Differential Cytotoxic Effects of Arsenic on Human and Animal Cells

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Human fibroblasts (HFW) were 10-fold more susceptible than Chinese hamster ovary (CHO-K1) cells to sodium arsenite. Comparison of cellular antioxidant enzyme activities showed that CHO-K1 cells contained 3- and 8-fold more glutathione-peroxidase and catalase activities, respectively, than HFW cells. Since vitamin E, methylamine, and benzyl alcohol could prevent, in part, the arsenite-induced killing of HFW cells, we suggest that arsenite can induce oxidative damage in HFW cells. We have also established arsenic-resistant cells, SA7 and CL3R, from CHO cells and from a human lung adenocarcinoma cell line (CL3), respectively. The arsenic resistance of SA7 cells was attributed mainly to elevation of glutathione S-transferase α levels, and that of CL3R cells was possibly due to an increase in heme oxygenase activity. Since induction of heme oxygenase is a general response to oxidative stress, we suspect that the differential toxicity of arsenic to human and animal cells could be due to arsenic's more efficient induction of oxidative damage in human cells. — Environ Health Perspect 102(Suppl 3):101-105 (1994).

Key words: arsenic, cytotoxicity, arsenic resistance, glutathione S-transferase, heme oxygenase, oxidative damage

Introduction

Arsenic is widely distributed in nature and is released into the environment through industrial processes and agricultural usage (1). According to epidemiologic studies and clinical observations, arsenic is associated with increased risk for certain types of human cancers, including epidermoid carcinomas of skin, lung cancers, and possibly liver cancers (2-4). However, treatment with the antiparasitic drug, mebendazole, has failed to induce tumors in most laboratory animals (5,6). This apparent contradiction has led to the suggestion that arsenic may act as a co-carcinogen (5,6). However, this phenomenon could also be due to metabolic differences between humans and experimental animals (7,8), or a combination of both. We have previously shown that cultured human skin fibroblasts (HFW) are more susceptible to the toxic effects of sodium arsenite than are Chinese hamster ovary (CHO) cells (9). However, the exact killing mechanism of sodium arsenite remains to be elucidated. Therefore, we further investigated what kinds of agents could differentially protect HFW and CHO cells from the killing effects of arsenic. We also established arsenic-resistant cells from human and animal cell lines and investigated their resistance mechanisms as an alternative approach to understand the toxic mechanisms of arsenic.

Materials and Methods

Cell Culture

Media and chemicals used for cell culture were purchased from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was obtained from HyClone Laboratories, Inc. (Logan, UT). CHO, SA7, and HFW cells were cultured as described previously (9,10). The culture conditions for CL3 cells were F12 medium supplemented with 10% FBS and antibiotics, and an atmosphere of 5% CO2/95% air. Arsenic-resistant CL3 cells (CL3R) were established by progressively increasing the concentration of arsenite in the culture medium. CL3R and SA7 cells were grown in the presence of 4 and 30 μM sodium arsenite, respectively.

Survival Assay

The fraction of CHO or HFW cells that survived after drug treatment was determined by the colony-forming method as described previously (9). The averaged colony forming efficiencies of untreated HFW and CHO-K1 cells were 40 and 90%, respectively. Since CL3 cells at very low cell density could not form colonies, the proliferation rates were used to analyze the sensitivity of CL3 and CL3R cells to drugs.

Preparation of Cell Extracts for Enzymatic Assays

Exponentially growing cells were washed twice with ice-cold phosphate-buffered saline and scraped off the culture dishes with a rubber policeman. Cells were spun at 1,000 × g for 10 min at 4°C and resuspended in an appropriate amount (approx. 4 × 10^6 cells/ml) of 0.1 M potassium phosphate buffer, pH 6.8, and transferred into Eppendorf tubes. The cells were sonicated three times (10-sec burst with 1-min intervals) with a Heat System-Ultrasonics W-380 sonicator. After centrifugation at 12,000 × g for 20 min at 4°C, the clear supernatant was kept on ice prior to enzymatic assays. Protein concentration in cell extracts was determined by the method of Bradford (11) using bovine serum albumin as the standard.

