Glutathione S-transferase \( \pi \) in an arsenic-resistant Chinese hamster ovary cell line

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INTRODUCTION

According to epidemiological evidence, inorganic arsenic compounds have been strongly suggested as human carcinogens (Leonard & Lauwerys, 1980; IARC, 1980; Chen et al., 1985). However, arsenic fails to induce tumors in experimental animals (IARC, 1980; Ishinishi et al., 1983). Although arsenic has been well demonstrated to be genotoxic in cultured mammalian cells (Nakamuro & Sayato, 1981; Larramendy et al., 1981; Lee et al., 1985), the molecular mechanism(s) underlying arsenic-induced genetic damage is still far from clear. To understand the toxic effects of arsenic in cells, we have previously isolated an arsenic-resistant cell line (SA7) from Chinese hamster ovary (CHO) cells (Lee et al., 1989). The resistance to arsenic was found to be associated with the elevated GSH levels and glutathione S-transferase (GST, EC 2.5.1.18) activities in SA7 cells.

GSTs are composed of a family of isoenzymes which are widely distributed in animal tissues (Mannervik, 1985). Their physiological function may be involved in the detoxification of xenobiotics (Jakoby, 1978; Berhane & Mannervik, 1990), synthesis of leukotriene \( \mathrm{C}_4 \) (Samuelsson, 1982), transport of haem (Haverly & Butler, 1982) and bilirubin (Kamisaka et al., 1975), and possibly in cell proliferation (Senjo & Ishibashi, 1988). Elevation of intracellular GST activities is frequently associated with acquired resistance to certain anti-cancer drugs (Batist et al., 1986; Robson et al., 1987; Lewis et al., 1988; Wang et al., 1989; Gupta et al., 1989). Furthermore, it is not unusual that overexpression of GST genes is accompanied with that of multiple-drug-resistance genes (Moscow et al., 1989; Cole et al., 1990). However, the roles of GST activities in drug resistance have not been well established in many instances. In this communication, we characterize the GST purified from SA7 cells and confirm its association with arsenic resistance.

MATERIALS AND METHODS

Cell culture and media

CHO, SA7 (arsenic-resistant), and SA7N (partial revertant) cells were cultured in McCoy's 5a medium supplemented with 10% (v/v) heat-inactivated fetal calf serum and antibiotics as described previously (Lee et al., 1989). SA7 and SA7N cells were 9- and 3-fold more resistant to sodium arsenite than parental CHO cells. All chemicals for cell culture were purchased from Gibco (Grand Island, NY, U.S.A.).

Survival assay

The survival fraction after drug treatment was determined by the colony-forming method as described previously (Lee et al., 1986). Briefly, \( 3 \times 10^4 \) cells were plated in a 60-mm Petri dish 18 h before experimental manipulation. The drug treatment was performed according to the protocols as indicated. At the end of the treatment, the cells were washed, trypsinized, replated at 200-2000 cells per 60-mm Petri dish (in triplicate), and incubated in normal medium for 7 days without changing the medium. The dishes were then fixed with 95% (v/v) ethanol, stained with a 10% (v/v) Giemsa solution, and the colony numbers were counted under a dissecting microscope. All the drugs were predissolved in distilled water and diluted with medium upon use.

Purification of GST

GST from SA7 cells was purified with a GSH-affinity column (Lee et al., 1989). N-terminal-sequence analysis of purified GST was performed using an automatic amino-acid sequencer (ABI gas/liquid phase Model 470A/900A).

Preparation of cell extracts

Crude extracts were prepared by scraping off the cells with a rubber policeman after washing twice with ice-cold phosphate-buffered saline. The cells were pelleted at 1000 g for 10 min at 4 °C and resuspended in an appropriate amount (approx. 4 × 10^7 cells/ml) of 0.1 M-potassium phosphate buffer, pH 6.8, and transferred into Eppendorf tubes. The cells were sonicated three times (a 10 s burst at intervals of 1 min) with a Heat System-Ultrasonics W-380 sonicator. After centrifugation at 12000 g for 20 min at 4 °C, clear supernatants were stored on ice.

Abbreviations used: CHO, Chinese hamster ovary; GST, glutathione S-transferase.

