Arsenite-induced apoptosis is prevented by antioxidants in zebrafish liver cell line

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Abstract
This study evaluated oxidative stress-induced apoptosis as a possible mechanism of arsenite toxicity in zebrafish liver cell line (ZFL cells). The heat shock protein 70 (HSP70), a chaperone protein, appears to provide protection against oxidative stress and apoptosis. Using the MTT assay, we demonstrated that survival of ZFL cells treated with arsenite for 24 h decreased in a dose-dependent manner. The possible mechanisms that promote the cytotoxicity of arsenite were addressed. Cell viability assays revealed that arsenite caused a dose-dependent increase in cell death, and pretreatment of the ZFL cells with antioxidants blunted these effects. Antioxidants such as N-acetyl-cysteine (NAC, 5 mM) and dithiothreitol (DTT, 80 μM) significantly prevented ZFL cells from arsenite-induced death. Nuclear staining was performed using 1 μg/ml Hoechst, and cells were analyzed with a fluorescent microscope. Arsenite (30 μM) induced massive apoptosis that was identified by morphology and condensation and fragmentation of the nuclei of the ZFL cells. Pretreatment with NAC or DTT before arsenite insult effectively protected the cells against oxidative stress-induced apoptosis from the arsenite. Using a transfected human hsp 70 promoter-enhanced green fluorescent protein (EGFP) reporter, pHsp70-EGFP, the induction of HSP70 by arsenite was observed. The induction of HSP70 by arsenite increased in a dose-dependent manner, and pretreatment of transfected ZFL cells with NAC or DTT before arsenite insult reduced EGFP expression. Taken together, our results provide evidence that stimulation of the heat shock response is a sensitive biomarker of arsenic exposure and that arsenite causes oxidative stress-induced apoptosis in ZFL cells.

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1. Introduction
Arsenic has been classified as the principal and most potent hazardous substance in according to the 2005 ATSDR/EPA and exists in a variety of chemical forms that can be absorbed easily by aquatic organisms. Organoarsenic compounds predominate in marine organisms (Francesconi et al., 1999). Whereas the main source of environmental arsenic exposure in most populations is drinking water, in which inorganic forms of arsenic (trivalent arsenite and pentavalent arsenite) (NRC, 1999). In the recent years, the term ‘Arsenicosis’ is being used to denote arsenic toxicity (Dhar et al., 2005).

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Epidemiological evidence strongly supports an association between chronic exposure to arsenite and increased risk for cancer of the skin, lung, liver, and prostate among residents of areas with high contents of arsenite in drinking water (Chen et al., 1992). Arsenite may interfere with the DNA repair system or DNA methylation state, cause DNA damage via the production of ROS (reactive oxygen species), inhibit p53 and telomerase activities, and promote cell proliferation and signal transduction pathways leading to the activation of transcription factors (Nakamuro and Sayato, 1981; Hamadeh et al., 1999; Matsui et al., 1999; Chou et al., 2001; Kitchin, 2001; Wang et al., 2001).

Although, reliable data for extrapolating environmental hazards such as heavy metals to humans are often obtained through laboratory rodent studies, laboratory rodents are expensive, and time consuming. As a surrogate for rodent experiments, the zebrafish and its cell lines offer an attractive alternative for evaluating the safety, toxicity, and mechanism of action of chemicals through low-cost, high-throughput screening. Although the toxicity of arsenite is well-known to many mammalian cells, arsenite-induced toxicity to aquatic animal species is still unclear. Arsenite toxicity has been reported in two fish cell lines (fin cells of Therapon jarbua and ovary cells of Tilapia), but the cytotoxicity was very particular according to each fish cell line (Wang et al., 2004). For example, although numerous studies have reported that arsenic compounds may damage cells by producing the oxygen radicals, hardly any protection of antioxidants, N-acetyl-cysteine (NAC) and dithiothreitol (DTT) to ovary cells of Tilapia implicates the disassociation of oxidative stress with the arsenite-induced reduction of the survival of ovary cells of Tilapia (Wang et al., 2004). With this discrepancy, more fish cell line models are needed for studying arsenite toxicity.

