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Glutathione depletion in human and in rat multi-drug resistant breast cancer cell lines

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Glutathione (GSH) is a major component of the cellular detoxification system. As the principal cellular non-protein sulfhydral, GSH maintains the redox state of cellular enzymes and is important in cellular defense against reactive oxygen species, including peroxides [1, 2]. Recent studies have demonstrated a central role of GSH in tumor cell responsiveness to a variety of antineoplastic treatments including radiation, the quinone-containing chemotherapy agent Adriamycin®, and DNA cross-link forming alkylating agents such as melphalan [3–5]. Tumor cell lines selected *in vitro* for resistance to a number of these agents have demonstrated alteration in GSH concentration or GSH-utilizing enzymes. Using buthionine sulfoximine (BSO) to inhibit the rate-limiting synthetic enzyme and thus deplete cellular GSH, these resistant cells may be sensitized to the respective chemotherapeutic agent [6, 7]. A study performed *in vivo* in tumor-bearing animals demonstrated differential effects of BSO on normal and tumor tissue, suggesting a potential clinical role for the combination of BSO with chemotherapy [8]. There was also a differential effect of BSO on GSH concentration between wild-type (WT) nad a melphalan-resistant variant of the same cell line.

We have examined a human breast cancer cell line and a subline selected for multi-drug resistance and found significant differences in responsiveness to BSO and in cellular GSH metabolism. Similar results were obtained in studies of a rat mammary tumor cell line with the phenotype of multi-drug resistance.

Methods

MCF-7 is a human breast cancer cell line and ADRr is a subline selected *in vitro* for resistance to Adriamycin which expresses the phenotype of multi-drug resistance [9]. These resistant cells were shown to have greatly increased expression of the MDR1 gene encoding the membrane-associated energy-utilizing drug-efflux pump protein P-glycoprotein, increased anionic glutathione-*S*-transferase (GST) and selenium-dependent glutathione peroxidase, and enhanced capacity to reduce GSH after peroxide challenge [10, 11]. MatB is a mammary carcinoma chemically induced in Fisher rats that can be grown both *in vitro* and *in vivo*. A subline selected in Adriamycin expresses the phenotype of MDR and similar molecular characteristics: enhanced expression of MDR1, significantly decreased GSH concentration, and increased expression of GST. Selenium-dependent glutathione peroxidase is not increased, but selenium-independent organic peroxidase due to a basic GST isoenzyme is elevated [12]. The cells used in these studies were all drug-free for at least 1 month, and retained these characteristics. Cells were maintained in RPMI 1640 medium (GIBCO) (human cell line) or Minimum Essential Medium alpha (MEM-alpha) (GIBCO) (rat cell line) with 5% fetal bovine serum plus penicillin and streptomycin, and 2 mM glutamine at 37° under 5% CO_2. The clonogenic assays of the human MCF-7 cell line were performed in 6-well Linbro plates using three different protocols. In the first two, cells were plated at a density of 200–400/well and left overnight to attach to the plastic surface. The next day the medium was replaced with fresh medium containing various concentrations of BSO. The BSO was maintained continually in the medium in one set of studies, or else was replaced after 24 hr by drug-free medium. In a third set of assays the cells were exposed as semi-confluent monolayers still in the logarithmic phase of growth, to various concentrations of BSO for 20 hr. The cells were then washed free of BSO, trypsinized, and replated at low density for clonogenic assay. In all assays, once the cells were treated and plated at low density they were incubated for 10 days, after which medium was removed, cells were fixed and stained with ethanol acetate and Coomassie blue, and visible colonies were counted. Results are presented as percent of untreated control surviving colonies. To study the effect of verapamil on cellular GSH concentration, cells in logarithmic growth were exposed to 10 μg/mL of verapamil over 2 days, harvested by trypsinization, and counted using a hemocytometer; then total cellular GSH was measured.

Glutathione was assayed according to the technique described in Ref. 13. Pre-confluent adherent cells were exposed to BSO for various times and were then harvested using trypsin–EDTA. After counting the cells, 4–5 × 10^6 cells of each cell line were lysed in distilled water and a solution of 5-sulfosalicylic acid was added resulting in a 3% final solution. After centrifugation, the supernatant was used to determine total glutathione (GSH + GSSG) concentration. γ-Glutamylcysteine synthetase (GGCS) was assayed in cytosolic preparations of the cell lines according to Seelig and Meister [14], and γ-glutamyltranspeptidase (GGT) was measured in whole cell homogenates as described by Szasz [15]. GST and selenium-dependent glutathione peroxidase (GSH-Px) were assayed as previously described using hydrogen peroxide as substrate [9, 16]. Selenium-independent peroxidase was assayed using cumene hydroperoxide as substrate. Protein was determined using the technique of Lowry *et al.* [17]. All biochemical assays were performed in triplicate in three separately grown samples.