Biochemical and Enzymatic Assays

Glutathione (GSH) levels were measured by a fluorometric method (12). GSH transferase activities were measured by the methods of Habig et al. (13) and Habig and Jakoby (14), using reduced GSH and 1-chloro-2,4-dinitrobenzene as substrates. GSH peroxidase activities, using hydrogen peroxide or cumene hydroperoxide as substrates, were analyzed according to Lawrence and Burk (15). GSH reductase activity was measured according to the method described by Calberg and Mannervik (16). Catalase activity was assayed by monitoring the decomposition of hydrogen peroxide, followed directly by measuring the decrease in absorbance at 240 nm (17). Postmitochondrial fractions, which were obtained by further spinning the cell extracts at 100,000 × g for 30 min, were used for assay of superoxide dismutase (18).

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Table 1. Comparison of several enzymatic and biochemical parameters in HFW and CHO-K1 cells.a

| Parameters                        | CHO-K1       | HFW         | Ratio, CHO/MFW |
|----------------------------------|--------------|-------------|----------------|
| Glutathione, nmol/mg of protein  | 14.5 ± 1.6a  | 10.9 ± 1.3  | 1.4            |
| Glutathione S-transferase, nmol of CDNB/min/mg of protein | 78.1 ± 3.4  | 87.8 ± 3.9  | 1.2            |
| Glutathione peroxidase, nmol of NADPH/min/mg of protein | 152 ± 1.5    | 50 ± 0.9    | 3.0            |
| With cumene peroxide             | 49.7 ± 2.7   | 20.8 ± 3.7  | 2.4            |
| With H2O2                        | 41.8 ± 2.7   | 30.6 ± 0.1  | 1.4            |
| Glutathione-reductase, nmol of NADPH/min/mg of protein | 61.0 ± 2.9   | 73.3 ± 0.7  | 1.2            |
| Catalase, umol H2O2/min/mg of protein | 28.7 ± 1.7  | 70.7 ± 3.5  | 0.4            |
| Superoxide dismutase, u/mg of protein | 0.24 ± 0.02  | 0.32 ± 0.02  | 0.75           |
| Proteins, mg/10^6 cells          |              |             |                |

aMean ± SD of a minimum of three experiments.

Protein Synthesis Analysis

Heme oxygenase has been reported to be an arsenic-induced 32-kDa stress protein (19). The synthesis of heme oxygenase can be easily detected using [35S]methionine incorporation, SDS polyacrylamide gel separation, and autoradiography. Briefly, the cells were labeled with [35S]methionine (specific activity >800 Ci/m mole; Amersham, Buckinghamshire, England) for the length of time indicated. At the end of labeling, the cell pellets were solubilized in an electrophoretic buffer containing 50 mM Tris, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 15% glycerol, and 0.005% bromophenol blue. The cellular polypeptides (100,000 cpm equivalent) were then analyzed on a 12.5% SDS-polyacrylamide gel (20), and visualized by autoradiography.

Western Blot Analysis

Antiserum against glutathione S-transferase (GST) was raised in New Zealand rabbits by subcutaneous injection of 100 to 225 μg of purified enzyme several times. Western blot analysis was performed according to the method described by Burnette (21). Alkaline phosphatase-conjugated anti-rabbit IgG was used as the second antibody to visualize the GST that has been transferred onto nitrocellulose paper.

Results

Cytotoxic Effects of Sodium Arsenite to Chinese Hamster Ovary Cells and Human Fibroblasts

As shown in Figure 1, the values of D37 for CHO-K1 cells and HFW cells were estimated to be 12.0 μM and 1.2 μM, respectively. Thus, CHO-K1 cells were 10-fold more resistant than HFW cells to sodium arsenite. In addition, there was apparently a shoulder on the survival curve of CHO-K1 cells treated with sodium arsenite. These results are consistent with those of our previous studies (9). Several possibilities have been proposed to explain the differential cytotoxicity of sodium arsenite to human fibroblasts and CHO cells. These included differences in GSH levels, membrane transportation, stress protein induction, and delay of cell-cycle progression (9). To further elucidate the killing mechanism of arsenic, the effects of several other factors on arsenic’s cytotoxicity were compared between these two cell types.

The effects of vitamin E, a well-known antioxidant (22,23), was investigated. As shown in Figure 2, pretreatment of cells with vitamin E partially protected HFW but not CHO-K1 cells from the killing effects of sodium arsenite.