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before GST-activity assay. Protein concentrations were determined by the Bradford method (1976) using BSA as a standard.

Analysis of GST

GST activities were measured by the modified methods of Habig et al. (1974) and Habig & Jakoby (1981). Briefly, assays were carried out in a mixture containing GSH (dissolved in 0.1 M-potassium phosphate buffer, pH 6.8), substrates as indicated, and purified GST in 0.1 M-potassium phosphate buffer, pH 6.5. Enzyme activity was determined by monitoring the absorbance at an appropriate wavelength with a Hitachi U-3120 dual-beam spectrophotometer at 37 °C.

Western-blot analysis

Antiserum was raised against GST in New Zealand rabbits which were subsequently injected several times with purified enzyme (108 ± 25 μg). Western-blot analysis was performed according to the method described by Burnette (1981). Alkaline-phosphatase-conjugated anti-(rabbit IgG) antibody was used as the secondary antibody to visualize the GST which was transferred onto nitrocellulose paper.

Electrophoretic analysis of metallothionein

Metallothionein was examined according to the method described by Otsuka et al. (1988) with a slight modification. Briefly, the cells were treated with various metal compounds and incubated in cysteine-free medium containing [35S]cysteine (5 μCi/ml, specific activity > 1000 Ci/mmol; Amersham International, Amersham, Bucks., U.K.) for 24 h. Afterwards, the cell pellets were disrupted in a lysis buffer (100 mM-Tris/HCl, pH 7.5, 0.15 mM-NaCl, 3 mM-MgCl2, 0.5% NP-40, 5 mM-β-mercaptoethanol), and centrifuged at 12000 g for 10 min. Clear supernatants were then incubated at 80 °C for 10 min. Heat-stable fractions were carboxymethylated by iodoacetatic acid and separated by SDS/PAGE (Laemmli, 1970). After exposure of X-ray film, the metallothionein was visualized on the autoradiogram.

Analysis of heat-shock-protein synthesis

[35S]Methionine incorporation was used to examine the synthesis of heat shock proteins. SA7N cells (5 × 106) were plated on a 60-mm Petri dish and incubated at 37 °C overnight before being subjected to heat shock or arsenic treatment. Heat shock was performed at 45 °C for 10 min. The cells were then either continuously labelled with 1 μCi of [35S]methionine/ml (specific activity > 800 Ci/mmol; Amersham) at 37 °C for 24 h or labelled with 5 μCi of [35S]methionine/ml for 1 h after incubation at 37 °C for 5 h. Arsenic treatment was performed by incubation of cells with 40 μM-sodium arsenite without medium change. The arsenic-treated cultures were labelled with [35S]methionine for either 24 h or 1 h, under conditions outlined above for heat-shock treatment. At the end of the labelling procedure, cell pellets were solubilized in electrophoretic buffer and the cellular polypeptides (100000 c.p.m. equivalent) were then analysed on a 12.5% SDS/polyacrylamide gel (Laemmli, 1970). The polypeptide composition was visualized by autoradiography.

RESULTS

Substrate specificities of the purified GST were summarized in Table 1. This GST showed a residual activity toward cumene hydroperoxide and no detectable activity towards bromosulphophthalein and trans-4-phenyl-3-butene-2-one respectively. However, the purified GST apparently catalysed the conjugation of GSH with ethacrynic acid (the specific substrate for Pi class GSTs) or 1-chloro-2,4-dinitrobenzene (a general substrate for all classes of GST). The specific activity of GST from SA7 cells towards ethacrynic acid is comparable with that of GSTπ reported by Mannervik et al. (1985). Furthermore, only six out of 50 amino acids from the N-terminus are different from those of rat GST P or human GSTπ (Fig. 1). These results clearly indicate that the GST elevated in SA7 cells belongs to a Class Pi isoenzyme.

