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GLUTATHIONE *S*-TRANSFERASE AND P-GLYCOPROTEIN IN MULTIDRUG RESISTANT CHINESE HAMSTER CELLS

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Abstract—Glutathione *S*-transferases (GSTs) have been reported to be elevated in some forms of hepatic carcinogenesis, in multidrug resistant (MDR) cells exhibiting elevated P-glycoprotein, and in cells resistant to alkylating agents independent of the MDR phenotype. The reported elevation of GST in association with the MDR phenotype and the overexpression of P-glycoprotein along with induction of GST in hepatic carcinogenesis suggest a correlation in the two mechanisms of cellular detoxification. To evaluate this hypothesis we examined the expression of GSTs in an MDR Chinese hamster fibroblast cell line overexpressing P-glycoprotein. We were unable to demonstrate concordant elevation of GST in these MDR cells. We conclude that GST expression is independent of P-glycoprotein expression in MDR Chinese hamster fibroblasts. The overexpression of GSTs in certain cells may provide an alternative mechanism for the development of drug resistance, either in association with or independent of P-glycoprotein overexpression, but is not essential for the MDR phenotype.

Cellular detoxification of exogenous toxins, antibiotics, carcinogens and anticancer agents involves detoxifying enzymes such as superoxide dismutase, catalase, glutathione peroxidase and glutathione *S*-transferases [1]. Glutathione *S*-transferases (GSTs, EC 2.5.1.18) catalyze the conjugation of a number of structurally distinct electrophilic compounds including several chemical carcinogens to glutathione (GSH), thereby playing a crucial role in general intracellular detoxification mechanisms [2, 3]. Multiple GST isozymes present in mammalian tissues arise from dimeric combinations of a number of distinct subunits which have been grouped into three major classes α, μ and π [4, 5]. π-Class GST, in particular GST-P, has been reported to be a marker for preneoplastic cells in chemical hepatocarcinogenesis in rats [6, 7]. GST-π is also reported to be elevated in human and rat hepatomas, gastrointestinal carcinomas [8, 9], and in an Adriamycin®-induced multidrug resistant (MDR) human breast cancer cell line (MCF-7) [10, 11] and some other drug-resistant cell lines [11–15]. The MDR phenotype may be associated with the induction and overexpression of a 170 kD membrane glycoprotein (P-glycoprotein) [16, 17]. P-Glycoprotein seems to function as a detoxifying membrane protein by binding drug or enhancing energy-dependent drug efflux [18]. Because P-glycoprotein overexpression also occurs in some forms of experimentally induced rat hepatocarcinogenesis [19, 20] even before the overexpression of GST-P, it has been implicated as a general drug detoxification mechanism. This is consistent with the known distribution of P-glycoprotein in organs such as the liver, kidney, and colon [21, 22]. Elevation of GST in some MDR cell lines along with the increased levels of P-glycoprotein suggests that the causes of MDR phenotype may be multifactorial [11, 13, 15]. GST isozymes may also be overexpressed during drug resistance to alkylating agents independent of the MDR phenotype [23–25]. To determine whether there is a concomitant overexpression of π-class GST or other GST isozymes in all MDR cell lines, we have compared GST expression in Adriamycin®-induced MDR Chinese hamster fibroblasts and the parent wild type cells from which they were derived.

MATERIALS AND METHODS

Cell lines. Chinese hamster V79-473 fibroblasts (V79) and the Adriamycin®-induced multidrug resistant LZ 632 (LZ) cells derived from V79 cells were cultured in Hams F10 medium containing 15% fetal calf serum and 1% penicillin–streptomycin. The LZ cells were cultured in medium containing 8 μg/mL of Adriamycin®. The LZ cells have been reported previously to express the MDR phenotype and are cross-resistant to vincristine, colcemid, and actinomycin D when compared to V79 cells [26]. Log phase V79 and LZ cells were harvested after treatment with 0.25% trypsin. The cells were centrifuged and washed three times with phosphate-buffered saline (PBS) before being processed for further analysis.

