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GLUTATHIONE *S*-TRANSFERASES AND GLUTATHIONE PEROXIDASES IN DOXORUBICIN-RESISTANT MURINE LEUKEMIC P388 CELLS

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Abstract—Energy-dependent rapid drug efflux is believed to be a major factor in cellular resistance to doxorubicin (DOX). However, several recent studies have demonstrated that cellular DOX retention alone does not always correlate with its cytotoxicity and suggest that mechanisms other than rapid drug efflux may also be important. In the present study, we have compared glutathione (GSH) *S*-transferase (GST), selenium-dependent GSH peroxidase and selenium-independent GSH peroxidase II activities in DOX-sensitive (P388/S) and resistant (P388/R) mouse leukemic cells. The GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) and ethacrynic acid (EA) was markedly higher in P388/R cells compared to P388/S cells. Purification of GST by GSH-affinity chromatography from an equal number of P388/S and P388/R cells revealed an increased amount of GST protein in P388/R cells. Immunological studies indicated that α and π type GST isoenzymes were 1.27- and 2.2-fold higher, respectively, in P388/R cells compared to P388/S cells. Selenium-dependent GSH peroxidase activity was similar in both the cell lines, whereas selenium-independent GSH peroxidase II activity was approximately 1.36-fold higher in P388/R cells compared to P388/S cells. These results suggest that increased GSH peroxidase II activity in P388/R cells may contribute to cellular DOX resistance by enhancing free radical detoxification in this cell line.

Doxorubicin (DOX§) is one of the most potent antibiotics used in chemotherapy of a variety of human malignancies [1]. However, the clinical effectiveness of DOX may often be limited by its dose-dependent cardiotoxicity and the emergence of drug-resistant tumor cells. Energy-dependent drug efflux has been shown to be responsible for cellular resistance of P388 mouse leukemic cells [2–5] and other cancer cell lines [6, 7] to DOX. Phenothiazine and verapamil, by blocking drug efflux, enhance DOX retention and cytotoxicity in resistant cells [8–11]. However, several recent studies suggest [12–14] that cellular drug retention alone does not always correlate with anthracycline cytotoxicity. Furthermore, recent studies from our laboratory on more than 84-fold DOX-resistant P388 mouse leukemic cells have shown that these cells continue to be >50-fold resistant even after efflux blocking by trifluoperazine [5, 15]. Taken together, these studies suggest that mechanisms besides that of rapid drug efflux may also contribute to DOX resistance of murine leukemic P388 cells. A complete understanding of the biochemical mechanism(s) of cellular resistance to DOX is, therefore, essential for the development of more effective therapies for the resistant tumors.

Glutathione (GSH) is a tripeptide thiol which serves many important biological functions. GSH plays a key role in the protection of cells against free radicals and electrophiles through non-enzymatic mechanisms as well as through GSH peroxidase [16, 17] and glutathione *S*-transferase (GST) [18] activities. GSH and GSH-related enzymes have been implicated recently in cellular resistance to chemotherapeutic agents in a variety of tumor cells. Elevated GSH levels have been reported in a number of drug-resistant tumor cells [19–22], and depletion of cellular GSH has been shown to enhance chemosensitivity [19, 20, 22]. Increased GST activity has been reported in tumor cells exhibiting resistance to chlorambucil [23–25], cyclophosphamide [26], melphalan [27], and doxorubicin [28, 29]. Doxorubicin resistance in a MCF-7 breast cancer cell line has been attributed to the increased GSH peroxidase activity [30]. In addition, the toxicity of 1,3-bis(2-chloroethyl)-1-nitrosourea is known to be influenced by the cellular GSH reductase levels [31]. These studies suggest that intracellular GSH and GSH-related enzymes may play an important role in cellular resistance to antitumor drugs including DOX. Since free radicals have been implicated in the cytotoxicity of DOX [32], intracellular levels of GSH-related enzymes may affect DOX toxicity. The present stud-

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§ Abbreviations: DOX, doxorubicin; P388/S, doxorubicin-sensitive P388 cells; P388/R, more than 84-fold doxorubicin-resistant P388 cells; GSH, glutathione; GST, glutathione *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 3,4-dichloronitrobenzene; EA, ethacrynic acid; and H_2O_2, hydrogen peroxide.

