Expression of Heme Oxygenase in Arsenic-resistant Human Lung Adenocarcinoma Cells

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ABSTRACT

We have established arsenic-resistant cells (CL3R) and their subclones from a human lung adenocarcinoma cell line (CL3). CL3R cells and their subclones were maintained in the presence of 4 μM sodium arsenite. They were 6-fold more resistant than CL3 cells to arsenite. Heme oxygenase was expressed in CL3R cells and their subclones, as demonstrated by electrophoretic analysis, Northern blotting, and enzyme activity assay. When CL3R15 cells were grown in arsenite-free medium, their arsenite resistance declined in parallel with their decreasing heme oxygenase activity. Tin-protoporphyrin, a heme oxygenase inhibitor, was found to increase the toxicity of arsenic to CL3R cells. Expression of heme oxygenase might therefore be involved in the mechanism of arsenic resistance. CL3R cells were also shown to be cross-resistant to oxygen-radical generating agents, such as menadione and Adriamycin. Furthermore, sodium arsenite treatment dose-dependently increased the dichlorofluorescein fluorescence in CL3 cells but not in CL3R15 cells. These results suggest that heme oxygenase plays an important role in reducing cellular oxidants that are enhanced by sodium arsenite treatment.

INTRODUCTION

Arsenic, a widely distributed toxic element in nature, is released into the environment through industrial processes and agricultural usage (1). Epidemiological studies and clinical observations strongly suggest that arsenic is closely associated with increased risk for certain types of human cancers, including epidermoid carcinomas of skin, lung cancers, and possibly liver cancers (2, 3). However, the failure of arsenic to induce tumors in most experimental animals has led to the suggestion that arsenic may act as a cocarcinogen (4). Alternatively, the differential carcinogenicity of arsenic can be attributed to metabolic differences between humans and experimental animals (5, 6), or a combination of both factors. A marked difference in the metabolism of arsenic in different species has already been reported (6, 7). To understand the toxic effects of arsenic in mammalian cells, we have previously isolated an arsenic-resistant cell line (SA7) from Chinese hamster ovary cells (8). The resistance to arsenic was found to be associated with elevated levels of GSH3 and GSTγ (8, 9). GSTγ has been demonstrated to facilitate the excretion of arsenic from SA7 cells (10). Recently, arsenic-resistant cells (CL3R) and their subclones derived from a human lung adenocarcinoma cell line (CL3) were established in our laboratory. Instead of GST overexpression, HO (EC 1.14.99.3) is expressed in CL3R cells and their subclones.

MATERIALS AND METHODS

Cell Culture. Media and chemicals used for cell culture were purchased from Gibco (Grand Island, NY). Fetal bovine serum was obtained from HyClone Laboratories, Inc. (Logan, UT). CL3 cells, kindly provided by Dr. P. C. Yang (National Taiwan University Hospital), were derived from a human lung adenocarcinoma from a 60-year-old male patient (11). CL3 cells were grown in F12 medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin), in a humidified atmosphere with 5% CO2.

Selection of Arsenic-resistant Cells. Arsenic-resistant cells, designated CL3R cells, were established by progressively increasing the concentration of sodium arsenite from 0.5 to 4 μM in the culture medium. CL3R cells were finally maintained in the presence of 4 μM sodium arsenite. Several arsenic-resistant subclones were isolated by the aid of cloning cylinders and maintained as CL3R cells. Six subclones (CL3R3, CL3R5, CL3R12, CL3R13, CL3R14, and CL3R15) were used for elucidating arsenic resistance mechanisms.

Resistance Assay. Due to the low colony-forming efficiency of CL3 cells, the susceptibility of CL3 and CL3R cells and arsenic-resistant subclones to sodium arsenite, menadione, and Adriamycin was assessed by their proliferation rates. In brief, cells were seeded at 1.5 × 10^5 cells/35-mm dish 1 day prior to experimental manipulation. The cultures were either treated with various concentrations of sodium arsenite or menadione continuously for 4 days, or treated with Adriamycin for 1 h and then incubated with normal medium for 4 days. At the end of treatment, cell numbers were counted with a hemocytometer.