Methylamine is a lysosomotropic amine that inhibits protein degradation by alkalinizing acidic vacular compartments (24); and benzyl alcohol is a membrane-perturbing agent that can inhibit the autophagic degradation of proteins by preventing the formation of autophagic vacuoles (25). As shown in Figure 3, these inhibitors reduced the toxicity of sodium arsenite to HFW cells, but not to CHO-K1 cells.

Several antioxidant activities, including GSH, GSH-transferase, GSH-peroxidase, GSH-reductase, catalase, and superoxide dismutase were measured in HFW and CHO-K1 cells (Table 1). All of these antioxidant activities, except superoxide dismutase, were higher in CHO-K1 cells than in HFW cells. In particular, CHO-K1 cells contained approximately 3-fold more GSH peroxidase and 8-fold more catalase activity than HFW cells.

Establishment and Characterization of Arsenic-resistant Cells

To understand the killing effects of arsenic on human and animal cells, our alternative approach was to establish arsenic-resistant cells. Arsenic-resistant CHO cells (SA7) and CL3 cells (CL3R) were established by progressively increasing the concentration of sodium arsenite in the culture medium as follows. The SA7 and CL3R cells were maintained in the presence of 30 M and 4 M sodium arsenite, respectively, and they are 9- and 6-fold more resistant than their parental cell lines, respectively (Table 2).

The resistance of SA7 cells to arsenic has been previously demonstrated to be associated with elevated GSH levels and

Table 2. Establishment of arsenic-resistant cell lines.a

| Parental cell lines | Initial conc., M | Final conc., M | As-resistant cells |
|---------------------|-----------------|----------------|-------------------|
| Chinese hamster ovary cells (CHO) | 5               | 30             | SA7               |
| Human lung adenocarcinoma cells (CL3) | 0.5           | 4              | CL3R              |

aAs-resistant cells were established by progressively increasing the concentration of sodium arsenite in culture medium.
glutathione S-transferase activities in SA7 cells (10). According to its substrate speci-

ficity and N-terminal amino acid sequence, the GST that is elevated in SA7 cells has

been identified to be a π class GST (26). As shown in our previous report, arsenic resistance of these cells was correlated well with their GST activity (26).

The recently established CL3R arsenic-resistant cells were routinely main-
tained in medium containing 4 μM sodium arsenite. The estimated ID₅₀ values for CL3 and CL3R cells were 2 M and 12 M, respectively (Figure 4). Protein synthesis in CL3 and CL3R cells was exam-

ined using a [³⁵S]methionine-labeling technique. As shown in Figure 5, sodium arsenite apparently induced the synthesis of a set of stress proteins including 90-, 70-, and 32-kDa proteins in CL3 cells. However, only the 32-kDa protein, which has been demonstrated to be heme oxygenase, was constitutively expressed in CL3R cells (Figure 6A). Western blot analysis demonstrated that there was no increase in the amount of GST in CL3 cells (Figure 6B).

Discussion

Our present data are consistent with our previous results showing that HFW cells were 10-fold more susceptible than CHO-K1 cells to sodium arsenite (9). Since an antioxidant, vitamin E, could protect HFW cells but not CHO-K1 cells from sodium arsenite-induced killing, the sensi-
tivity of HFW cells to sodium arsenite may be partly attributed to oxidative damage. Numerous recent studies have reported that arsenic compounds may damage cells through the production of oxygen radicals (27–29). Our present results also showed that the cytotoxicity of sodium arsenite to HFW cells, but not to CHO-K1 cells, was apparently inhibited by a lysosomotropic amine (methylamine) and a membrane-perturbing agent (benzyl alcohol). Both agents are inhibitors of autophagic degra-
dation of proteins that are possible sources of cellular ferric iron (30,31). Iron ions usually participate as catalysts in Fenton-like reactions in which hydroxyl radicals will be generated. Since arsenic, generally considered to be a sulfhydryl reagent, can interact with sulfhydryl groups of functional mole-
cules, this may result in denaturation of proteins and enzymes. Thus, treatment of

Figure 2. Effects of vitamin E on cytotoxicity of sodium arsenite to HFW and CHO-K1 cells. Cells were incubated with 100 M vitamin E for 4 hr and subsequently treated with various concentrations of sodium arsenite for 6 hr (HFW) or 12 hr (CHO-K1). The relative survival was determined by colony-forming assay. Data are expressed as mean SD of three experiments.

