-AFC were added to the caspase buffer to final concentrations of 10 mM and 2.5 μM respectively to assay DEVD-caspase activity in lysate samples.

Harvesting of cells

Cells were harvested from supernatants that contained nonadherent cells by centrifugation at 900 g for 10 minutes and the pellet washed twice with phosphate buffered saline (PBS, pH 7.4). The remaining adherent cells in the wells were treated with 0.8ml of

fresh lysis buffer for at least 20 min and the contents were pipetted out and added to the pellets obtained above. The pellets were resuspended and allowed to stand overnight at 4 °C for lysis to complete. Lysed cell material was then transferred to microcentrifuge tubes (Sorenson BioScience) and spun at 12000 g for 6 min at 4 °C to sediment debris.

Cell viabilities were checked on replicate wells harvested in the same manner except that adherent cell fractions were washed gently *in situ* with RPMI alone, harvested with trypsin-EDTA and washed twice with PBS. Cells resuspended in 0.5 ml of medium were counted using a haemocytometer with equal volumes of 0.3% trypan blue.

Measurement of apoptosis using the DEVD-caspase assay

Apoptosis was measured using a DEVD-caspase activity assay. DEVD-caspase activity collectively refers to both caspase-3 and caspase-7 activity as both caspases recognise the common tetrapeptide motif DEVD (Krzyzowska *et al.* 2002).

The DEVD-caspase assay was performed on cell lysates with the caspase activity buffer described above. In this assay, caspases cleave the fluorogenic substrate zDEVD-AFC creating a fluorescent AFC molecule whose fluorescence was measured. One millilitre of caspase activity buffer was added to 50 μl of sample in disposable semi-micro cuvettes (Greiner, Austria), incubated for 18 h at 20 °C and the fluorescence measured in a Perkin-Elmer LS50 spectrofluorometer at 490 nm using 400 nm incident light and a 5nm slit width (Ho *et al.* 2000). Raw caspase activities expressed as fluorescent units were converted into DEVD-caspase activity units. One unit of DEVD-caspase activity is defined as 1 fluorescence unit per mg of protein per hour of caspase assay. EDTA from the lysis buffer was present in the reaction mix at a final concentration of 1.25 mM, sufficient to prevent inactivation of the caspase activity by trace metals (Stennicke & Salvesen 1997).

Protein concentrations were then determined by a modified Lowry method using detergent-compatible protein assay reagents (Bio-Rad Laboratories) and fraction V bovine serum albumin serially diluted over the range 0.1 to 3.2 mg/ml to create a standard curve. This enabled expression of DEVD-caspase activity per mg of protein in the lysates, thus reflecting varied cell growth in different wells. Optical densities were plotted and the line of best fit determined. The resulting linear equation (optical density = slope of line * protein concentration) was used to convert optical densities of lysate samples into protein concentrations (in mg/ml).

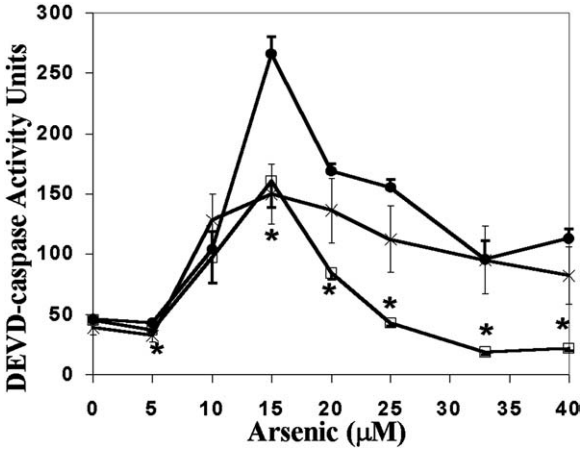

Figure 1. Shows DEVD-caspase activity induced in 2.3D cells stimulated with arsenic trioxide in the presence of no added zinc (closed circles) or 75 μM $ZnSO_4$ (open squares). Thick error bars show standard deviation for each mean. Asterisks show which DEVD-caspase activities differed significantly from the nil added zinc activity of the control at the same arsenic concentration ($P < 0.02$). The middle curve with cross-hair points shows the mean DEVD-caspase activity of 13 experiments using 2.3D cells stimulated in triplicate with arsenic trioxide and the thin error bars indicate standard errors for each mean.

