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Glutathione S-Transferases in Nitrogen Mustard-Resistant and -Sensitive Cell Lines

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SUMMARY

Tumor cell resistance to alkylating agents was studied by examining Walker 256 rat mammary carcinoma cells differentially sensitive to nitrogen mustards. A resistant subpopulation (WR) was selected by exposure to chlorambucil. WR cells showed approximately a 15-fold resistance to the cytotoxic effects of nitrogen mustards and elevated glutathione *S*-transferase (GST) activity when compared to the sensitive parent cell line (WS). To extend these findings, the GSTs from WR and WS were purified by affinity chromatography on *S*-hexylglutathione coupled to epoxy-activated agarose. Substrate specificity experiments using purified GSTs demonstrated different profiles of enzyme activity for WR and WS and suggested differential isoenzyme expression in these two cell lines. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis revealed that the major GST present in both WR and WS was a 26,000-Da subunit that was immunologically distinct from the rat liver GSTs. This GST subunit cross-reacted with antibodies against anionic human placental GST. In addition, three GST forms common to rat liver (29,500, 28,500 and 27,500 molecular weight) were also identified. Overexpression of the 29,500-Da protein was observed in WR cells. These data suggest that differential expression of GST subunits may contribute to the nitrogen mustard-resistant phenotype.

Development of acquired resistance to anticancer drugs by an initially drug-responsive tumor is a primary cause of treatment failure and patient relapse. In order to design more efficacious treatment regimens, it is important to understand the molecular basis of cellular resistance. Alterations in drug-catabolizing enzymes have been proposed to contribute to the resistant phenotype by allowing the more rapid and efficient detoxication of cytotoxic anti-cancer agents.

The GSTs have been implicated recently in mediating cellular resistance to several classes of anticancer drugs (1–3). The GSTs are a family of detoxication isoenzymes which catalyze the conjugation of glutathione with a diverse range of hydrophobic and electrophilic substrates (4). Elevated levels of GST activity have been reported in cell lines expressing resistance to chlorambucil (1) and cyclophosphamide (2), as well as in a multidrug-resistant cell line (3). Although little is known about tumor cell GSTs, these enzymes have been characterized extensively in a variety of rat tissues, most notably in rat liver (for review, see Ref. 5). The rat cytosolic GSTs are homo- and heterodimeric proteins with subunit molecular weights ranging from 22,000 to 30,000. At least seven different subunit monomers have been identified with overlapping substrate specificities. In the present study, we report on the characterization of the cytosolic GSTs from a tumor cell line demonstrating resistance to nitrogen mustards.

Materials and Methods

Cell culture. Walker 256 rat mammary carcinoma cells were maintained as described (6). Resistant cells (WR), selected from the sensitive parent cell line (WS) following exposure to chlorambucil (7), possess an approximately 15-fold resistance to alkylating agents (6). Resistance was maintained by routine exposure to chlorambucil (20 μg/ml) every 6 months. WR and WS cells were grown as suspension cultures in Dulbecco's minimal essential medium supplemented with 4 mM L-glutamine, penicillin/streptomycin, and 10% fetal calf serum.

Purification of Walker cell GST. Walker cells (5–8 × 10^9, for both WR and WS) were harvested during mid-log growth by centrifugation. All steps were performed at 4°. The cells were homogenized in 3 volumes of 10 mM Tris-HCl, pH 7.8 (buffer A). A cell cytosol was prepared by spinning the homogenate at 10,000 × *g* for 20 min followed by ultracentrifugation at 100,000 × *g* for 65 min. The resulting microsome-free supernatant was dialyzed against buffer A and then applied to a column of *S*-hexylglutathione coupled to epoxy-activated agarose (Sigma). The packing was soaked in buffer A for 2 hr at room temperature. The settled resin was washed with the same buffer and poured into a 1.5 × 10 cm column. The column was packed under gravity and washed sequentially with 60 ml of 50 mM Tris/0.5 M NaCl (pH 9.6), 60 ml of 50 mM sodium acetate/0.5 M NaCl (pH 4.5) and equilibrated overnight against buffer A. After sample application, the column was washed with buffer A, followed by the same buffer fortified with 0.2 M NaCl (8). Walker cell GSTs were eluted with buffer A containing 0.2

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ABBREVIATIONS: GST, glutathione *S*-transferase; SDS, sodium dodecyl sulfate.

M NaCl and 0.005 M *S*-hexylglutathione. Fractions exhibiting GST activity were combined, dialyzed overnight against buffer A, and concentrated using the Pro-Dimem system (Bio-Molecular Dynamics).