Table 1. Specific activities of GST purified from SA7 cells

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Specific activity (μmol/min per mg of protein)</th>
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<tbody>
<tr>
<td>1-Chloro-2,4-dinitrobenzene</td>
<td>59.12 ± 0.09</td>
</tr>
<tr>
<td>Ethacrynic acid</td>
<td>3.94 ± 0.94</td>
</tr>
<tr>
<td>Bromosulphophthalein</td>
<td>N.D.</td>
</tr>
<tr>
<td>trans-4-Phenyl-3-butene-2-one</td>
<td>N.D.</td>
</tr>
<tr>
<td>Cumene hydroperoxide</td>
<td>0.84 ± 0.16</td>
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</tbody>
</table>

Fig. 1. N-terminal sequence of GST purified from SA7 cells

The N-terminal sequence of GST purified from SA7 cells was determined on an automatic amino-acid sequencer. Amino-acid sequences of rat GST P and human GSTπ were deduced from their respective cDNA clones (Suguoka et al., 1985; Kano et al., 1987).

Fig. 2. Effects of GST inhibitors (a, b) and heavy-metal (c) pretreatment on arsenic resistance of SA7 and SA7N cells

(a) SA7 cells were treated with the GST inhibitor, ethacrynic acid (○) or in combination with 125 μM-sodium arsenite (●) for 6 h. At the end of treatment, the relative survival rate was analysed by the colony-forming method as described in the Materials and methods section. (b) SA7 cells were treated with GST inhibitor, Cibacron Blue (Δ), or in combination with 125 μM-sodium arsenite (▲) for 6 h. The relative survival rate was then determined as in (a). (c) SA7N cells were pre-incubated without drug (○), with 30 μM-sodium arsenite (▲), 0.5 μM-cadmium acetate (●), or 300 μM-zinc sulphate (■) for 24 h respectively. At the end of incubation, cells were challenged with various concentrations of sodium arsenite for 18 h. The relative survival was then determined as described. Bars indicate s.d. of three independent experiments.
Table 2. Induction of GST activities by heavy metals in SA7N cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GST activity (µmol/min per mg of protein)</th>
<th>Increase (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.39 ± 0.08</td>
<td>1.0</td>
</tr>
<tr>
<td>As (30 µM)</td>
<td>1.16 ± 0.20*</td>
<td>3.0</td>
</tr>
<tr>
<td>Zn (300 µM)</td>
<td>1.49 ± 0.09*</td>
<td>3.8</td>
</tr>
<tr>
<td>Zn (600 µM)</td>
<td>1.47 ± 0.23*</td>
<td>3.7</td>
</tr>
<tr>
<td>Cd (0.5 µM)</td>
<td>1.81 ± 0.16**</td>
<td>4.6</td>
</tr>
<tr>
<td>Cd (1.0 µM)</td>
<td>1.01 ± 0.15*</td>
<td>2.6</td>
</tr>
<tr>
<td>Cd (1.0 µM)</td>
<td>1.37 ± 0.29*</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Because of the relative abundance of GSTπ in SA7 cells, this GST isoenzyme can be easily isolated to show a homogeneous band on a SDS/polyacrylamide gel (Lee et al., 1989). Only a single sharp peak was observed when the isolated GSTπ was subjected to analysis with an Aquapore RP-300 reverse-phase column (4.6 mm × 220 mm) attached to a Hitachi D-6000 h.p.l.c. system according to the method described by Ostlund Farrants et al. (1987) with a slight modification (results not shown). These results indicate that the affinity-purified enzyme is mainly composed of GSTπ.

Several experiments were conducted to aid understanding of whether GSTπ is involved in arsenic resistance in SA7 cells. First, two known GST inhibitors, ethacrynic acid and Cibacron Blue, were used to reduce the GST activities in SA7 cells. By treating the SA7 cells with sodium arsenite in combination with ethacrynic acid or Cibacron Blue, their arsenic resistance was apparently decreased [Figs 2(a) and 2(b)]. Alternatively, we could re-elevate the GST activities in the partial revertant SA7N cells, which have a lower level of GST and are more susceptible to arsenic than SA7 cells, by the presence of several heavy-metal compounds. As shown in Table 2, treatment of SA7 cells with sublethal doses of sodium arsenite, cadmium acetate, or zinc sulphate increased the GST activity by 3- to 4-fold. By using Western-blot analysis, the increased GST activities by these compounds could be confirmed by GSTπ antibody but not antibodies against other classes of GST (results not shown). Furthermore, the arsenic resistance of SA7N cells was obviously re-acquired by pretreatment with these metal compounds (Fig. 2c).