Preparation of cell extract. Cells were sonicated at 4° in 0.5 mL of 10 mM potassium phosphate buffer, pH 7.0, containing 1.4 mM 2-mercaptoethanol. The sonicate was centrifuged at 27,000 *g* for 40 min, and the supernatant fraction was used for enzyme assays and Western blotting for GSTs.

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Table 1. Glutathione levels and enzyme activities in V79 and LZ cell extracts

Cell extracts	GSH (nmol/mg protein)	GST (units/mg protein)*		GSH peroxidase (units/mg protein)†
		CDNB	Ethacrynic acid	
V79	3.86 (2)	60.5 ± 4 (3)	6.98 ± 0.2 (3)	7.41 (2)
LZ	3.03 (2)	45.6 ± 1 (3)	5.86 ± 0.1 (3)	5.0 (2)

Values are means of two or three determinations, as indicated by the number in parentheses. Standard deviations are shown for the values with three determinations.

* One unit of GST activity is defined as the amount required to catalyze the conjugation of 1 nmol of the electrophilic substrate to glutathione per min at 25°.

† One unit of peroxidase activity is defined as the amount required to utilize 1 nmol of NADPH per min at 37° for the reduction of oxidized glutathione formed by reducing the cumene hydroperoxide substrate in a coupled reaction.

Enzyme assays. GST activites towards 1-chloro-2,4-dinitrobenzene (CDNB) and ethacrynic acid were determined according to the method of Habig *et al.* [27]. GSH peroxidase activity was determined according to the procedure described by Awasthi *et al.* [28]. The protein content was determined by the method of Bradford [29], using bovine serum albumin as the standard. One unit of GST activity is defined as the amount required to catalyze the conjugation of 1 nmol of GSH to the electrophilic substrate per min at 25°. The rate of conjugate formation was monitored at 340 nm for CDNB and at 270 nm for ethacrynic acid. One unit of GSH peroxidase activity is defined as the amount required to utilize 1 nmol of NADPH per min at 37° for the reduction of oxidized glutathione formed by reducing the cumene hydroperoxide substrate in a coupled reaction.

Estimation of glutathione content. Cells were washed several times with PBS and sonicated in 500 μL of 10 mM potassium phosphate buffer, pH 7. The extract was centrifuged for 10 min at 10,000 *g*, and the supernatant fraction was used for the measurement of non-protein thiols, primarily GSH, according to the method of Beutler [30].

Immunoblotting for GSTs. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed in a Bio-Rad mini-electrophoresis cell essentially by the method of Laemmli [31]. Western blotting was done according to the method of Towbin *et al.* [32], with slight modifications as described previously [33]. Polyclonal antibodies specific for the α, μ and π classes of human GSTs were raised in rabbits according to conventional procedures, as described by us in a previous study [34]. Peroxidase conjugated anti-rabbit IgG was used as the secondary antibody, and the blot was developed using H_2O_2 and 4-chloro-1-naphthol as the substrates.

Preparation of plasma membranes. Plasma membrane fractions were purified from cells by the method of Riordan and Ling [35]. Membrane proteins were estimated by the method of Lowry *et al.* [36], using bovine serum albumin as the standard.

Immunoblotting for P-glycoprotein. SDS-polyacrylamide gel electrophoresis was performed according to the procedure of Fairbanks *et al.* [37]. Western blotting was done essentially according to the method of Towbin *et al.* [32]. C219 monoclonal antibody (Centocor, Inc., Malvern, PA) against P-glycoprotein was used as the primary antibody. Alkaline phosphatase conjugated goat anti-mouse IgG was used as the secondary antibody, and blots were developed using 5-bromo,4-chloro,3-indocyl phosphate (BCIP) and P-nitroblue tetrazolium chloride (NBT) as substrates.