The final DEVD-caspase activity was calculated by dividing the fluorescent AFC activity (in fluorescent units per ml) by the protein concentration (in mg/ml) measured in each lysate sample. This method is used to correct for the effect of growth inhibition (Mundle *et al.* 1999; Haridas *et al.* 2001; Kwon *et al.* 2001; Feng & LeBlanc 2003).

Results

Butyrate and Zinc controls

Controls with sodium butyrate caused activation of DEVD-caspase activity in the concentration range 4 to 12 mM. The activity of $ZnSO_4$ alone is shown in Figures 1 and 2 at the points marked 0 μM arsenic trioxide, and is not significantly different from baseline activity in medium alone.

Response to arsenic

The morphological changes in cells treated with 5 μM As_2O_3 were withdrawal of cell processes. At 10 μM

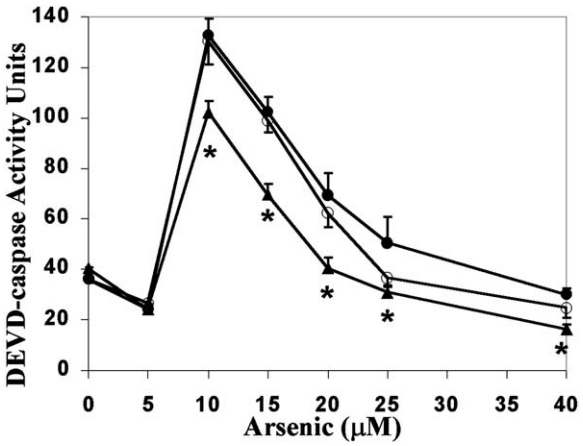

Figure 2. Shows DEVD-caspase activity induced in 2.3D cells stimulated with arsenic trioxide in the presence of no added zinc (closed circles), 25 μM $ZnSO_4$ (open circles) or 50 μM $ZnSO_4$ (triangles). Error bars show standard deviation for each mean. Asterisks show, at the same arsenic concentration, the significant differences in DEVD-caspase levels between 50 μM zinc and when no zinc was added ($P < 0.02$).

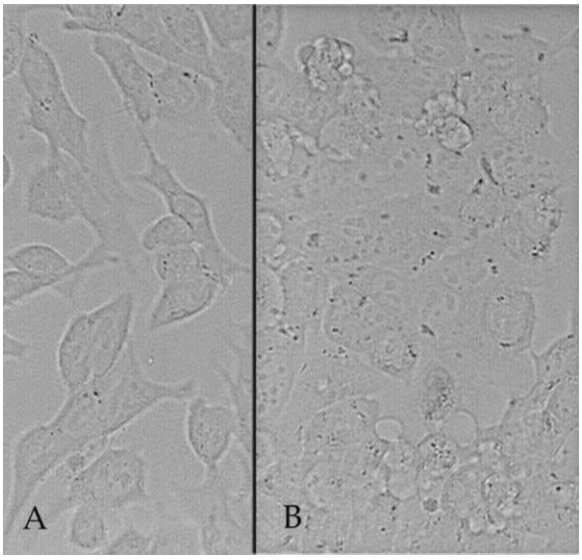

Figure 3. (A): Unstained control 2.3D cells photographed with phase contrast *in situ* on the culture plate showing the normal phenotype including extended cellular processes. (B): Cells treated with 10 μM As_2O_3 showing withdrawal of cell processes and membrane blebbing characteristic of early apoptosis.

and above, cells began to lose adherence, and microscopic examination of cells showed condensation and fragmentation of chromatin and blebbing of cell membranes in proportion to the concentration of arsenic (see Figures 3 and 4). Staining with trypan blue showed decreasing viability with increasing arsenic concentration.