Biochemical and Enzymatic Assays. GSH levels were measured by a fluorometric method (12). GST activities were measured by the methods of Habig et al. (13) and Habig and Jakoby (14). GST peroxidase activity and GSH reductase were analyzed according to the methods of Lawrence and Burk (15) and Calberg and Mannervik (16), respectively. Catalase activity was assayed by monitoring the decomposition of hydrogen peroxide (17). Postmitochondrial fractions were used for assay of SOD (18). Protein concentration in cell extracts was determined by the method of Bradford (19) using bovine serum albumin as the standard.

Analysis of Heme Oxygenase. Heme oxygenase was analyzed by 35S-methionine labeling, Northern blotting technique, and activity assay. An arsenic-induced M, 32,000 stress protein identified to be HO can be easily detected using 35S-methionine incorporation, sodium dodecyl sulfate-polyacrylamide gel separation, and autoradiography (20). Briefly, the cells were labeled with 35S-methionine (specific activity, >800 Ci/mmol; Amersham) for 24 h. The cellular polypeptides (100,000 cpm equivalent) were then analyzed on a 12.5% sodium dodecyl sulfate-polyacrylamide gel (21), and visualized by autoradiography.

An oligomer complementary to nucleotides 83–116 of HO mRNA, 5'TTCTGAAGGTCTCCTAGAACCTGACTTCTTT (22), was synthesized as a probe to detect the levels of HO mRNA in CL3 and arsenic-resistant subclones by Northern blotting techniques (23). Total cellular RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform extraction procedure (24).

Heme oxygenase activity was analyzed by a HPLC technique as described by Bonkovsky et al. (25) and Lincoln et al. (26) with modification. Microsome fractions were first prepared from the parental and arsenic-resistant cells by centrifugation of cell lysates at 105,000 × g for 1 h (26). An aliquot of the microsome fraction (500 μg protein equivalent) was added into a 100-μl reaction mixture containing 1 mM NADPH, 0.5 mM defereroxamine, 1 μM hemin, 0.05 mg/ml bovine serum albumin, 0.1 M potassium phosphate (pH 7.4), and perfused rat liver homogenate (200 μg protein equivalent) as a source of biliverdin reductase. The reaction was carried out at 37°C for 6 min, and stopped by addition of an equal volume of ethanol-dimethyl sulfoxide (95/5, v/v) containing 100 nmol mesoporphyrin IX (Porphyrin Products, Logan, UT) which served as an internal control for estimation of bilirubin production. After standing on ice for 2 min, the reaction mixture was centrifuged at 10,000 × g for 5 min, and 100 μl of clear supernatant were injected onto an HPLC Nova-Pak C18 column (3.9 × 150 mm). The chromatography conditions were...
as follows: 80% solvent A/20% solvent B for 2 min; a linear gradient from 20 to 100% solvent B for 10 min (2 to 12 min); and then 100% solvent B for 6 min (12 to 18 min). A flow rate of 1.5 ml/min was maintained throughout, and the porphyrins were monitored at 405 nm. Solvent A was composed of 56% 0.1 M ammonium phosphate solution, pH 3.5, and 44% methanol; solvent B was pure methanol.

**Effect of Heme Oxygenase Inhibitor on Arsenic Resistance of CL3R15 Cells.** Since treatment with SnPP, a HO inhibitor, significantly reduced the proliferation rate, this experiment used the colony forming method. CL3R15 cells, which have a plating efficiency of 30–40%, were plated at 600 cells/60-mm dish. After overnight incubation, the cells were treated with a combination of 20 μM sodium arsenite and various concentrations of SnPP for 24 h. Afterwards, the cells were incubated in drug-free medium for 9 days. The colonies (>50 cells) were fixed, stained, and counted as described previously (27).

**Effect of Arsenite-free Medium on Arsenic Resistance of CL3R15 Cells.** CL3R15 cells grown in normal medium without sodium arsenite for 1, 3, and 7 days were replated at 600 cells/60-mm dish. After overnight incubation, they were treated with various concentrations of sodium arsenite for 24 h. Their survival rate was determined by the colony forming method described above.