We have developed transgenic zebrafish lines with response elements such as aryl hydrocarbon and heat shock to indicate exposure to specific toxicants (Seok et al., 2004; 2006). Since, aquatic organisms such as fish are particularly susceptible to environmental toxicants, whole fish and fish cell cultures have long been used in standardized toxicity tests (Bols et al., 1985; Powers, 1989). Because, whole fish are inconvenient, time consuming, difficult to reproduce, and require sacrificing the organisms, for several decades fish cell lines have been developed as potential surrogates for an entire fish for studying the cytotoxicity and the genotoxicity of single compounds or environmental samples (Castano et al., 2003).

The aim of this investigation was to analyze the mechanisms involved in HSP70 induction by arsenic, using zebrafish liver cell line (ZFL cells). Therefore, in this study, the relationship of arsenic exposure and the levels of HSP70 expression were investigated in correlation with oxidative stress-induced apoptosis. Also an attempt was made to determine whether arsenic-induced apoptosis was modulated by antioxidants, NAC and DTT.

2. Materials and methods

2.1. Materials

Sodium m-arsenite (NaAsO₂), NAC, DTT, trypan blue (TB), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Hoechst 33258 were purchased from Sigma (St. Louis, MO). All tissue-culture products were from Gibco (Grand Island, NY).

2.2. Cell culture

We cultivated adult zebrafish liver cell line (ATCC CRL-2643) in ZFL medium consisting of Leibovitz’s L-15, Dulbecco’s Modified Eagle’s Medium (DMEM), and Ham’s F12 (50:35:15) supplemented with 10 mg/ml insulin, 5% fetal bovine serum, and 50 ng/ml epidermal growth factor (EGF) at 28 °C, as described by Ghosh et al. (1994). Cells were grown in 75 mm² tissue culture plates and the culture medium was changed every other day.

2.3. Arsenite treatment

After pretreatment with or without anti-oxidative modulators such as 5 mM NAC and 80 μM DTT in serum-free L-15 medium for 2 h, 5 × 10⁶ ZFL cells undergoing exponential growth were treated with various concentrations of arsenite for 24 h. Arsenite was dissolved in deionized water to create stock solutions of 800 times the indicated concentration (0–300 μM) of arsenite in 5 ml serum-free L-15 medium and then washed before conducting the bioassays. All experiments were carried out in three replicates.

2.4. Cell viability assay

2.4.1. MTT assay

The MTT test assesses cell metabolism based on the ability of the mitochondrial succinate–tetrazolium reductase system to convert the yellow compound MTT to a blue formazan dye. The amount of dye produced is proportional to the number of live metabolically active cells. The assay was carried out as previously described (Mosman, 1983). Briefly, the cells were seeded in 24-well microplates and incubated overnight. Then the cells were treated arsenite as explained in arsenite treatment. 5 mg/ml MTT was dissolved in phosphate-buffered saline (PBS), 50 μl of this stock solution was added to culture wells, and plates were incubated for 4 h at 37 °C. Supernatant was removed and 500 μl of DMSO was added to each well, and then transferred into 96-well microplate before reading the optical density at 580 nm with an EL800 microplate reader (BIO-TEK Instrument, Winoo-ski, VT).
2.4.2. Trypan blue (TB) exclusion test

Cell viability was determined by the TB exclusion test when NAC was employed due to NAC interference with the MTT assay (Desole et al., 1997). A viable cell will exclude acidic dye such as TB, and so its uptake is indicative of irreversible membrane damage preceding cell death. In brief, the cells were seeded in 24-well microplates and incubated overnight. After arsenite treatment (as explained in arsenite treatment), the cells were loaded with TB dye on a hematocytometer slide at the ratio 1:1 (v/v) and analyzed by light microscopy. The percentage of dead cells was determined by counting a total of 150–200 cells per independent experiment.