Table 1. GST and GSH peroxidase activities in 14,000 *g* supernatant fractions of P388/S and P388/R cells

Enzyme	Cell line	
	P388/S	P388/R
GST activity (units*/mg protein)		
1-Chloro-2,4-dinitrobenzene	54.0 ± 8.0 (6)	97.0 ± 9.0 (6)
3,4-Dichloronitrobenzene	2.3 ± 0.5 (3)	1.4 ± 0.7 (3)
Ethacrynic acid	11.0 ± 4.0 (6)	24.0 ± 3.0 (6)
GSH peroxidase activity (units†/mg protein)		
Cumene hydroperoxide	88.2 ± 9.6 (3)	120.6 ± 4.6 (3)
Hydrogen peroxide	73.8 ± 1.8 (3)	77.7 ± 3.8 (3)

Values are means ± SD; the number of determinations is given in parentheses.
* One unit of enzyme catalyzed the conjugation of 1 nmol of CDNB to GSH/min at 25°.
† One unit of enzyme converted 1 nmol GSH to oxidized glutathione/min at 37°.

ies were designed, therefore, to investigate the role of GSH-related enzymes, particularly GSH peroxidase and GST, in cellular resistance to DOX.

MATERIALS AND METHODS

Materials. All the chemicals used in this study were similar to those described by us previously [33].

Cell lines and culture. P388/S and P388/R mouse leukemic cell lines were obtained in 1978 from Dr Randall K. Johnson and Dr Arthur D. Little, Cambridge, MA, and maintained in DBA/2 mice by weekly i.p. transplantation. A >84-fold DOX-resistant cell line was isolated by serial cultivation of the P388/R cell line in methylcellulose containing gradually increasing drug concentrations. This resistant subline has been maintained in drug-free medium for the past several years. The IC_{50} values for the current *in vitro* passages of the sensitive and resistant cell lines in soft agar were 0.075 and 10.5 μM respectively. Log-phase cultures of P388/S and P388/R cells were maintained as suspension cultures in RPMI 1640 medium supplemented with fetal bovine serum (10%), penicillin (100 I.U./ml), streptomycin (100 μg/ml), and 2-mercaptoethanol (10 μM)

Preparation of cell extract. Cells were washed three times with 10 mM potassium phosphate, pH 7.0, containing 150 mM sodium chloride (PBS) and sonicated twice in 10 mM potassium phosphate, pH 7.0, containing 1.4 mM 2-mercaptoethanol (buffer A) for 30 sec with an interval of 5 min between each sonication. The cell sonicate was centrifuged at 14,000 *g* for 40 min, and the supernatant fraction was used for enzyme assays and immunological studies.

Enzyme assays. GST activity with 1-chloro-2,4-dinitrobenzene (CDNB), 3,4-dichloronitrobenzene (DCNB), and ethacrynic acid (EA) was determined according to the method of Habig *et al.* [34]. Selenium-dependent and -independent GSH peroxidase activities were determined according to the procedures described by Beutler [35] and Awasthi *et al.* [36]. The protein content was determined according to the method of Bradford [37].

Immunological studies. Antibodies against α, μ, and π classes of human GSTs were raised in New Zealand albino rabbits as described by us previously [36]. To assure that these antibodies were specific to their respective antigens, immunoblotting studies were performed using apparently homogenous preparations of GST α, μ (GST Ψ), and π. In immunoblotting, antibodies raised against α, μ, and π class human GSTs cross-reacted only with their respective antigens (data not shown). Immunotitrations and Western blotting were performed according to the procedures of Batist *et al.* [28] and Towbin *et al.* [38] respectively.

Purification of GST from sensitive and resistant P388 *cells.* GST from P388/S and P388/R cells was purified according to our previously described protocol [39]. Briefly, the 14,000 *g* supernatant fraction after dialysis against buffer A was subjected to GSH-affinity chromatography [40]. The affinity column was pre-equilibrated with 22 mM potassium phosphate, pH 7.0, containing 1.4 mM 2-mercaptoethanol (buffer B) at a flow rate of 10 ml/hr, and this flow rate was maintained throughout the affinity chromatography. After washing the column thoroughly with buffer B, GST was eluted with 5 mM GSH in 50 mM Tris–HCl, pH 9.6, containing 1.4 mM 2-mercaptoethanol.