**Detection of Dichlorofluorescein Fluorescence in Cells.** The method of Huang et al. (28) with modification was adopted to determine the increase in intracellular oxidants in arsenite-treated CL3 and CL3R15 cells. The logarithmically growing cells were treated with sodium arsenite for 24 h, and harvested by trypsinization. An aliquot of cells (1.5 × 10⁶ cells) was resuspended in 2 ml of phosphate-buffered saline containing 80 μM nonpolar 2',7'-dichlorofluorescein diacetate. The reaction was carried out at 37°C for 30 min in the dark, and stopped by centrifugation at 200 × g for 5 min. The cell pellets were resuspended in 3 ml of phosphate-buffered saline. The relative fluorescence intensity of DCF, which is formed by peroxide oxidation of its nonfluorescent precursor (nonfluorescent 2',7'-dichlorofluorescein), was determined at an emission wavelength of 525 nm by using an excitation wavelength of 475 nm.

**RESULTS**

CL3R cells and their 6 arsenic-resistant subclones were approximately 6-fold more resistant than their parental cell line (Fig. 1). The estimated ID₅₀, values for CL3 and CL3R cells were 2 and 12 μM sodium arsenite, respectively. Most (98%) of the CL3 cells contained 46 chromosomes. However, >90% of the CL3R cells and the subclones contained 48 chromosomes, and no double minutes or homogeneous staining regions were observed in CL3R cells.

As summarized in Table 1, levels of GSH in CL3R cells were double those in CL3 cells, but there was no apparent difference in activities of GST and GSH peroxidase. Catalase and SOD activities in CL3R cells were 30 and 60%, respectively, of those in parental CL3 cells. By Western blot analysis, there was no overexpression of GST or multiple drug-resistant glycoprotein (data not shown).

The protein compositions of CL3 cells and arsenic-resistant subclones were examined by [³⁵S]methionine labeling. When the cells were continuously labeled with [³⁵S]methionine for 24 h, a Mr 32,000 protein was overexpressed in these arsenic-resistant cells (Fig. 2A). This Mr 32,000 protein comigrated with arsenic-induced Mr 32,000 stress protein (data not shown), which has been identified to be HO.

By using a synthetic oligonucleotide as a probe in Northern blot analysis, a significant amount of HO mRNA was detected in arsenic-resistant subclones, whereas almost none was detected in parental cells (Fig. 2B). However, treatment of CL3 cells with 50 μM sodium arsenite for 30 min induced a tremendous increase in HO mRNA levels (Fig. 2B, Lane 2).

To confirm the expression of HO, its activity was measured by HPLC. By using a Nova-Pak CL column, hemin (substrate), mesoporphyrin IX (internal control), and bilirubin (final product) could be well separated (Fig. 3). As shown in Table 2, CL3R cells and their subclones contained HO activity at a range from 49.2 to 67.9 pmol bilirubin/min/mg microsomal protein, while no detectable HO activity was observed in CL3 cells. Similar to the results of our Northern blot analysis, HO activity in CL3 cells was induced from 0 to 29.4 pmol bilirubin/min/mg microsomal protein by treatment of CL3 cells with 10 μM sodium arsenite for 6 h.

As shown in Table 3, HO activity rapidly declined when the cells were cultured in normal medium. Their resistance to arsenite also significantly decreased (Fig. 4A). The lethal effects of sodium arsenite to CL3R15 cells were also significantly increased by incubating the cells with sodium arsenite and SnPP, an inhibitor of HO (Fig. 4B). Under the same treatment conditions, SnPP did not decrease GSH.

![Fig. 1. Cytotoxic effect of sodium arsenite on parental CL3 cells, CL3R, and subclones of CL3R cells. Cells were treated with various concentrations of sodium arsenite for 4 days. The cell numbers were determined by hemocytometer. Average of 3 independent experiments. ○, CL3; ●, CL3R; ◆, CL3R3; ▲, CL3R5; ■, CL3R12; ○, CL3R13; ▼, CL3R14; ▽, CL3R15 cells.](image)