2.4.3. Apoptosis assay

Apoptosis of ZFL cells was analyzed with Hoechst 33258. The cells were seeded in 6-well microplates and incubated overnight. Then the cells were treated with arsenite as explained in arsenite treatment. Cells were fixed in 4% paraformaldehyde in PBS for 30 min, washed with PBS, and stained with 1 μg/ml Hoechst 33258 in PBS for 30 min. Stained cells were washed twice with PBS. The changes in nuclei were observed with a fluorescent microscope (Olympus, Melville, NY) through a UV filter. Apoptotic cells were identified by morphology and by condensation and fragmentation of their nuclei. The percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cells counted, multiplied by 100. Three separate experiments were conducted, and at least 300 cells were counted for each experiment.

2.4.4. Transient transfection studies with a human hsp70-regulated plasmid vector

For construction of the human hsp70 promoter-enhanced green fluorescence protein (EGFP) reporter, pHsp70-EGFP, the vector was constructed by fusing a

Fig. 1. Dose-response curve between arsenite concentration and survival rates of ZFL cells. Cells were treated with the indicated concentration of arsenite for 24 h, and cell-survival rates were determined using the MTT assay. The results are expressed as the percentage of the survival rate of control cells. All values are represented as means ± S.D. of at least three independent experiments.

Fig. 2. Arsenite-induced alterations in the morphology of ZFL cells. ZFL cells were exposed to arsenite (0, 10, and 30 μM) for 24 h in the absence or presence of NAC (5 mM) or DTT (80 μM). NAC or DTT was added 2 h before the addition of arsenite. Swollen and multinucleated ZFL cells were observed after arsenite treatment (10, and 30 μM).

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portion of a heat shock response element sequence, an inverted CAAT-box, and a TATA-box. This oligonucleotide (Genbank accession no. X04676) was ligated into the pEFGP-N1 vector (Clontech, Sparks, MD), using AscI and NheI sites (Seok et al., 2006). One day before transfection, 1.0 × 10^5 ZFL cells per well were plated in 500 μl growth medium without antibiotics, resulting in 90–95% confluence at the time of transfection. Lipofectamines² 2000 (Invitrogen, Carlsbad, CA) was used according to the manufacturer’s recommendations for the transient transfection studies. The cells were observed with a Nikon TE-2000 confocal microscope (Nikon, Tokyo, Japan) with a laser that emits at 488 nm. The display merged the differential interference contrast (DIC) image with the EGFP expression fluorescence image.

2.4.5. Statistical analysis

Data are represented as means ± SD. Data were subjected to statistical analysis with the general linear model procedure (SAS, Inc., Cary, NC). Two-way analysis of variance was used to compare the mean values between the mock-treated and pretreatment experimental groups. A significance level of \( P < 0.05 \) was used in all statistical analyses.

3. Results

3.1. Exposure of ZFL cells to arsenite

To study the cellular toxicity of arsenite in ZFL cells, we exposed the cells to different concentrations of arsenite and examined the correlation between arsenite concentration and cytotoxicity at designated time points. As shown in Fig. 1, the survival rate of arsenite-treated ZFL cells was expressed as a percentage of the survival rate of mock-treated control cells for 24 h using the MTT assay. Arsenite-induced ZFL cell death was dose-dependent; survival rates decreased as arsenite concentration increased (0–140 μM). A range of arsenite concentrations (10, 30, 60, and 100 μM) was selected to examine arsenite-induced apoptosis and EGFP expression in a human hsp70 promoter.

3.2. Effects of arsenite on general morphology of ZFL cells

Control ZFL cells attached firmly to the substratum with random orientations (Fig. 2). Following treatment with 10 μM arsenite for 24 h, ZFL cells apparently retracted and detached from the substratum. ZFL cells showed severe retraction and more rounding after treatment with 30 μM arsenite for 24 h. Multinucleated and apparently swollen cells appeared in the ZFL cells treated with 30 μM arsenite for 24 h. Cells were pretreated with an antioxidant, 5 mM NAC or 80 μM DTT, to examine whether oxidative stress could be involved in the arsenite-induced cytotoxicity in ZFL cells. Pretreatment with NAC or DTT effectively inhibited the arsenite-induced alterations in the morphology of ZFL cells. DTT pretreatment did not protect against arsenite-induced alterations as much as NAC. Multinucleated and apparently swollen cells were not observed in the ZFL cells treated with 30 μM arsenite for 24 h after 5 mM NAC pretreatment.