RESULTS AND DISCUSSION

Total GST activities of the wild type V79 cells and the MDR LZ cells measured using CDNB as the substrate are presented in Table 1. The MDR LZ cells consistently exhibited 1.5 to 2.0-fold lower GST activity than the V79 cells in a series of comparative determinations. A similar pattern was observed when ethacrynic acid, a preferred substrate for the π-class isozymes, was used as the electrophilic substrate, suggesting lower activity of π-class isozyme(s) in the LZ cells. The GSH level and GSH peroxidase activity were also slightly lower for the LZ cells than for the wild type V79 cells. These results demonstrate that overexpression of GST or enhanced levels of GSH and GSH peroxidase activity are not associated with this MDR cell line and that the overexpression of GST(s) may not be essential to the mechanism(s) for the development of MDR phenotype.

Western blots of the crude supernatant fraction developed by using the antibodies against the π class GST of human placenta also indicated slightly lower levels (Fig. 1) of the π class or GST-P type isozyme(s) in LZ cell as compared to the wild type V79 cells. These findings are consistent with the GST enzyme activities.

Antibodies directed against α and μ class of GSTs indicated minimal expression of these GSTs in the V79 and LZ cells by Western blot analysis (data not shown). These data indicate that GSTs other than π were not significantly different in the V79 and the MDR LZ cells. Figure 2 demonstrates that the Adriamycin®-resistant MDR LZ cells overexpress the P-glycoprotein in comparison to the drug-sensitive wild type V79 cells. These findings are consistent with the previously reported presence of double minutes and P-glycoprotein gene amplification in the LZ cells [17, 26].

On the basis of these results we suggest that concordant elevation of P-glycoprotein expression and

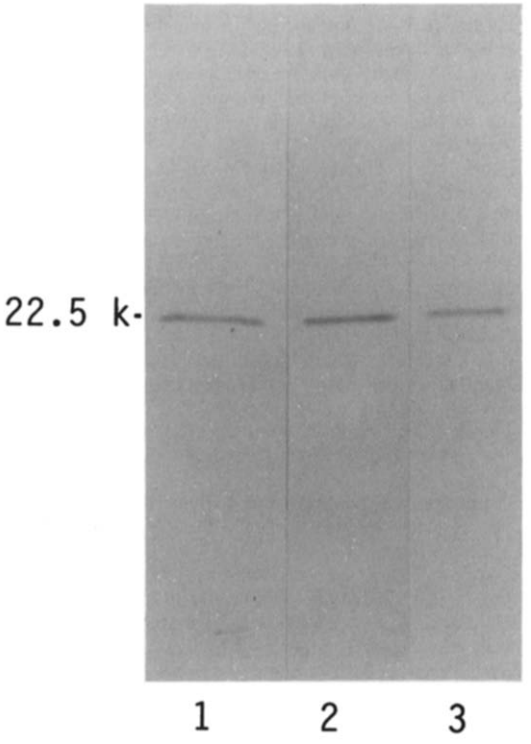

Fig. 1. Western blot of cell protein (25 μg) from V79 cells (lane 2) and LZ cells (lane 3), developed using antibody raised against human placental GST π. Lane 1 is the positive control, which was purified GST π from human placenta.