RESULTS AND DISCUSSION

Data on GST activity in 14,000 *g* supernatant fractions of P388/S and P388/R cells towards CDNB, EA, and DCNB are presented in Table 1. GST activity towards CDNB and EA was 1.8- and 2.2-fold higher, respectively, in P388/R cells than in P388/S cells (Table 1). GST activity towards DCNB, however, was lower in P388/R cells compared to P388/S cells. It is well-documented that multiple isoenzymes of GST arise from the dimeric combination of a number of distinct subunits [41]. Multiple isoenzymes of GST have been grouped into three major classes, α, μ, and π, based on their structural, catalytic, and immunological properties [41]. Even though, α, μ, or π class GST isoenzymes have overlapping substrate specificities, certain substrates are preferentially utilized by isoenzymes of a particular class. For example, π class GST iso-

Table 2. Purification of glutathione *S*-transferase from P388/S and P388/R cells*

	GST activity (units†)	Protein (mg)	Specific activity (units/mg protein)	Yield (%)
14,000 *g* Supernatant				
P388/S	0.345	6.39	0.054	
P388/R	0.565	6.58	0.086	
Affinity chromatography				
P388/S	0.298	0.058	5.13	86.3
P388/R	0.518	0.109	4.75	91.6

* 40×10^6 P388/S and P388/R cells were used for enzyme purification.
† One unit of enzyme catalyzed the conjugation of 1 μmol of CDNB to GSH/min at 25°.

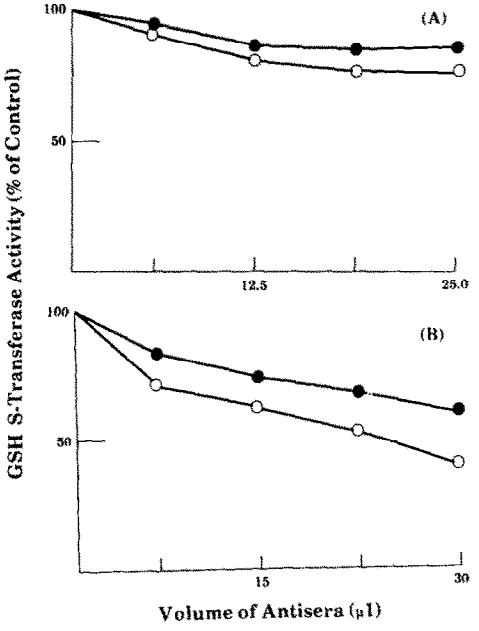

Fig. 1. Immunotitration studies using antibodies raised against (A) GST α of human liver and (B) GST π of human placenta with 14,000 *g* supernatant fractions of P388/S (○) and P388/R (●) cells. Approximately 160 μg of cytosolic protein from P388/S and P388/R cell supernatant fractions was used in immunotitration studies. The specific activities of GST in P388/S cells in experiments (A) and (B) were 0.059 and 0.052 μmol/min/mg protein respectively. GST activities in P388/R cells in experiments (A) and (B) were 0.093 and 0.090 μmol/min/mg protein respectively.

enzymes show preference in expressing activity towards EA [41, 42]. Therefore, comparatively higher EA conjugating activity in P388/R cells would suggest overexpression of π class GST isoenzyme in this cell line. The intracellular GSH concentrations were found to be similar in P388/S and P388/R cells (about 14.4 nmol/mg protein).