Antibody Production. Antibodies to affinity purified rat liver GST (Sigma) were raised in rabbits using conventional methods (9). Prior to antibody production, rat liver GST was dialyzed against phosphate-buffered saline, analyzed by SDS-polyacrylamide gel electrophoresis, and found to be free of protein contaminants. Female New Zealand White rabbits were immunized subcutaneously at 10 injection sites (100 μl each) with a total of 0.5 mg of GST. Two subsequent injections were given in an identical manner at 3- to 4-week intervals. Blood was obtained by cardiac puncture and centrifuged at 2500 rpm for 7 min to separate serum from blood cells.

Antibodies to anionic human placental GST were provided courtesy of Dr. Gerald Batist, Montreal General Hospital Research Institute, Montreal, Canada.

Gel electrophoresis. SDS-polyacrylamide slab gel electrophoresis was performed according to the method of Laemmli (10) using a 12% polyacrylamide resolving gel. Gels were stained overnight with 0.1% Coomassie blue and destained in 20% methanol/10% acetic acid.

Immunoblot analysis. Proteins were electrophoretically transferred from polyacrylamide gels onto nitrocellulose membranes (Bio-Rad Transblot System) according to the method of Towbin *et al.* (11). Electroblotting was conducted for 16 hr at 30 V in 25 mM Tris base/192 mM glycine, pH 8.3, containing 20% methanol. All incubations were for 1 hr at 25° with intermediate rinses in buffer B (50 mM Tris base, pH 7.5, 400 mM NaCl) containing 0.05% Tween-20. Nonspecific binding was blocked by placing membranes in buffer B containing 3% bovine serum albumin. Membranes were incubated with primary antibody (antisera against either rat liver GST or anionic human placental GST) at optimum dilutions (1:500) in buffer B containing 3% bovine serum albumin. Membranes were then incubated with horseradish peroxidase-conjugated IgG. For visualization of cross-reacting bands, membranes were developed with buffer B containing H_2O_2 and either 4-chloro-1-napthol or 3,3′-diaminobenzidine.

Enzyme assays. GST activity was assayed spectrophotometrically using 1-chloro-2,4-dinitrobenzene as a substrate according to the method of Habig *et al.* (12). The specific activities toward various other substrates were determined according to published procedures (12–14). Protein determinations were made using the Bio-Rad protein assay with bovine gamma globulin as standard.

Results

The results of affinity purification of Walker cell GSTs from cytosol are shown in Table 1. Application of the cytosol to the *S*-hexylglutathione affinity column resulted in elution of the cytosolic Walker cell GSTs as a single peak with specific activities of 2.06 and 3.10 μmol/min/mg for WS and WR, respectively. This single chromatographic step produced greater than a 170-fold purification for both WS and WR. Less than 20% of the applied cytosolic GST activity eluted in the flow-through fractions, suggesting that most of the activity bound to the *S*-hexylglutathione column. However, for both WR and WS, only 30–35% of the total activity was recovered from the column, suggesting that some GST activity remained bound to the affinity column. Alternatively, an irreversible loss of enzyme activity may have occurred as previously suggested (15). No greater recovery of activity could be achieved by using a glutathione (instead of *S*-hexylglutathione) affinity matrix.[1]

Differences in the substrate specificities of the GSTs from various tissues are indicative of differences in isoenzyme content (16). As shown in Table 2, although GSTs from WR and WS were both active against the same range of substrates, there were marked differences in the individual activity profiles. Only WR GST was active against bromosulfophthalein. Differences also existed in the specific activities of WR and WS for 3,4-dichloronitrobenzene, ethacrynic acid, and *trans*-4-phenyl-3-buten-2-one. WR and WS showed comparable activity against *p*-nitrophenyl acetate. No activity was detected in either WS or WR using 1,2-epoxy-3-(*p*-nitrophenoxy)propane as a substrate. These data differ from earlier reported substrate specificities (1) since, in the present study, purified GST preparations, rather than crude cellular extracts, were used.

SDS-polyacrylamide gel electrophoresis revealed that the affinity purified GST preparation contained several subunits. Fig. 1 shows that the major GST subunit (molecular weight = 26,000) present in the affinity purified preparation from Walker cells (WR and WS) does not co-migrate with GST subunits present in rat liver. Both WR and WS contain a subunit with a molecular weight of 28,500. WR cells also contain a GST subunit that corresponds to molecular weight 29,500. This protein is not visible by Coomassie blue staining in WS.