Results

Table 1 lists the biochemical features of the human cell lines examined. The GSH concentration of the cells determined in logarithmic phase of growth in both cell lines was found to be significantly lower in ADRr compared to WT. In the MCF-7 cell line this cannot be explained on the basis of reduced synthesis since the activity of GCS was increased significantly ($P < 0.001$). Both GSH-Px and GST were increased in the ADRr cells compared to the sensitive cell line, and GGT activity was conversely significantly ($P < 0.001$) decreased in activity. In the MatB rat mammary carcinoma cell lines, GSH was also decreased significantly in the ADRr subline compared to the WT cells (1.8 ± 0.09 vs 5.2 ± 0.4) nmol/10^6 cells), while GST activity was increased significantly (11.8 ± 0.5 vs 8.7 ± 1.2). Molecular characterization of the isozymic composition of GST demonstrated that members of both the pi and alpha gene families were increased (data not shown). The latter proteins have organic peroxidase activity that results in the elevated peroxidase activity with cumene hydroperoxide. MDR1 expression was greatly enhanced in these cells as well.

Figure 1 demonstrates the changes in GSH concentration in the MCF-7 WT and ADRr cell lines treated with 20 μM BSO for various periods of time. There was a more rapid decline in cellular GSH concentration observed in the MCF-7/ADRr compared to the WT cells. At maximal depletion the remaining GSH concentration, expressed as a percentage of basal GSH concentration, was 25% in WT

Table 1. Biochemical characterization of MCF-7 sensitive WT and resistant ADRr cell lines

Cell line	GSH	GST	GSH-Px	GGCS	GGT
WT	15.9 ± 4.4*	4.0 ± 0.5	2.2 ± 0.5	1.2 ± 0.08	1.0 ± 0.04*
ADRr	8.3 ± 0.7	152.5 ± 2.0*	18.5 ± 1.0*	1.9 ± 0.02*	0.7 ± 0.004

GSH is expressed as nmol/10^6, and GGCS as μmol/60 min/mg protein; all others are expressed as nmol/min/mg protein. Values are means ± SEM, from three separate experiments each done in triplicate.

* Significantly higher than corresponding cell line ($P < 0.001$) using unpaired Student's *t*-test.

and 14% in the resistant cells. The effect of exposure to verapamil on total cellular GSH concentration in the MCF-7 cells was quite striking. After 48 hr of exposure in 10 μg/mL of verapamil, in three separate experiments, the total cellular GSH concentration in the WT cells changed from 11.2 ± 3.5 to 6.7 ± 1.2 nmol/10^6 cells, and in the ADRr cells from 8.6 ± 1.7 to 4.8 ± 2.1 nmol/10^6 cells. These were marked decreases in the GSH content in both cell lines. On the other hand, there was no change in either GSH-Px or GST after verapamil treatment (data not shown). In the MatB cells, there was also a significant depletion of cellular GSH observed ranging from 25 to 43%.

The results of clonogenic assays performed in the MCF-7 WT and ADRr cell lines using continuous and 24-hr exposure of cells at low density to BSO are shown in Fig. 2, A and B. As can be seen in these conditions, there was a cytotoxic effect that was more prominent in the ADRr cells. Figure 2C shows the clonogenic assay performed when cells were exposed to various concentrations of BSO for 20 hr while at high cell density, before being plated at low cell density. Under these conditions the ADRr cells were also more sensitive to BSO, but the difference was less marked.

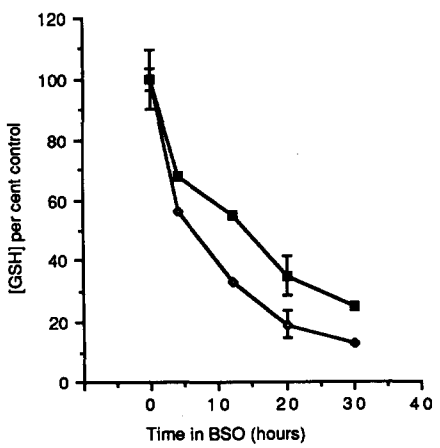

Fig. 1. Concentration of total GSH determined in cells [WT (●), ADRr (○)] after exposure to 20 μM BSO for various times. The protocol most commonly used in other studies is the 20 to 24-hr exposure. Multiple duplicates were made for this time point and the bars demonstrate the standard error. See Table 1 for control values.