In order to confirm that the acquired arsenic resistance of SA7N cells was dependent on GSTπ activity, the susceptibility of SA7N cells to sodium arsenite was determined after pretreatment with various concentrations of zinc sulphate because of its minimal toxicity. As shown in Fig. 3, both GST activity and arsenic resistance were dose-dependently increased by zinc sulphate pretreatment. An increase in GSTπ expression was confirmed by Western-blot analysis (Fig. 4). These results support the hypothesis that arsenic resistance of the cells is well correlated with their GSTπ activities.

Since both zinc and cadmium are potent inducers of metallothionein, a [35S]cysteine-labelling method was used to determine the metallothionein levels. Pretreatment of SA7N cells with zinc sulphate or cadmium acetate at a concentration that shows a significant protective effect against arsenic toxicity causes only a very slight increase in metallothionein level (Fig. 5). No induction of metallothionein could be detected by pretreating SA7N cells with sodium arsenite at 30 µM (Fig. 5). Because of the very low induction of metallothionein in SA7N cells by these compounds, the metallothionein induced by zinc sulphate in human fibroblasts was used as a control. Thus, it is unlikely that metallothionein is involved in the protective effects of arsenite, zinc and cadmium pretreatment against arsenic toxicity.

As shown in Figs. 2(c) and 6(a), pretreatment of SA7N cells for 24 h with sodium arsenite apparently increased arsenic resistance. As a comparison, the SA7N cells were heated at 45 °C for 10 min, incubated at 37 °C for 24 h, and then challenged with 75 µM-sodium arsenite for another 24 h. Under these experimental conditions, heat shock did not protect the cells from the toxicity of sodium arsenite (Fig. 6a). By using Western-blot analysis, although treatment with sodium arsenite caused a significant increase of GSTπ accumulation, heat shock did not apparently alter the levels of GSTπ (Fig. 6b).

As shown in Fig. 7 (lanes 1-3), sodium arsenite pretreatment and heat shock could enhance the synthesis of different sets of heat-shock proteins when the cells were labelled with [35S]methionine for 1 h five hours after heat shock, or during...
SA7N cells were treated with 30 μM-sodium arsenite, or 0.5 μM-cadmium acetate, or 300 μM-zinc sulphate for 24 h. During the drug treatment, [35S]cysteine (5 μCi/ml) was added to the culture medium. At the end of treatment, metallothionein (MT) was electrophoretically analysed (see the Materials and methods section). Lanes 1 and 2 represent polypeptides of human fibroblasts treated with 0 and 100 μM-zinc sulphate; lanes 3–6 are samples from SA7N cells treated with no drug, sodium arsenite, cadmium acetate and zinc sulphate respectively.

**Fig. 5. Effect of sodium arsenite, cadmium acetate and zinc sulphate pretreatments on metallothionein synthesis in SA7N cells**

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**Fig. 6. Effect of heat-shock and sodium arsenite pretreatment on arsenic resistance and GSTn induction in SA7N cells**

(a) SA7N cells were either heated at 45 °C for 10 min and then incubated at 37 °C for 24 h or pre-treated with 40 μM-sodium arsenite for 24 h. Afterward, the cells were challenged with 75 μM-sodium arsenite for another 24 h. The relative survival rate was then determined as described in the Materials and methods section. Bars indicate S.D. of three independent experiments. (b) SA7N cells were treated by heat shock or with sodium arsenite as described in (a). The levels of GSTn after treatment were determined by Western-blotting technique. Lanes 1–3 were Coomassie Blue stained gel; 4–6 were detected by antiserum against GSTn. Lanes 1 and 4, control cultures; 2 and 5, 24 h after heat shock; 3 and 6, 40 μM-sodium arsenite for 24 h.

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**DISCUSSION**

GSTs are a family of isoenzymes (Habig et al., 1974), which are classified as Alpha, Mu and Pi forms according to their substrate specificities and amino-acid sequence (Mannervik et al., 1985). Comparing the specific activities of GSTn from several mammalian species, the SA7 GST could be classified as a Pi family isoenzyme because of its high specific activity towards ethacrynic acid and its sequence identity.

