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Overexpression of Glutathione S-Transferase and Elevation of Thiol Pools in a Multidrug-Resistant Human Colon Cancer Cell Line

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SUMMARY

A human colon cancer cell line with acquired multidrug resistance (MDR) was assayed for the intracellular GSH level and the activity of GSH-S-transferase (GST), which catalyzes the conjugation reaction of electrophilic drugs with GSH. The GSH level and GST activity (as measured with 1-chloro-2,4-dinitrobenzene) were elevated in the resistant cells by 1.7-fold and 2-fold, respectively. This elevated catalytic activity of the resistant cells was reflected in a 2-fold increase in GST-π mRNA, which was not the result of gene amplification. In addition, buthionine sulfoximine, a specific inhibitor of GSH synthesis, significantly increased Adriamycin sensitivity in both the MDR and the parental cells, affecting the former more than the latter. The effects seen with buthionine sulfoximine were not seen with puromycin and actinomycin D. A dramatic overexpression of *mdr*1, a P-glycoprotein gene responsible for the MDR phenotype, was also observed in the MDR cells. In contrast, none of these products (i.e., *mdr* P-glycoprotein, GSH level, total GST activity, GST-π gene copy, and GST-π mRNA level) was elevated in HeLa cells resistant to cisplatin and some alkylating agents, supporting the notion that the acquisition of cisplatin resistance differs from the mechanism of MDR. These results indicate that the intrinsic GSH level and GST-π activity affect anthracycline resistance *per se* and not MDR in the human colon cancer cells.

GSH (L-γ-glutamyl-L-cysteinglycine) accounts for the majority of the intracellular nonprotein sulfhydryl or thiol content. It participates in many important cellular functions, including protection from free radical damage, detoxification of xenobiotics, and synthesis of DNA precursors (for review, see Refs. 1 and 2). Evidence has accumulated concerning the role of intrinsic GSH as a modulator of cellular responses to drugs and irradiation (3–9). This was confirmed by studies showing that the elevation of cellular GSH is associated with the development of induced resistance to Melphalan, nitrogen mustard, or *cis*-diamminedichloroplatinum(II) (cisplatin) (10–15). In addition, depletion of intracellular GSH by treatment with BSO, a potent inhibitor of γ-glutamylcysteine synthetase (16, 17), renders both normal and cancer cells more sensitive to drugs and/or radiation (7, 12, 18–22). GSTs catalyze the coupling reaction of intracellular electrophiles with GSH (for reviews, see Refs. 23 and 23a). The multifunctional isozymes of GST, which are found in virturally all tissues, are presumed to be involved in the detoxification of a variety of noxious xenobiotics, including certain anticancer drugs and their metabolites. Elevation of GSTs is associated with the acquired resistance of cells to certain anticancer drugs (24). A number of studies have confirmed the generality of this phenotype (11, 25–28). In the majority of cases, the elevated expression of GST-α has been firmly correlated with increased resistance to alkylating agents. This was directly supported by studies in which the transfection of a GST-α cDNA into COS cells led to an unstable overexpression of GST-α, resulting in an increased resistance of cells to alkylating agent (29). Thus, the GSH/GST levels in cancer cells may be of particular relevance to the emergence of resistance to alkylating agents. However, the role of GSH/GSTs in resistance to other drugs, such as doxorubicin, is less clear.

We have previously reported an ADR human colon cancer cell line that had developed an enhanced P-glycoprotein-mediated efflux mechanism, showing a typical MDR phenotype (30). This cell line is cross-resistant to a variety of drugs, including puromycin and actinomycin D. Although it is logical to assume that P-glycoprotein plays a vital role in determining

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ABBREVIATIONS: GST, glutathione-S-transferase; BSO, DL-buthionine-S,R-sulfoximine; MDR, multidrug resistance (resistant); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ADR, Adriamycin-resistant; CPR, cisplatin-resistant.

the MDR phenotype (for review, see Ref. 31), quantitatively the drug efflux did not correlate with drug sensitivity in this MDR cell line. Therefore, we attempted to look at the other possible mechanisms that may mediate the MDR phenotype. Of great interest, as first reported by Batist *et al.* (10), is the overexpression of GST-π in MDR human breast cancer cells, although those authors did not measure other potentially important products. Nakagawa *et al.* (32) have recently demonstrated that overexpression of human GST-π by transfection of the gene into transformed NIH3T3 cells conferred resistance to Adriamycin and ethacrynic acid but not to cisplatin and the alkylating agent Melphalan. *mdr* P-glycoprotein overexpression was not detected in these resistant transfectants. These results suggest a potential role for GSH and GST-π in the development of MDR. In this report, we show evidence that confirms part of this hypothesis. Because GSH/GSTs affect only Adriamycin resistance in our MDR cells, the results suggest that the level of GSH/GSTs is important in anthracycline resistance *per se* and not MDR. The results also reveal an overexpression of GST-π and *mdr* P-glycoprotein in the MDR cells.