To determine if the increased GST activity in P388/R cells was due to an increase in GST protein, we purified GST from an equal number of P388/S and P388/R cells using GSH-affinity chromatography (Table 2). This one-step affinity chromatography has been used successfully to obtain apparently homogenous preparations of GST from several tissues [39, 42, 43]. In gel filtration over a calibrated column of Sephadex G-100, affinity purified GST from P388/R and P388/S cells showed the presence of a single protein peak which was coincident with the GST activity peak corresponding to a M_r value of approximately 48,000 (data not presented). These results indicated that affinity purified GST preparation was probably free from detectable amounts of other proteins. From 40×10^6 P388/S and P388/R cells, 58 and 109 μg of purified GST protein, respectively, were obtained (Table 2). The total purified GST protein was approximately 1.9-fold more in P388/R cells. The percentage yields of the purified GST obtained from P388/S and P388/R cells were similar (Table 2). Taken together, these results suggested that the elevated GST activity in P388/R cells was due to the increased GST protein content rather than to enzyme activation.

To quantitate the amounts of various classes of GST isoenzymes in these cell lines, immunotitration and Western blotting studies were performed using antibodies raised against α, μ, and π classes of human GST isoenzymes. In immunotitration studies with antibodies against GST α, when equal amounts of cytosolic protein (160 μg) from P388/S and P388/R cell supernatant fractions were used, 25 μl of the antibody precipitated 23 and 18% of total GST activity in P388/S and P388/R cells respectively (Fig. 1A). These results indicate that α type GST isoenzyme constitutes less than 25% of total GST protein in P388/S cells, and P388/R cells have about 1.27-fold more α type antigen compared to P388/S cells. However, a clearcut difference in the level of α type GST protein was not observed in Western blot analysis (data not shown). These data suggest that Western blotting is not sensitive enough to detect a difference of about 27% in the levels of GST α between these cell lines. When antibodies raised against a μ class GST isoenzyme (GST Ψ of human liver) were used in immunotitration studies, a significant precipitation of GST activity did not occur in either of these cell lines (data not shown). Western blotting using antibodies raised against GST Ψ also showed similar results (data not shown). These results suggest that μ class GST isoenzymes are either absent or present at a very low concentration in these cell lines. Immunotitrations with antibodies raised against GST π indicated that in P388/S cells more than 65% of total GST activity was accounted for by

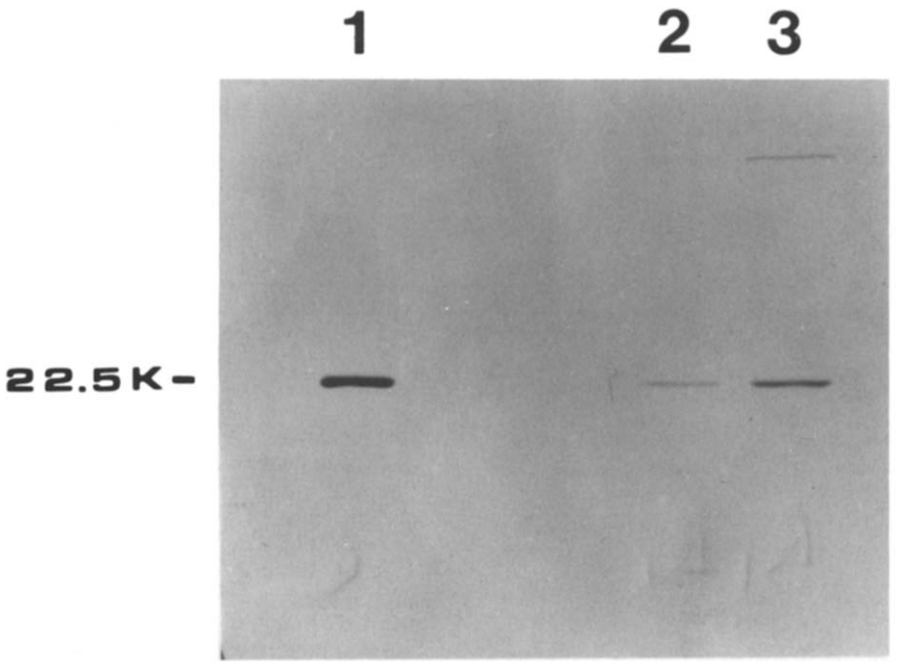

Fig. 2. Western blot analysis using antibodies raised against GST π of human placenta. Aliquots of 14,000 g supernatant fractions of P388/S and P388/R cell lines containing 50 μg of cytosolic protein were used for polyacrylamide gel electrophoresis. Lane 1 contained 5 μg of purified GST π from human placenta; Lanes 2 and 3 contained aliquots from P388/S and P388/R cell supernatant fractions respectively.

this class of GST isoenzyme, and P388/R cells had about 2.2-fold more GST π (Fig. 1B). Similar to immunotitration studies, Western blot analysis also revealed the presence of comparatively more GST π type antigen in P388/R cells (Fig. 2, lane 3) when compared to that in P388/S cells (Fig. 2, lane 2).