Discussion

MCF-7/ADRr cells, like other drug-resistant variants, differ in terms of growth kinetics from their WT counterparts. We have shown previously by flow cytometry that the ADRr cells contain a higher population of cells in the G1 phase of the cell cycle (59.6 vs 50.3% in WT cells) [18]. While higher GSH values have been reported in S versus G1 phase human T-1 cells (1.6-fold) [19], the difference in the GSH content between the ADRr and the WT cells cannot be explained by kinetic factors alone. Thus, there appear to be intrinsic differences in cellular GSH concentration. The fact that a similar decrease in cellular concentration was observed in the MatB cell line, which was selected under the same conditions and exhibits similar molecular changes, suggests that this may be a more generalized phenomenon.

The effects of inhibition of GSH synthesis on cellular concentration is determined by utilization, either oxidation to GSSG which can efflux from cells, or conjugation and excretion as a GSH conjugate of an electrophilic molecule. The findings in this study are consistent with enhanced utilization of GSH in the ADRr cell line. Since both the selenium-dependent GSH-Px and the GST activities were increased in the resistant MCF-7 cells, it is likely that they are involved directly in the enhanced GSH utilization. It is curious that this is occurring in the absence of any chemotherapeutic agent, suggesting that there has been a constitutive alteration in the expression of these metabolic pathways in these cells. It will be of interest to determine the substrates for both of these enzymes under drug-free conditions. The level of GGT activity may also affect GSH concentration since it acts as a "salvage pathway" at the membrane for γ-glutamyl moieties. The decrease in this activity in the ADRr cell line may further contribute to GSH depletion and to increased cellular sensitivity to BSO.

The finding of diminished GGT concurrent with enhanced GST activity was unexpected since there are a number of examples of apparently coordinated expression of these two components in other experimental systems. In the MCF-7 cell line studied here, changes in both phase I and phase II xenobiotic metabolic components strongly parallel those described in chemically initiated rat liver hepatocytes, including elevated placental form of GST, and decreased inducibility of cytochrome P4501A1 [20]. However, a consistent finding in the transformed hepatocytes is increased expression of both GGT and GST [21]. Similarly *in vitro* transformation of rat liver epithelial cells by transfection with activated H-*ras* results in enhanced expression of both GST and GGT [22], while in human colon tissue we have found consistent elevation of both GGT and GST-P (particularly GST) in tumors compared to normal adjacent mucosa [23]. A recently reported doxorubicin-resistant subline of HL-60 was shown to have significantly diminished GSH concentration and GGT activity while the GST and GSH-Px were not altered with respect to the WT control cell line [24], so it is possible

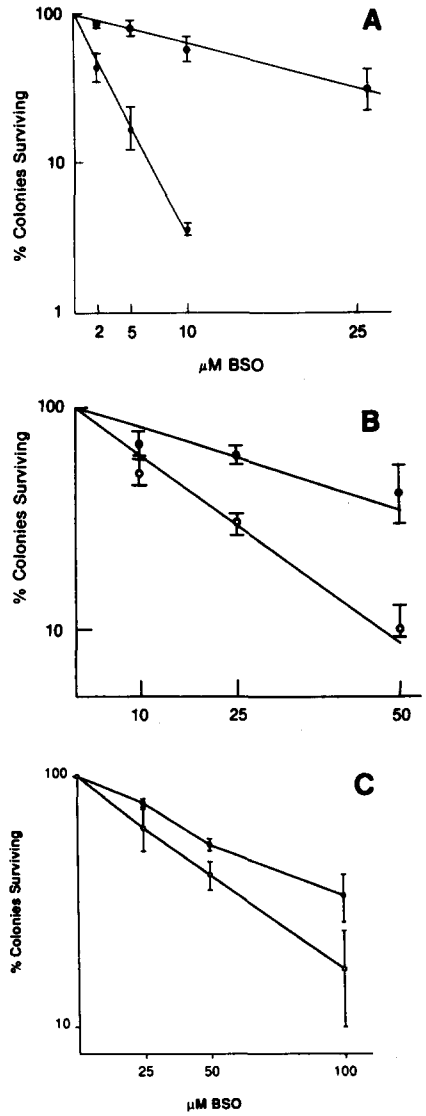

Fig. 2. Results of clonogenicity assays performed under three different conditions. In panels A and B, cells were plated at low cell density the night before treatment with BSO [WT (●), ADRr (○)]. In panel A, the BSO was added at the doses indicated and left in the medium throughout the clonogenic assay. In panel B, the BSO was maintained in the medium for 24 hr, and then was replaced by drug-free medium. In panel C, cells in semi-confluent monolayers were exposed to BSO at the indicated doses for 24 hr, after which they were plated at low cell density for clonogenic assay [WT (●), ADRr (○). The data are presented as the percent of colonies surviving at each BSO dose level compared to the untreated controls. Results are means ± SEM, from three separate experiments, each done in triplicate.