Antibodies generated in rabbits cross-reacted with rat liver GSTs and Walker cell cytosol, as determined by enzyme-linked immunosorbent assay (17). To identify the constituent GSTs of Walker cells common to rat liver, affinity purified proteins were separated by SDS-gel electrophoresis, transferred to nitrocellulose, and incubated with the rat liver GST antibodies. Western blot analysis revealed that at least two of the GST subunits present in rat liver were also present in Walker cells. As is evident in Fig. 2 (*lanes a–c*), both WR and WS express similar levels of the M_r 28,500 GST (co-migrating with rat liver Yb class GSTs) and very low levels of M_r 27,500 (Ya class, not visible by protein staining on SDS gels). In contrast, a marked difference existed between WR and WS in the levels of expression of the 29,500-Da GST subunit. Only low levels of this 29,500-Da subunit (co-migrating with rat liver Yc class GSTs) were evident in WS, whereas this subunit was overexpressed in WR.

The most prominent subunit present in affinity purified

TABLE 1

Affinity purification of Walker cell GSTs from cell cytosol

	Total volume	Total protein	Total activity	Specific activity	Yield	Fold purification
	ml	*mg*	*μmol/min*	*μmol/min/mg*	%	
WS						
Cytosol	33.0	498.3	6.4	0.0138		
Affinity purified	4.0	0.96	1.97	2.060	33	171
WR						
Cytosol	34.0	387.0	6.7	0.0171		
Affinity purified	3.4	0.68	2.10	3.100	31	179

TABLE 2

Comparison of substrate specificities of affinity purified Walker cell GSTs[a]

Substrate	WS	WR	WR/WS
1-Chloro-2,4-dinitrobenzene	2.19	3.10	1.42
3,4-Dichloronitrobenzene	0.0651	0.0318	0.49
Ethacrynic acid	0.1942	0.0849	0.44
p-Nitrophenyl acetate	0.0091	0.0073	0.80
1,2-Epoxy-3-(*p*-nitrophenoxy)propane	ND[b]	ND	
Bromosulfophthalein	ND	0.0040	
trans-4-Phenyl-3-buten-2-one	0.0149	0.0063	0.42

[a] Values are expressed as specific activity (μmol/min/mg).
[b] ND, no detectable activity.

[1] Unpublished data.

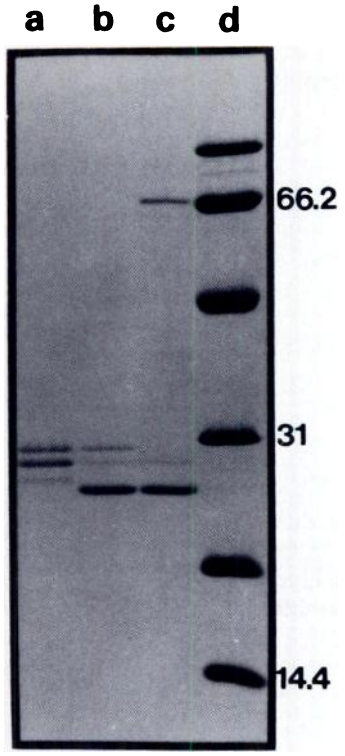

Fig. 1. SDS-polyacrylamide gel electrophoresis of affinity purified GSTs from rat liver (*lane a*), WR (*lane b*), and WS (*lane c*). *Lane D* shows molecular weight markers. Proteins are stained with Coomassie blue.

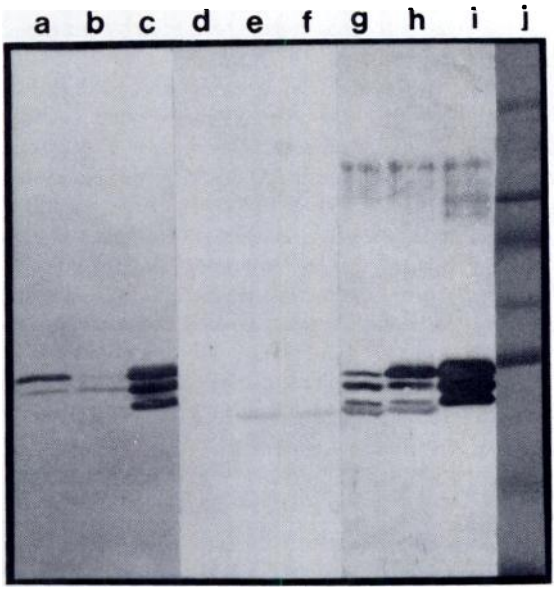

Fig. 2. Western blot analysis of affinity purified GSTs from rat liver (*lanes c, d,* and *i*), WR (*lanes a, e,* and *h*), and WS (*lanes b, f,* and *g*). Nitrocellulose membranes were incubated with antibodies raised against rat liver GSTs (*lanes a–c*), antibodies raised against human placental GST (*lanes d–f*), or both antibodies (*lanes g–i*). *Lane j* shows molecular weight markers (12,400, 18,400, 29,000, 36,000, 43,000, 55,000, and 95,500). Staining was achieved as described in Materials and Methods.

