Glutathione S-transferase π in an arsenic-resistant Chinese hamster ovary cell line

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A glutathione S-transferase (GST) was purified from an arsenic-resistant Chinese hamster ovary cell line, SA7. The SA7 GST was shown to catalyse the conjugation of glutathione and ethacrynic acid, a specific substrate for Pi class GST. Its N-terminal amino-acid sequence has 80 % identical residues to that of rat GST P and human GST π . Thus, the GST purified from SA7 cells belongs to the Pi family. Treatment with Cibacron Blue or ethacrynic acid, which are GST inhibitors, significantly decreased the resistance of SA7 cells to sodium arsenite. On the other hand, pretreatment of SA7N cells, a partial revertant of SA7 cells, with sublethal doses of sodium arsenite, cadmium acetate or zinc sulphate resulted in re-elevation of GST activities and the cells regained the arsenic resistance. The regained arsenic resistance was well correlated with the levels of GST π which were induced dose-dependently by zinc sulphate. Heat-shock treatment (45 °C for 10 min) did not increase GST π expression or arsenic resistance of SA7N cells. The results indicate that GST π is possibly involved in the mechanism of arsenic detoxification.

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 tumours 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 C₄ (Samuelsson, 1982), transport of haem (Havery & Beutler, 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, 3×10^5 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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Table 2. Induction of GST activities by heavy metals in SA7N cells

SA7N cells were treated with sodium arsenite or zinc sulphate or cadmium acetate at the concentrations indicated for 24 h. GST activity was determined at 37 °C using 1-chloro-2,4-dinitrobenzene and GSH as substrates. Results are expressed as mean $\pm$ S.D. of three experiments. Statistically difference between the culture with and without treatment as shown: * $P < 0.01$; and ** $P < 0.001$, according to Student's *t*-test.

Treatment	GST activity ($\mu\text{mol/min per mg of protein}$)	Increase (fold)
Control	0.39 ± 0.08	1.0
As (30 μM)	$1.16 \pm 0.20^*$	3.0
As (60 μM)	$1.49 \pm 0.09^*$	3.8
Zn (300 μM)	$1.47 \pm 0.23^*$	3.7
Zn (600 μM)	$1.81 \pm 0.16^{**}$	4.6
Cd (0.5 μM)	$1.01 \pm 0.15^*$	2.6
Cd (1.0 μM)	$1.37 \pm 0.29^*$	3.5

Fig. 3. Dose-dependent increase of GST activities and arsenic resistance of SA7N cells by zinc pretreatment

(a) SA7N cells were incubated with various concentrations of zinc sulphate (0–400 μM) for 24 h. The crude cell extracts were prepared and assayed for GST activity using 1-chloro-2,4-dinitrobenzene as substrate. (b) Zinc-sulphate-pretreated SA7N cells were subsequently challenged with 0 (○) or 100 μM -sodium arsenite (●) for 24 h. The relative survival rate was determined by the colony forming method as described. Bars indicate S.D. of three independent experiments.

Fig. 4. Western-blot analysis of GST π induction in SA7N cells by treatment with zinc sulphate

SA7N cells were treated with zinc sulphate (0–400 μM) for 24 h as described in Fig. 3. Cell lysates (containing 10 μg of cellular proteins) were loaded on to a 12.5% SDS/polyacrylamide gel. The GST levels were analysed by Western-blotting technique after electrophoresis (lanes 7–12). Lanes 1–6 were a corresponding gel stained with Coomassie Blue. Lanes 1 and 7, control culture; 2 and 8, zinc sulphate 50 μM ; 3 and 9, 100 μM ; 4 and 10, 200 μM ; 5 and 11, 400 μM ; 6 and 12, 0.5 μg of purified GST π .

Because of the relative abundance of GST π 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 GST π was subjected to analysis with an Aquapore RP-300 reverse-phase column (4.6 mm $\times$ 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 GST π .

Several experiments were conducted to aid understanding of whether GST π 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 SA7N 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 three compounds could be recognized by GST π 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 GST π 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 GST π 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 GST π activities.

Since both zinc and cadmium are potent inducers of metallothionein, a [^{35}S]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 GST π accumulation, heat shock did not apparently alter the levels of GST π (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 [^{35}S]methionine for 1 h five hours after heat shock, or during

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

SA7N cells were treated with 30 μ M-sodium arsenite, or 0.5 μ M-cadmium acetate, or 300 μ M-zinc sulphate for 24 h. During the drug treatment, [35 S]cysteine (5 μ Ci/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 μ M-zinc sulphate; lanes 3–6 are samples from SA7N cells treated with no drug, sodium arsenite, cadmium acetate and zinc sulphate respectively.

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

(a) SA7N cells were either heated at 45 $^{\circ}$ C for 10 min and then incubated at 37 $^{\circ}$ C for 24 h or pre-treated with 40 μ M-sodium arsenite for 24 h. Afterward, the cells were challenged with 75 μ M-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 GST π after treatment were determined by Western-blotting technique. Lanes 1–3 were Coomassie Blue stained gel; 4–6 were detected by antiserum against GST π . Lanes 1 and 4, control cultures; 2 and 5, 24 h after heat shock; 3 and 6, 40 μ M-sodium arsenite for 24 h.

Fig. 7. Induction of heat shock proteins by heat shock and sodium arsenite in SA7N cells

SA7N cells were treated with heat shock or sodium arsenite as described in Fig. 6. Protein synthesis was examined by [35 S]methionine incorporation. [35 S]Methionine labelling was performed either for 1 h at the 5th hour after treatment (lanes 1–3) or for 24 h after heat shock or during sodium arsenite treatment (lanes 4–6). Lanes 1 and 4, control cultures; 2 and 5, heat shock; 3 and 6, sodium arsenite. Induced proteins are indicated with $\circ$ and $\blacksquare$.

polypeptide bands with molecular masses of 45 kDa and 32 kDa (Fig. 7, lanes 4–6). They were enhanced in heat-shock or sodium-arsenite-treated cells respectively. Thus, the arsenic resistance conferred by sodium arsenite pretreatment is unlikely to be caused by the expression of heat shock proteins.

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 GST π 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 GST π in preventing the toxic effects of arsenic is completely unclear, our experimental results suggest a close association between the GST π 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 GST π 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 GST π 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

sodium arsenite treatment. However, if SA7N cells were continuously labelled with [35 S]methionine for 24 h after heat shock or during the sodium arsenite treatment, there was no significant accumulation of most heat shock proteins, except for two

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 arsenite, 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 & Lauwerys, 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 π 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 π in arsenic resistance is necessary to provide a better understanding of the toxic mechanism of arsenic.

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