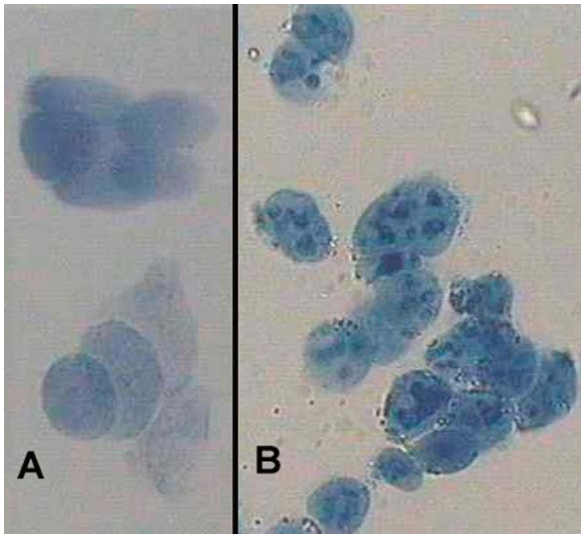

Figure 4. Cells were gently lysed and stained to reveal intracellular structures — (A): Control 2.3D cells showing no condensation of nuclear material. (B): Cells treated with 10 μM As_2O_3 showing nuclear changes of chromatin condensation and fragmentation, characteristic of early apoptosis.

DEVD-caspase activation occurred over a range of As_2O_3 concentrations from 10 μM to 40 μM. The peak activity was between 10 μM and 20 μM As_2O_3 (Figure 1). Concentrations between 25 μM and 40 μM induced lower DEVD-caspase activity. Experiments using As_2O_3 at 80 μM resulted in cell death without measurable DEVD-caspase activation.

Cultures with higher cell concentrations, reflected by higher protein concentrations, required increased concentrations of arsenic to induce maximal DEVD-caspase activity. This relationship was positive but low, with a coefficient of correlation of 0.27.

Effects of $ZnSO_4$ on arsenic-induced DEVD-caspase activity

Adding either 50 μM or 75 μM of $ZnSO_4$ to As_2O_3 at a range of concentrations produced a statistically significant decrease in DEVD-caspase activity at 10 μM arsenic and above (P values from < 0.05 to < 0.01) in each of three experiments. Figure 1 shows results from a typical experiment with 75 μM of $ZnSO_4$ and its nil-zinc control, superimposed on the mean response of 13 experiments using arsenic without zinc present. Figure 2 shows a second series of experiments using 25 μM or 50 μM $ZnSO_4$ with the same concentrations of arsenic.

These results indicate a beneficial role of high concentrations of $ZnSO_4$ in reducing the level of DEVD-

caspase activity induced by arsenic. The decrease in DEVD-caspase activity was greater with 75 μM than with 50 μM zinc. The addition of 25 μM of $ZnSO_4$ did not significantly decrease arsenic-induced DEVD-caspase activity.

Discussion

Chronic arsenic toxicity affects most organ systems of the body and is associated with malignancies (Hall 2002; Ratnaike 2003). The nervous system manifestations are toxic delirium, encephalopathy, seizures and peripheral neuropathy due to demyelination and severe axonal loss (Le Quesne 1982; Campbell & Alvarez 1989; Abernathy *et al.* 1999; Hall 2002).

Our study confirms a report of apoptosis caused by As_2O_3 in a neuroblastoma cell line (Akao *et al.* 1999). We have established that apoptosis, measured by DEVD-caspase activity, occurs maximally within a narrow range of 10 μM to 20 μM of As_2O_3. Although still significantly above the zero arsenic control, there was diminishing DEVD-caspase activity at 25 μM and 33 μM arsenic, which may reflect that intracellular damage is occurring due to arsenic toxicity. At arsenic concentrations of 40 μM and above, no significant activation of DEVD-caspase occurred and the cells showed very low viability. Indeed, as arsenic concentrations increased, DEVD-caspase activity peaked and then decreased while viability declined continuously, accompanied by cell changes characteristic of apoptosis such as cell membrane blebbing and condensation and fragmentation of chromatin.

Shen's group (1997) treated APL patients with arsenic trioxide and achieved serum concentrations ranging from 5.5 μM to 7.3 μM (Shen *et al.* 1997). Our experiments required a higher level of arsenic to effect apoptosis in the neuroepithelial cell line we used *in vitro*.