that continuous exposure to doxorubicin disassociates the commonly coordinated expression of GGT and GST through an undetermined mechanism. Alternatively, since we have not measured the level of mRNA encoding GGT, it is possible that the alteration is at the level of the protein which is situated on the outer surface of the cell membrane.

The effects of verapamil on GSH are of note given the many examples of verapamil sensitization to Adriamycin in multi-drug resistant tumor cell lines, and in these cells in particular [25–27]. Although the verapamil has a major inhibitory effect on the P-glycoprotein, and in so doing has been demonstrated to enhance intracellular drug accumulation, it is possible that GSH depletion also plays a role. In HL-60 cells selected for multi-drug resistance, GSH may play an operative role in the effects of verapamil by altering intracellular drug distribution [24]. Here again we have seen an analogous effect in two distinct cell lines selected for the MDR phenotype in Adriamycin.

Our data demonstrate differential sensitivity in terms of clonogenicity of the MCF-7 WT and ADRr cell lines. BSO was most toxic to both cell lines when they were treated while at low cell density and were chronically exposed. The latter finding is consistent with reports in some cell lines that chronic GSH depletion inhibits the GSH/glutaredoxin-mediated conversion of ribonucleotides to deoxyribonucleotides [28]. Both the degree of BSO toxicity and the difference between these two cell lines varied with the assay protocol, which suggests caution in interpreting various studies. When the cells were permitted to adhere to the plate prior to treatment (Fig. 2, A and B), it is possible that the WT cells, which grow faster with a doubling time of 36 versus 17 hr [18], had already divided to doublets by the time the BSO was added. The slower growing ADRr cells may still be single cells at that time and, therefore, be more sensitive to BSO. On the other hand, when the cells were treated prior to low density plating (Fig. 2C), there was still a differential effect of BSO, although it was smaller. While this may be a function of the greater GSH depletion in the ADRr cells, it is also possible that in these cells the target protein for BSO (GGCS) has greater affinity for the drug. Finally, the enhanced toxicity of the combination of verapamil and BSO described in the same cell line studied here may, at least in part, be related to the effects of both agents on cellular GSH levels [29].

In summary, the effects of GSH depletion in a human breast cancer cell line and a multi-drug resistant subline (ADRr) were determined in a number of experimental conditions. The ADRr cells contained lower GSH concentration which cannot be explained solely on the basis of differences in cell kinetics, and yet the rate-limiting synthetic enzyme γ-glutamylcysteine synthetase was increased 2-fold. Inhibition of GSH synthesis by BSO resulted in more rapid and more pronounced GSH depletion in ADRr compared to the wild-type cells, suggesting that enhanced GSH utilization and efflux in the resistant cells account for the lowered basal concentration. In addition, the γ-glutamyl moiety salvage enzyme γ-glutamyltranspeptidase was reduced markedly in the ADRr cell line. Since these cells have overexpression of the efflux pump protein P-glycoprotein, we examined the effects on cellular GSH of inhibition of the pump's function by verapamil. We found that verapamil significantly depleted cellular GSH. In a rat mammary carcinoma cell line selected in Adriamycin for multi-drug resistance, a similar molelcular phenotype has been described including diminished cellular GSH concentration. Verapamil treatment of these cells also resulted in significant depletion of cellular GSH. These results are consistent with the recent report [29] that combined treatment of BSO and verapamil has an additive effect on cytotoxicity. It is likely that decreased basal GSH concentration is due to oxidation and conjugation of it in

reactions catalyzed by the enhanced peroxidase and GST found in these cells.