Although the mechanism(s) of GSTn in preventing the toxic effects of arsenic is completely unclear, our experimental results suggest a close association between the GSTn level and arsenic resistance. First, the GST inhibitors, ethacrynic acid and Cibacron Blue, are capable of decreasing the arsenic resistance in SA7 cells. Secondly, the GSTn level in the partial-revertant SA7N cells could be re-elevated by pretreatment with sublethal doses of sodium arsenite itself, as well as zinc sulphate and cadmium acetate. This pretreatment of SA7N cells with heavy-metal compounds generates arsenic resistance in the partial-revertant cells simultaneously. The current results demonstrate a good correlation between the levels of GSTn and arsenic resistance.

Although metallothionein is involved in the detoxification of numerous heavy metals (Cherian & Nordberg, 1983; Hamer, 1986), no available evidence shows that metallothionein could protect the cells from the cytotoxic effects of arsenic. Our previous results showing that arsenic-resistant SA7 cells were not...
cross-resistant to nickel, platinum and cadmium indicate that metallothionein is probably not involved in arsenic resistance (Lee et al., 1989). Since the induction of metallothionein by pretreatment with arsenic, zinc and cadmium compounds at sublethal doses is minimal, the regained arsenic resistance in SA7N cells is apparently not related to the metallothionein expression.

Treatment of cells with heat shock or sodium arsenite could transiently induce the synthesis of so-called heat shock proteins (Li, 1983; Lindquist, 1986). Recently, numerous studies have uncovered the biological functions of heat shock proteins, such as acting as protein chaperones (Ang et al., 1991; Gething & Sambrook, 1992). However, our results do not support the involvement of heat shock proteins in arsenic resistance.

By using a 24-h-long labelling process, we have shown that sodium arsenite can increase the synthesis of a 32 kDa stress protein. This 32 kDa protein has been identified as haem oxygenase (Keyse & Tyrrell, 1989). Since the induction of haem oxygenase is a general response to oxidant stress in mammalian cells (Applegate et al., 1991), arsenic may induce oxidative damage through an unknown mechanism. Numerous recent studies have reported that arsenic compounds may cause cell damage through the production of oxygen radicals (Burdon et al., 1987; Yamanaka et al., 1989; Blair et al., 1990). Haem oxygenase could protect the cells from oxidant stress by rapidly reducing cellular haem pools and consequently inhibiting the generation of oxygen radicals. Our current experiments, however, can not exclude the possibility that haem oxygenase is also involved in the mechanism of arsenic resistance.

In general, GSTs have at least three functions: (1) to catalyse the conjugation of glutathione on the sulphur atom of cysteine to various electrophiles; (2) to act as a selenium-independent GSH peroxidase; and (3) to bind with high affinity to a variety of hydrophobic compounds such as haem, bilirubin, polycyclic aromatic hydrocarbons, and dexamethasone (reviewed by Waxman, 1990). How GSTs are involved in arsenic detoxification is not completely clear. It could be simply proposed that GSTs catalyse the conjugation of GSH and inorganic arsenic. Nevertheless, the GSH–arsenic complexes have never been identified. On the other hand, methylation has been demonstrated to be a major mechanism for the detoxification of inorganic arsenic by mammals (Buchet et al., 1981; Vahter, 1981). Recently, GSH has been shown to stimulate the methylation of inorganic arsenic (Hirata et al., 1988; Buchet & Lauwers, 1988; Georis et al., 1990), but the underlying mechanism has not been completely elucidated. Whether GSTs participate in the methylation steps is an interesting question which remains to be answered.

Alternatively, the relative high abundance of GST\(\xi\) in SA7 cells implies that it could possibly act 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 upon metabolic transformation of arsenic. In fact, GSTs have been proposed to serve as potent binders of lipophilic toxins (Jakoby, 1978; Smith & Litwack, 1980; Listowsky et al., 1988). Therefore, further investigation of the role of GST\(\xi\) in arsenic resistance is necessary to provide a better understanding of the toxic mechanism of arsenic.

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