Walker cell GST preparations (WR and WS) did not cross-react with rat liver GST antibodies. However, as shown in Fig. 2 (*lanes d–f*), this protein did cross-react with antibodies raised against the anionic GST found in human placenta. Fig. 2 (*lanes g–i*) demonstrates the results from a double labeling experiment in which nitrocellulose membranes were incubated and stained using anionic antibodies followed by rat liver antibodies. This technique allowed the identification of all the Coomassie-stained bands as well as those GSTs not visible by conventional protein staining procedures.

Discussion

Purification of the GSTs from Walker cells by *S*-hexylglutathione affinity chromatography isolated a total of four GST subunits. Resistant cells contained elevated levels of a 29,500-Da GST co-migrating with Yc class rat liver GSTs which was present in the drug-sensitive parent cell line at low levels. These data suggest that the Yc class GST is overexpressed in the resistant cells.

The mechanism by which this GST subunit is overexpressed remains to be elucidated. The GSTs of rat liver can be induced with a number of xenobiotics, including phenobarbital and 3-methylcholanthrene (18). This induction appears to be specific for certain GST subunits (19) and is mediated by an increase in transcriptional activity (20, 21). At this time, there are no other reports of drug-induced overexpression of Yc subunits. Low doses of cyclophosphamide have been shown to produce a transient elevation in mouse bone marrow GST and protect against the effects of subsequent lethal doses of the same drug (22). In WR cells, increased levels of GST resulted from further exposure to chlorambucil, and this was concomitant with increased resistance (1).

Whether or not the increase in Yc-type GSTs in the WR cells is due to induction of transcriptional activity remains to be determined. Alternatively, the overexpression of GST in WR cells may result from the amplification of the gene or genes coding for the specific GST(s) affected. Such an occurrence would be analogous to the overexpression of dihydrofolate reductase in methotrexate resistance (23). Although karyotype analysis in WR and WS (24) showed chromosomal differences, (including centromere-minus chromosomes and limited homogeneously staining regions in WR), there was no definitive indication of gene amplification in the WR cells.

Previous studies have shown that melphalan is a substrate for the GST-catalyzed conjugation with glutathione (25). These studies have since been extended to show that chlorambucil can also serve as a substrate for GST.[2] The low level of glutathione conjugation observed in the absence of GST for both studies (25)[3] suggested that enzymatic catalysis was essential for the efficient detoxication of these compounds. Such spontaneous conjugation is limited by the "hard" [high charge density and polarization (27)] nature of the electrophilic alkyl carbonium ion and the contrasting "soft" [diffuse electron density which is polarizable (28)] nucleophilic thiol group of glutathione. Thus, the increased rate of conjugation provided by GST involvement would effectively protect cells from alkyl carbonium ion toxicity, a process further enhanced by elevated GST in WR cells.

The significance of a GST that is immunologically related to the anionic human placental GST is not known. A similar GST, not found in other tissues, has been identified in rat placenta (29). Interestingly, this form has been shown to be

[2] D. M. Dulik, O. M. Colvin, and C. Fenselau, submitted for publication.
[3] D. M. Dulik, O. M. Colvin, and C. Fenselau, submitted for publication.

elevated in preneoplastic lesions, suggesting that it may be a tumor marker (30).

The elevation of detoxifying enzymes in the resistant line would seem to provide compelling evidence for a mechanism by which resistance to mustards may be expressed. Using 1-chloro-2,4-dinitrobenzene as a substrate, the WR cells have been shown to have between a 2- (1) and 5-fold (31) increase in bulk GST activity. However, the specific overexpression of the subunit co-migrating with Yc is considerably greater and correlates more accurately with the quantitative resistance. Moreover, recent data have shown that WR cells which have reverted to a sensitive phenotype no longer have elevated GST or overexpress certain GST subunits (data not shown). This would be consistent with a defined role for GST in maintaining resistance; however, other reported changes may be of relevance. For example, earlier studies with WR cells have shown alterations in a number of cellular properties including glutathione reductase activity (32), O^6 guanine alkyl transferase activity (33), and cyclic AMP-binding proteins (6). In addition, it remains to be determined whether or not the elevation in GST activity is a direct response to drug treatment and specific to the GSTs or part of a more generalized cellular response to stress. In either case, differential expression of GST isoenzymes may contribute to a more efficient detoxication of cytotoxic chemotherapeutic agents.

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