3.3. Cell viability assay

After the DTT pretreated cells were treated with arsenite, the MTT assay was performed to determine cellular survival rates. As presented in Fig. 3a, DTT pretreatment for 2 h significantly \( (P < 0.05) \) increased the survival rate of the ZFL cells treated with 60 and 100 μM arsenite for 24 h. Cell viability was assessed by TB exclusion for NAC pretreatment experiments as NAC interferes with MTT. NAC pretreatment for 2 h increased the survival rate of the ZFL cells treated with 60 μM arsenite for 24 h.

![Fig. 3.](image-url)

Fig. 3. Pretreatment with NAC or DTT before arsenite insult effectively protected the cells against oxidative-stress-induced cell death. MTT assay determined the cellular survival rate. (a) Arsenite-induced cell death in a time course. This effect can be countered by pretreatment with 80 μM DTT. The cell viability percentage was calculated as the ratio of apoptotic cells to total cells counted, multiplied by 100. (b) Cell viability was assessed by trypan blue exclusion when 5 mM NAC was employed, due to NAC interference with the MTT assay. Results are expressed as mean ± S.D. for triplicate samples. *Significantly different from control \( (P < 0.05) \).
and significantly \((P < 0.05)\) increased the survival rates of the ZFL cells treated with 100 \(\mu\)M arsenite for 24 h com-
pared with control cultures that were not pretreated (Fig. 3b).

**Fig. 4.** ZFL cells were exposed to arsenite (0, 10, and 30 \(\mu\)M) for 24 h in the absence or presence of NAC (5 mM) or DTT (80 \(\mu\)M). NAC or DTT were added 2 h before the addition of arsenite. Nuclear staining was performed using 1 \(\mu\)g/ml Hoechst and cells were analysed with a fluorescence microscope. (a) Representative morphological changes of control ZFL cells, pretreated with 5 mM NAC or 80 \(\mu\)M DTT induced massive apoptosis in ZFL cells. Pretreatment with NAC or DTT before arsenite insult effectively protected the cells against oxidative stress-induced apoptosis by arsenite. (b) Arsenite-induced apoptosis in a time course. This effect can be countered by 5 mM NAC or 80 \(\mu\)M DTT pretreatment. The percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cells counted, multiplied by 100. Gray bars indicate DTT and black bars indicate NAC pretreatment. Results are expressed as mean ± S.D. for triplicate samples. Reproducibility was confirmed in three separate experiments. *Significantly different from control \((P < 0.05)\).
3.4. DNA fragmentation induced by arsenite in ZFL cells and arsenite-induced apoptosis is correlated with oxidative stress

Arsenite induced massive apoptosis in ZFL cells. Apoptotic cells were identified by Hoechst staining of condensation and fragmentation of the nuclei (Fig. 4a, upper right panel). Pretreatment with NAC or DTT before arsenite insult effectively protected the cells against oxidative stress-induced apoptosis (Fig. 4a, lower panel). In the absence of NAC or DTT protection, the cell death caused by arsenite ($P < 0.05$) was dose-dependent (Fig. 4b).

3.5. Human HSP70-driven EGFP reporter assays

Arsenite inducibility of a pHhsp70 construct expressing the EGFP gene under the control of the exogenous human hsp70 promoter was tested in transfected ZFL cells using a transient expression system. The 30 μM arsenite caused stronger EGFP fluorescence than the 10 μM arsenite (Fig. 5). These results indicate that the induction of expressed human HSP70 protein by arsenite in ZFL cells was increased in a dose-dependent manner. Transfected cells were pretreated with the antioxidants 5 mM NAC or 80 μM DTT. EGFP fluorescence was absent or reduced in the transfected arsenite-treated ZFL cells in the presence of NAC or DTT (Fig. 5).