**Table 1 Comparison of several enzymatic and biochemical parameters of CL3 and CL3R cells**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CL3</th>
<th>CL3R</th>
<th>CL3R/CL3</th>
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<tr>
<td>GSH (nmol/mg protein)</td>
<td>50.7 ± 5.5</td>
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<td>GSH S-transferase (nmol CDNB/min/mg protein)</td>
<td>73.3 ± 5.7</td>
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<tr>
<td>GSH peroxidase (nmol NADPH/min/mg protein)</td>
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<tr>
<td>With CaO⁴⁺</td>
<td>37.0 ± 0.6</td>
<td>36.2 ± 2.8</td>
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<tr>
<td>With H₂O₂</td>
<td>13.5 ± 2.5</td>
<td>3.8 ± 0.2</td>
<td>0.3</td>
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<tr>
<td>Superoxide dismutase (units/mg protein)</td>
<td>250.5 ± 12.5</td>
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<tr>
<td>Proteins (mg/10⁶ cells)</td>
<td>0.32 ± 0.07</td>
<td>0.32 ± 0.06</td>
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Fig. 2. Constitutive expression of heme oxygenase in CL3R cells and the subclones. A, cells were continuously labeled with [35S]methionine for 24 h as described in "Materials and Methods." Protein composition was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. B, total cellular RNAs were prepared from CL3 cells, CL3 cells treated with 50 μM sodium arsenite for 30 min and incubated with drug-free medium for 2 h (Lane CL3Ars), and CL3R cells and their subclones. Northern blot analysis of heme oxygenase mRNA was performed as described in "Materials and Methods." Glyceraldehyde phosphate dehydrogenase mRNA was used as a loading control. Arrow, position of HO protein or mRNA.

levels (data not shown). However, after treatment of CL3R15 cells with 20 μM SnPP for 24 h, HO activity became undetectable (Table 3). These results imply that HO plays a role in arsenic resistance.

As shown in Fig. 5, CL3R cells were cross-resistant to the active oxygen-generating agents menadione and Adriamycin. A DCF fluorescence method was therefore adopted to assess the formation of cellular oxidants by sodium arsenite. As shown in Fig. 6, sodium arsenite treatment dose-dependently enhanced DCF fluorescence in CL3 cells, whereas no enhancement was noticed in CL3R15 cells. These results imply that HO expression can efficiently eliminate the accumulation of arsenite-enhanced oxidative activity.

DISCUSSION
The present results show that CL3R cells and 6 subclones with constitutively expressed HO were 6-fold more resistant than parental CL3 cells to arsenite. The level of HO was demonstrated by [35S]methionine labeling, Northern blot analysis, and enzymatic activity assay. Our results also suggest that HO plays a potential role in the
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mechanism of arsenic resistance. Heme oxygenase is responsible for heme degradation (29). Heme and heme-containing proteins may provide iron for the catalysis of reactions leading to the formation of reactive free radicals (30, 31). Therefore, the inducible HO may counteract oxidative stress by decreasing the heme pool and producing the strong antioxidants biliverdin and bilirubin (29, 32). Reactive free radicals (30, 31). Therefore, the inducible HO may provide iron for the catalysis of reactions leading to the formation of oxidants in several different cell systems (27, 37, 38). Thus, our results support the view that arsenite may induce oxidative damage through an unknown mechanism.

Numerous recent studies have reported that arsenic compounds may damage cells through the production of oxygen radicals (34–36). In support of this view, our present studies have also revealed that CL3R cells are cross-resistant to Adriamycin and menadione, both of which are oxygen radical-generating agents. Furthermore, by using the DCF fluorescence method, we have shown that sodium arsenite treatment can significantly enhance levels of cellular oxidants in parent CL3 cells but not in arsenic-resistant CL3R15 cells. The DCF fluorescence method has been used to assess the generation of cellular oxidants in several different cell systems (27, 37, 38). Thus, our results support the view that arsenite may induce oxidative damage through an unknown mechanism.

![Figure 4](image1.png)

**Fig. 4.** A, decrease in arsenite resistance of CL3R15 cells by incubation in arsenite-free medium. CL3R15 cells were grown in arsenite-free medium for 0 (B), 1 (C), 3 (L), and 7 (E) days. B, effect of SnPP on arsenite resistance of CL3R15 cells. CL3R15 cells were treated with SnPP alone (L) or with a combination of 20 µM sodium arsenite and SnPP for 24 h (B). The relative survival was determined according to the cells' colony-forming efficiency as described in "Materials and Methods." Bars, SD of 3 independent experiments.

![Figure 5](image2.png)

**Fig. 5.** Cross-resistance of CL3R cells to menadione (A) and Adriamycin (B). CL3 (C) and CL3R (B) cells were treated with various concentrations of menadione for 4 days or Adriamycin for 1 h as described in "Materials and Methods." The relative survival was determined by hemocytometer counter. Bars, SD of 3 independent experiments.

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**REFERENCES**


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