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

Te-Chang Lee and I-Ching Ho
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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 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 arsenic metabolism 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.

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.

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1 This study was supported in part by Academia Sinica and by a grant from the National Science Council, Republic of China.

2 To whom requests for reprints should be addressed.

3 The abbreviations used are: GSH, glutathione; DCF, 2',7'-dichlorofluorescein; GST, glutathione S-transferase; HO, heme oxygenase; HPLC, high pressure liquid chromatography; SsPP, tin-protoporphyrin; SOD, superoxide dismutase.

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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 with a Hitachi 4010 fluorescence spectrophotometer.

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 GSTκ 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 arsenite-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 C₁₈ column, hemin (substrate), meso-porphyrin 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.

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>
</tr>
</thead>
<tbody>
<tr>
<td>GSH (nmol/mg protein)</td>
<td>50.7 ± 5.5</td>
<td>100.8 ± 1.0</td>
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<tr>
<td>GSH S-transferase (nmol CDNB/min/mg/protein)</td>
<td>73.3 ± 5.7</td>
<td>72.7 ± 13.8</td>
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<tr>
<td>GSH peroxidase (nmol NADPH/min/mg protein)</td>
<td>37.1 ± 5.7</td>
<td>37.7 ± 6.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Catalase (μmol H₂O₂/min/mg protein)</td>
<td>37.0 ± 0.6</td>
<td>36.2 ± 2.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Superoxide dismutase (units/mg protein)</td>
<td>13.5 ± 2.5</td>
<td>3.8 ± 0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Proteins (μg/10⁶ cells)</td>
<td>250.5 ± 12.5</td>
<td>159.0 ± 25.4</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>0.32 ± 0.07</td>
<td>0.32 ± 0.06</td>
<td>1.0</td>
</tr>
</tbody>
</table>
HEM OXYGENASE IN ARSENIC-RESISTANT CELLS

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 CL3As), 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

Table 2. Heme oxygenase activity in CL3, CL3R, and arsenic-resistant subclones
Heme oxygenase activity was analyzed as described in "Materials and Methods." Values represent the average ± SD, and the numbers of individual experiments are given in parentheses.

Table 3. Effects of arsenite-free culture and SnPP on heme oxygenase activity in CL3R15 cells
CL3R15 cells were incubated in sodium arsenite-free medium for a time period as indicated or treated with SnPP for 24 h. Afterward, heme oxygenase activity was analyzed as described in "Materials and Methods." Values represent the average ± SD, and the numbers of individual experiments are given in parentheses.

Fig. 3. HPLC chromatogram for heme oxygenase assay. The enzymatic activities of HO were analyzed as described in "Materials and Methods." A, chromatographic profile of authentic compounds (50 pmol for each), H, hemin; MP, mesoporphyrin; and BR, bilirubin. B, assay of HO activity from CL3 cells. C, assay of HO activity from CL3R15 cells. The ordinate scale is arbitrary units of absorbance at 405 nm.
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A 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).

Arsenic as well as many sulfhydryl reaction agents is a potent inducer of HO in a variety of cells (20, 32). Acute administration of sodium arsenite has previously been shown to markedly increase rat hepatic microsomal HO activity while simultaneously increasing bilirubin production and decreasing cytosolic free heme (33). Although HO is usually transiently expressed in most circumstances, it is constitutively expressed in arsenic-resistant cells that are maintained in the presence of sodium arsenite.

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.

**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 (■), 1 (○), 3 (□), and 7 (□) days. B, effect of SnPP on arsenite resistance of CL3R15 cells. CL3R15 cells were treated with SnPP alone (□) or with a combination of 20 μM sodium arsenite and SnPP for 24 h (■). The relative survival was determined according to the cells’ colony-forming efficiency as described in “Materials and Methods.” Bars, SD of 3 independent experiments.**

**Fig. 5. Cross-resistance of CL3R cells to menadione (A) and Adriamycin (B). CL3 (○) and CL3R (■) 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.**

**Fig. 6. Induction of DCF fluorescence by sodium arsenite. CL3 (open bars) and CL3R (solid bars) cells were treated with various concentrations of sodium arsenite for 24 h. DCF fluorescence was determined as described in “Materials and Methods.” Bars, SD of 3 independent experiments. Asterisks, significant difference between control and sodium arsenite-treated cultures (P < 0.05; **P < 0.01).**

Catalase and SOD, 2 major oxygen radical scavengers, were 70 and 40% lower, respectively, in CL3R cells than in parental CL3 cells. Since catalase is a hemoprotein with a prosthetic heme group, decreased catalase activity may be due to heme degradation by HO that is expressed in CL3R cells. Our results indicate that HO can supplement the decreased SOD and catalase activities and plays an important function in protecting cells from oxidative damage. In addition, depletion of intracellular GSH by treatment with buthionine sulfoximine has been shown to increase the susceptibility of cells to arsenite (8, 39). Therefore, the 2-fold increase in GSH levels in CL3R cells may contribute in part to arsenic resistance.

Oxidative processes have recently been suggested to play an important role in metal carcinogenesis (40). Numerous reports have shown that Cr, Ni, Fe, and Cu can induce DNA damage via the generation of oxygen radicals (41–43). If sodium arsenite can produce oxidative stress in cells, further investigation of the underlying mechanism by which sodium arsenite induces oxygen radicals will help us to understand the carcinogenicity of arsenic in humans.

**ACKNOWLEDGMENTS**

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**REFERENCES**


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