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A comparison of levels of glutathione transferases, cytochromes P450 and acetyltransferases in human livers

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Soluble glutathione (GSH) transferases (GSTs) (EC 2.5.1.18) catalyse a number of GSH-dependent reactions including the detoxification of electrophilic metabolites of xenobiotics. In rat, mouse and human, GSTs have been clearly demonstrated to consist of a supergene family including three multigene families referred to as alpha, mu and pi, with considerable homology in each family across the species. The hepatocyte is the richest source of GSTs in all these species. In the human liver, alpha enzymes predominate; mu enzymes are present in 30–40% of the population; and the pi enzyme is restricted to the biliary epithelial cell [1, 2]. In the alpha family, two subunits, referred to variously as Y1 and Y4 [3], B1 and B2 [4] or αx and αy, [5] have been identified. In the mu family there are two expressing alleles: one referred to as GST μ [6] and the other as GST ψ [7] and a null allele, the inheritance of the last named giving rise to mu-deficient individuals in the population [8].

Cytochrome P450 monooxygenases (P450) ("unspecific monooxygenase", EC 1.14.14.1) are the major enzymes involved in the oxidation of drugs, carcinogens and steroids. As in the case of the GSTs, a supergene family of these enzymes exists and the individual proteins are characterized by distinct catalytic specificities [9]. In recent years, considerable insight has been gained regarding the nature of the human P450s [10, 11] and their wide variation among individuals. In some cases, the nature of the variation is genetic polymorphism (e.g. debrisoquine 4-hydroxylation); while in other cases the variation seems to be due to induction of a specific P450 (e.g. induction of phenacetin O-deethylation by cigarette smoking or consumption of char-broiled meat [12, 13]).

N-Acetylation of arylamines is a major route in their biotransformation and, by affecting their disposition, may play a modulatory role in the metabolic activation of these compounds. O-Acetylation and N-O-transacetylation of *N*-hydroxy metabolites also occur and lead directly to activation and DNA binding [14]. The enzymes involved are controlled by two genetic loci designated "monomorphic" and "polymorphic", N-acetylation reactions in humans being controlled principally by the polymorphic locus [15]. This has two major alleles designated as "*r*" and "*s*", giving rise to rapid acetylator (*rr* and *rs*) and slow acetylator (*ss*) phenotypes [16]. In addition, these same polymorphic isozymes probably participate in O-acetylation and N,O-acetylation reactions [17, 18]. In Caucasian populations rapid and slow metabolizers occur with similar frequency [15].

Obviously, all three of these enzymes, the GSTs, the P450s and acetyltransferases, can be involved in the overall metabolism of drugs and carcinogens and the relative levels of catalytic activity towards certain substrates and their products may be expected to influence the balance between bioactivation and detoxification [19]. Thus, it would be of interest to compare levels of some of these enzymes in human tissues to determine if levels of some of the enzymes might vary in concert, particularly since certain chemicals such as phenobarbital and polycyclic hydrocarbons induce several GSTs and P450s in experimental animal models.

In the present paper, we analysed 22 human liver samples for GST subunits by reverse phase HPLC. Fifteen of these liver samples were also assayed for activities of the cytochrome P450 mixed function oxygenase family and for N- and O-acetyltransferases; and values obtained compared to determine to what extent levels of any one of these drug metabolizing enzymes correlated with another.

Materials and Methods

Human livers were obtained from organ donors through Tennessee Donor Services (Nashville, TN, U.S.A.) and CHU Necker (Paris, France). Analysis of GSTs in homogenates was determined by the method in Ref. 5. Total cytochrome P450 was determined spectrally according to the method in Ref. 20, debrisoquine 4-hydroxylase and phenacetin *O*-deethylase activity according to the methods in Ref. 21 (gas chromatography and mass spectrometry) and in Ref. 22 (thin-layer radiochromatography), respectively and methods for *N*-acetyltransferases and *O*-acetyltransferase were as described in Ref. 23.

Results and Discussion

Table 1 shows the results of all the assays involved. Correlations between sulphamethazine and *N*-acetyl-2-aminofluorene *N*-acetyltransferase activity ($r = 0.94$) and *N*-hydroxy-2-aminofluorene-*O*-acetyltransferase activity ($r = 0.88$), have already been published [23]. However, no correlations were seen in comparisons made between levels of activity in any of the other drug metabolizing enzymes examined (see Table 2).

All samples contain both αx and αy but the levels of these two subunits are not related to each other (see Table 2). Thirty to forty per cent of the samples contained mu family enzymes. GST π was never detected in these assays.

Since all members of this sample contain GST αx and αy, each expressed at a level independent of the other, it appears that GST αx and αy are not allelic but products of different gene loci. A smaller sample of eight individuals has been analysed previously and both GSTs αx and αy were present in all individuals [24].

A correlation between *N*- and *O*-acetyltransferase levels has already been reported [23] and while it was of interest to see to what extent acetyltransferase levels might correlate with other drug metabolizing enzymes, it was not surprising