GSH peroxidase activities in P388/S and P388/R cells were also determined (Table 1). There are two types of GSH peroxidases in mammalian tissues [16, 17]. GSH peroxidase is a selenium-dependent enzyme and uses both H_2O_2 and lipid hydroperoxide, such as cumene hydroperoxide, as substrates [16, 17]. On the other hand, selenium-independent GSH peroxidase II activity is expressed by some of the isoenzymes of GST only towards lipid hydroperoxides [17, 36, 43].

GSH peroxidase activity towards H_2O_2 was comparable in P388/S and P388/R cells (Table 1), indicating similar levels of selenium-dependent GSH peroxidase in these cell lines. On the other hand, GSH peroxidase activity with cumene hydroperoxide was approximately 1.36-fold higher in P388/R cells compared to P388/S cells (Table 1), indicating elevation of GSH peroxidase II activity in P388/R cells. Increased GSH peroxidase II activity in P388/R cells is consistent with elevated levels of α class GST (1.27-fold) in these cells (as evidenced by immunotitration studies, Fig. 1A) because GSH peroxidase II activity has been suggested to be maximally expressed by this class of GST isoenzymes [41, 43].

Energy-dependent rapid efflux has been shown to be a major factor for cellular resistance to DOX in our >84-fold resistant P388 cells [5]. Treatment of these cells with efflux blockers, however, does not cause complete reversal of resistance [5]. The results of the present study taken together with those of other investigators [29, 44] suggest that cellular DOX resistance of P388/R cells is multifactorial.

GSH regulates activities of several enzymes (such as GSH peroxidase and GST) which are important for the protection of cells against oxidants and electrophilic xenobiotics [16–18]. In the present study we observed an increase in GSH peroxidase II activity in P388/R cells. This activity, therefore, may contribute to DOX resistance of P388/R cells by enhanced detoxification of free radicals in these cells as free radicals have been implicated in the cytotoxic activity of DOX [32].

The role of GST has received much attention recently because of several reports on the elevated levels of this enzyme in drug-resistant tumor cells. Overexpression of α (Yc-type subunits) and μ (Yb-type subunits) class GST isoenzymes has been reported in Walker 256 rat mammary carcinoma cell lines with acquired resistance to chlorambucil [23, 24]. A 45-fold increase in π type GST has been reported in a multi-drug-resistant MCF-7 breast cancer cell line selected for resistance to DOX [28] with a positive correlation between the degree of resistance and elevation of GST π. Elevation in the levels of α and π class GST isoenzymes has been shown in a Chinese hamster ovary cell line resistant to bifunctional nitrogen mustards [25, 45]. We have demonstrated recently induction of π type GST isoenzyme in a human plasma cell line resistant to melphalan [27]. In the present study we also observed an increase in α and π class GST isoenzymes in P388/R cells. Overexpression of π class GST has been

reported previously in 5- to 7-fold DOX-resistant murine P388 leukemic cells [29]. However, these investigators did not find any significant increase in the levels of α class GST in their resistant subline [29]. Interestingly, in a different 37-fold DOX-resistant P388 mouse leukemic cell line, the GST activity was shown to be only about 33% of that in the sensitive cell line [44]. These results, in conjunction with other studies [24, 27, 29, 44], suggest that the expression of GST isoenzymes is under complex regulation and may vary with the degree of resistance, origin of cell line, and nature of the chemotherapeutic agent. Further studies, however, are needed to substantiate these hypotheses. Studies are also required to establish the physiological significance of increased π class GST isoenzyme in our more than 84-fold DOX-resistant P388 cell line.

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