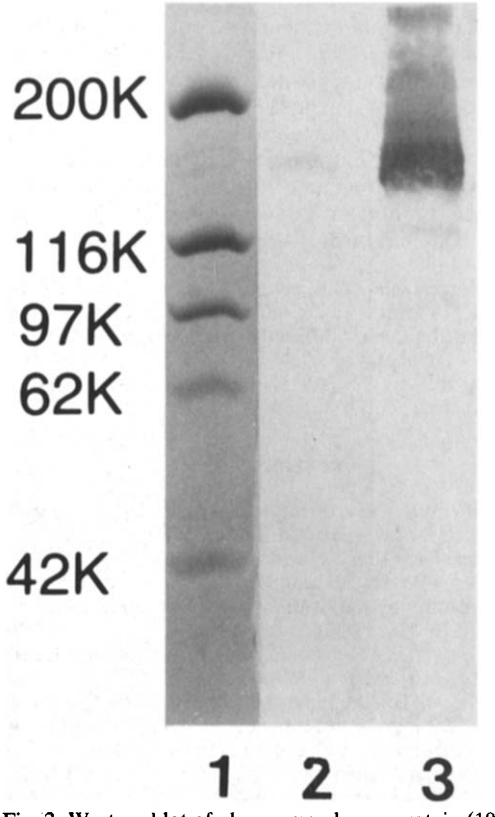

Fig. 2. Western blot of plasma membrane protein (100 μg) from wild type V 79 cells (lane 2) and MDR LZ cells (lane 3), demonstrating the presence of P-glycoprotein using C219 monoclonal antibody. Lane 1 shows molecular weight markers.

GSTs, particularly π-class GST, is not a universal feature of all MDR cell lines and may be species dependent. The human GST-π is located on chromosome 11 [38]. This is the same chromosome in MCF-7 Adriamycin®-resistant cells which contains homogeneously staining regions resulting from P-glycoprotein gene amplification [10]. Therefore, the high overexpression of GST-π type mRNA and protein in human MCF-7 Adriamycin®-resistant cells may be secondary to these chromosomal changes since the normal location of the P-glycoprotein gene is on chromosome 7 [39].

It is probable that overexpression of a specific GST isoenzyme does contribute towards the development of MDR in some cell lines, but this is not a universal phenomenon. Several factors, including the nature of wild type cells, their species of origin, and the series of events resulting in the MDR phenotype may dictate the mechanisms by which MDR is developed. That the differential overexpression of GST isoenzymes may be cell line or species dependent is also suggested by the different isozymes of GST induced in association with drug resistance to alkylating agents. Recently, it was reported that drug resistance to nitrogen mustards in Chinese hamster ovary cells is associated with gene amplification, mRNA overexpression, and increased activity of the α class of GST [40]. Rat Walker 256 chlorambucil-resistant cells have been reported to overexpress the Yc or α-class subunit(s) of GST [41]. In nitrosourea-resistant 9L rat gliosarcomas, a μ-type GST appears to be overexpressed [42]. We have reported recently the induction of a π-class GST in Sultan human myeloma cells resistant to melphalan [14]. The Sultan human myeloma cells resistant to melphalan and MCF-7 Adriamycin®-resistant cells [10] both overexpress an immunochemically similar π-class GST since they cross-react with the same antibody. However, these isozymes appear to be functionally different because the overexpressed GST π of the Sultan myeloma cell line does not express glutathione peroxidase activity, whereas that of the MCF-7 cell line strongly expresses this activity. Therefore, it appears that depending on the drug toxin exposed, and cells or tissues studied, different isozymes of GST and other detoxifying agents such as P-glycoprotein may be induced singly or in combination. This would also explain the differential response of GST-P to hepatocarcinogens such as 2-acetylaminofluorene and peroxisome proliferators. The former carcinogen induces GST-P, whereas the latter does not [6, 7, 43]. It should be pointed out that interspecies differences do exist in the makeup of GST isoenzymes of similar organs/tissues among mammals. For example, a GST-P-like isozyme constitutes the bulk of the GST activity of mouse liver [44] in contrast to minimal or nondetectable expression of GST-P in normal rat liver [45]. The overexpression of GST-P in rat liver during chemically induced hepatocarcinogenesis is

well documented but has not been demonstrated for the mouse model. Alterations in the expression of GSTs seen in similar tissues or derived cell lines in response to drugs during the development of resistance may not be expected to be similar in different species. Possible extrapolation of different animal model studies to evaluate the mechanisms of drug resistance in human tumors, therefore, must be approached cautiously.

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