Figure 3. Effects of methylamine and benzyl alcohol on cytotoxicity of sodium arsenite to HFW and CHO-K1 cells. Cells were simultaneously treated with various concen-

trations of sodium arsenite and methylamine (2 mM) or benzyl alcohol (20 mM) for 6 hr. The relative survival was determined by colony-forming assay. Data are expressed as mean ± SD of three experiments.

Figure 4. Cytotoxic effect of sodium arsenite to parental CL3 and CL3R cells. Cells were treated with various concentrations of sodium arsenite for 4 days. The cell numbers were determined by hemocytometer. Bars indicate SD of three independent experiments.

Figure 5. Induction of stress proteins in CL3 and CL3R cells by sodium arsenite. The cells were treated with various concentrations of sodium arsenite for 6 hr. Protein synthesis was examined by [³⁵S]methionine incorporation. [³⁵S]methionine-labeling was performed during the last hour of sodium arsenite treatment. Protein composition was examined by SDS-polyacrylamide gel electrophoresis and autoradiography.

Figure 6. (A) Constitutive expression of heme oxygenase in CL3R cells. Both CL3 and CL3R cells were labeled with [³⁵S]methionine for 48 hr. Protein composition was exam-

ined by SDS-polyacrylamide gel electrophoresis and autoradiography. (B) Levels of GST in CHO-K1, CL3, and CL3R cells. The levels of GST in these three cell lines were analyzed by Western blotting after electrophoresis (lower part). The upper part shows the Coomassie blue-
stained gel.
cells with sodium arsenite may increase the level of denatured proteins, accelerate cellular autophagic degradation, and promote cell injury through the release of iron and the generation of oxidative damage.

The susceptibility of cells to oxidative damage often depends on their antioxidant defense capacity (32-34). CHO-K1 cells contain very high GSH-peroxidase and catalase activities that catalyze dismutation of H₂O₂. Hydrogen peroxide is an important reactive oxygen intermediate and also a source of hydroxyl radicals, very active molecules responsible for most biological damage (35). Therefore, the high levels of GSH-peroxidase and catalase in CHO-K1 cells certainly play an important role in protecting CHO-K1 cells from oxidative damage induced by sodium arsenite. Although our previous results have demonstrated a good correlation between the levels of GST π and arsenic resistance (10,26), the role of GST π in arsenic resistance remains to be elucidated further. The relative abundance of GST π in SA7 cells implies that it possibly acts as a carrier or binder to eliminate certain injurious substances induced by arsenic treatment; i.e., GSTs may trap these active radicals or deleterious molecules produced during metabolic transformation of arsenic. In fact, GSTs have been proposed to serve as potent binders of lipophilic toxins (36-38). Alternatively, GSTs may catalyze the conjugation of GSH and inorganic arsenic or may participate in the methylation of inorganic arsenic.

The present results show that CL3R cells are 6-fold more resistant than CL3 cells to arsenite, and that heme oxygenase is overexpressed in CL3R cells. We have confirmed the overexpression of heme oxygenase by Northern analysis in an preliminary study (data not shown). The induction of heme oxygenase is a general response to oxidant stress in mammalian cells (39). Heme oxygenase is proposed to protect the cells from oxidant stress by rapidly reducing cellular heme pools and consequently inhibiting the generation of oxygen radicals. Our preliminary studies have also revealed that CL3R cells are cross-resistant to atriadmycin and menadione, both of which are oxygen radical-generating agents (data not shown). Therefore, we suspect that arsenic may induce oxidative damage through an unknown mechanism. These findings also support the view that the differential toxic effects of arsenic on human and animal cells could be due to different efficiencies of inducing oxidative damage.

Cellular prooxidant states have recently been shown to exert a growth-promoting effect and to participate in radiation and chemical-induced carcinogenesis, especially in tumor promotion and progression (40-43). However, the molecular mechanisms of oxygen radical carcinogenesis are far from clear. Thus, additional studies are necessary to elucidate the role of arsenic-induced oxidative damage in arsenic's genotoxicity and carcinogenicity in humans.

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