4. Discussion

In the present study, the cytotoxicity of arsenite to ZFL cells was explored. Morphological alterations are considered to be the primary indications of cytotoxicity and its underlying mechanisms.

Fish are particularly susceptible to environmental toxicants because they absorb contaminants across the gill epithelium and bioconcentrate it in the food chain. Although much of the previously published research has focused on arsenite toxicity in vivo, arsenite induces more dramatic and distinct toxicity with lower doses and shorter exposure time in fish cells, especially ZFL cells. For example, the toxic concentration of arsenite in Tilapia mossambica under a 7-day exposure was 10 mg/l (about 77 μM) (Suhammadatna et al., 2002), and the ovary cells of Tilapia showed indications of subacute exposure of arsenite at much lower doses (0.125–10 μM). Arsenite induced mitotic arrest and disturbed the cell cycle in the Tilapia cells (Wang et al., 2004).

Although the distinct arsenite-induced toxicity with low doses (10–30 μM, 1.3–3.9 mg/l) in ZFL cells was observed, the concentrations of arsenite that caused cytotoxicity was very high compared to concentrations of arsenite in the environment. Recently, the US Environmental Protection Agency to reduce the limit for the acceptable level of arsenic in drinking water by 80%, from 50 μg/l to 10 μg/l, according to many risks associated with these low levels of arsenic such as those found in drinking water in many areas of the United States, may also pose a substantial carcinogenic risk to humans for many tumors (Lewis et al., 1999; Morales et al., 2000). Much lower concentrations of arsenic in vitro systems to mimic chronic exposure should be warranted.

We have been developing a sentinel animal system that uses transgenic zebrafish with an easily assayable reporter
gene under the control of pollutant-inducible DNA response elements (e.g. aryl hydrocarbon, heat shock response elements) (Seok et al., 2004; 2006) for the detection of hazardous environmental chemicals. In the in vitro zebrafish cell line model, ZFL cells defend against toxic heavy metals with oxidative stress. Numerous studies have reported that arsenic compounds may damage cells by producing oxygen radicals (Burdon et al., 1987; Blair et al., 1990). Our study also provides evidence of the oxidative damage caused by arsenite in ZFL cells. The antioxidants, NAC and DTT, can protect ZFL cells from arsenite-induced death. This finding, combined with the results of the apoptosis analysis, implies that oxidative damage may be strongly associated with arsenite-induced apoptosis of ZFL cells. The amount of oxidative damage and genetic mechanisms of apoptosis induced by arsenite in ZFL cells are currently clarified by our further work.

Mosser et al. (2000) demonstrated that the chaperone function of HSP70 is necessary for protection against stress-induced apoptosis. Heat shock proteins have been proposed as molecular biomarkers for toxicity associated with physical and chemical stress (Sanders, 1993; Ryan and Hightower, 1994). A number of studies have also shown a relationship between the extent of hsp gene expression and the amount of the damage inflicted on cells. hsp gene expression has been used as an assay to evaluate chemical and heavy metal ion toxicity in fish cells (Pipkin et al., 1987; Hightower and Renfro, 1988; Aoki et al., 1990; Edwards et al., 1990; Goering et al., 1992). According to Lau et al. (2004) HSP70 was up-regulated in cultured lung cells treated with arsenite, causing oxidative stress-induced apoptosis. Our ZFL cell model confirmed this toxicity process and even showed that the use of a human promoter in zebrafish cell lines can produce an effective biosensor for human risks from environmental pollutants.

In conclusion, we have shown that arsenite induces apoptosis in ZFL cell line and oxidative stress induced HSP70 is preventable by antioxidants. Also the induction of a human HSP70-driven reporter gene may prove useful as a part of discovering the mechanism of cytotoxicity of arsenite. Further studies on the accumulation and transformation of arsenite with the zebrafish, danio rerio are warranted.

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