Arsenic causes apoptosis by a variety of mechanisms. One mechanism is through direct activation of caspase-8, which then initiates the apoptotic process (Cohen 1997). Apoptosis also occurs by arsenic producing reactive oxygen species that inactivate enzymes and damage DNA molecules by direct chemical attack on their structure (Pelicano *et al.* 2002; Shen *et al.* 2003).

In this study we demonstrated a previously unreported finding that zinc decreased arsenic-induced apoptosis. Both 50 μM (Figure 2, triangles) and 75 μM of $ZnSO_4$ (Figure 1, open squares) caused a significant decrease in DEVD-caspase activity. Lower concentrations of $ZnSO_4$ did not show this effect (Figure 2, open circles), either because the concentration was insufficient or because of the differences set out below, relating to the cellular uptake of arsenic and zinc. As_2O_3 enters cells by simple diffusion, proportional to its extracellular concentrations and independent of energy consumption (Huang & Lee 1996). The highly charged, hydrophilic ion Zn^{++} does not cross biological membranes as easily and the entry of zinc is determined by the availability and concentration of membrane-spanning zinc transport proteins (Kambe *et al.* 2004). In contrast to arsenic, zinc may be lost easily from within the cell, as it is not tightly bound inside the cell, but labile (Cousins 1986) while arsenic is highly reactive, binding to sulfhydryl groups in intracellular and membrane proteins (Menzel *et al.* 1999; Zhang *et al.* 2000).

The findings of our study may be of potential value to those suffering from chronic arsenic exposure, though further study relating to zinc is necessary. It may be of interest to note that the zinc tolerance test uses a fasting oral dose of zinc of 50 mg with no ill effects and induces concentrations in serum of 43 μM +/− SD of 7 μM (Sullivan *et al.* 1979). Doses of 150 mg for short periods of time are within prescription limits (Sweetman 2002). The concentration of zinc in our experiments that best prevented apoptosis (75 μM) is thus equivalent to an 85 mg oral dose of elemental zinc.

Zinc has been demonstrated to prevent apoptosis, though not induced by arsenic (Zalewski *et al.* 1993; Sunderman 1995; Fraker & Telford 1997; Ho *et al.* 2000). Zinc may act at several points in the cascade of enzymes involved in the activation of caspases. At relatively low physiological concentrations zinc blocks the activation of caspase-3 by cytochrome c in cell-free systems (Truong-Tran *et al.* 2000) possibly by affecting the activity of caspase-6, which zinc inhibits completely at 10 μM (Stennicke & Salvesen 1997; Takahashi *et al.* 1996). Caspase-6 initiates the dissolution of the cell nuclear membrane via cleavage of lamins and also cleaves and activates the proenzyme form of caspase-3 (Srinivasula *et al.* 1996). Since arsenic is known to affect the mitochondrial membrane potential (Larochette *et al.* 1999; Lorenzo *et al.* 1999; Susin *et al.* 2000), the point at which zinc is acting in our cells may be after the arsenic-induced release of mitochondrial factors such as cytochrome c.

Zinc also manifests antioxidant properties inside cells (Vallee & Falchuk 1993), which may directly

oppose the oxidising effects of arsenic. Zinc may also act to stabilise the cytoskeleton, preventing arsenic-induced disruption of cell membranes (Pfeiffer & Cho 1980; Johanning & O'Dell 1989). Zinc may also reduce arsenic-induced DEVD-caspase activity by inducing the synthesis of metallothionein, a metal-binding protein that binds seven zinc ions per protein molecule (Durnam & Palmiter 1981; Vallee & Falchuk 1993). Metallothionein has the potential to scavenge for arsenic and reduce its damaging effects by binding up to six As(III) moieties per molecule (Toyama *et al.* 2002).

In summary, we have shown that arsenic induces apoptosis in a neuronal cell line, 2.3D, and that zinc reduces arsenic-induced apoptosis as measured by DEVD-caspase activity. This beneficial effect of zinc suggests a possible role for zinc in arsenic toxicity, as a preventative or as a therapeutic agent.

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