Materials and Methods

Chemicals and medium. Dulbecco's modified Eagle's medium, fetal bovine serum, and penicillin/streptomycin were obtained from GIBCO (Gaithersburg, MD). Platiamine (cisplatin) was purchased from Farmitalia Carloerba Ltd. BSO and Adriamycin were purchased from Sigma (St. Louis, MO). Other chemicals and suppliers are indicated below.

Cell cultures and determination of resistance. Human colon cancer SW620 parental and MDR cells (30) and HeLa parental and CPR cells (33) were maintained as previously described. The acquired resistance of the cells to drug is defined as the ratio of ID_{50} (the drug concentration inhibiting cell survival by 50%) of resistant cells to that of parental cells. Cytotoxicity was assayed by the MTT colorimetric method (34).

Isolation of membrane proteins and SDS-PAGE. Cellular membrane proteins were isolated according to the method described by Riodan and Ling (35). In brief, cells were washed with phosphate-buffered saline and disrupted using a Dounce homogenizer. The extent of cell breakage was checked under the phase-contrast microscope. The following differential centrifugation was applied: nuclear spin, 300 × *g* for 10 min; mitochondrial spin, 4,000 × *g* for 10 min; and membrane spin, 35,000 × *g* for 30 min. The membrane pellet was resuspended in 0.5% Triton X-100 at 4° for 30 min and separated on 10% SDS-PAGE.

Cellular GSH content. Total cellular GSH content was measured using the enzyme recycling assay (36). After 5×10^6 cells were suspended in 300 μl of phosphate-EDTA solution (125 mM KH_2PO_4, 6.3 mM EDTA, pH 7.5), cells were sonicated at 4° (120 W, 30 sec) and then 100 μl of 12% 5-sulfosalicylic acid were added to each sample tube. The solution was mixed and allowed to precipitate for 2–3 hr, on ice. After centrifugation at 10,000 × *g* for 15 min, protein-free lysates were obtained. The enzyme reaction was carried out in a 1-ml cuvette, in 175 mM KH_2PO_4, 6.3 mM EDTA, 0.21 mM NADPH, 0.6 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (Sigma), 0.5 unit/ml glutathione reductase (Sigma), at 25°. The absorbance at 412 nm (A_{412}) was monitored for 3 min at 20-sec intervals, and the GSH content was calculated from the rate of change in A_{412} on the basis of the standard curve in each experiment.

GST activity. Cells (1×10^7) were washed with phosphate-buffered saline and resuspended in 500 μl of 10 mM Tris·HCl, pH 7.8, containing 0.2 M NaCl. After sonication at 4° (120 W, 30 sec), they were centrifuged in a microfuge at 12,000 rpm for 30 min. The supernatant was assayed for total GST activity using 1-chloro-2,4-dinitrobenzene (Sigma), according to the method of Habig and Jakoby (37), and for GST-α activity using cumene hydroperoxide (Sigma), according to the method of Stockman *et al.* (38).

Hybridization probes. A 0.75-kilobase *Eco*RI fragment was cut out from pGPi-2 (39) (a kind gift from Dr. M. Muramatsu, University of Tokyo, Japan) and used as a probe for GST-π gene. The human *mdr* gene probe was a full-length human *mdr*1 cDNA clone cut with *Bam*HI and *Sal*I from pGEM3Zf(−)-*mdr*1 (a generous gift from Dr. P. Borst, Netherlands Cancer Institute). Rat β-actin cDNA (a gift from Dr. S. N. Cohen, Stanford University) was used to probe the human gene, as an internal control. Probes were purified by electrophoresis on NA45 membranes (Schleicher & Schuell) and radiolabeled by the random priming method, with [α-^{32}P]dCTP (40), to a specific activity of 10^8 cpm/μg of DNA.

RNA and DNA blot hybridizations. All solutions and procedures involving the isolation of DNA and RNA, electrophoresis, blot transfers, and hybridizations were carried out by standard methods (41). For Northern RNA blots, 10 μg of total RNA from the cell lines were fractionated by electrophoresis on 1% agarose containing 6.7% formaldehyde. RNA was transferred to Hybond-N filters (Amersham), UV-cross-linked with Stratalinker (Stratagene), and hybridized at 42° for 16 hr in hybridization buffer (6 × standard saline citrate, 50% deionized formamide, 10 × Denhardt's solution, 10 mM EDTA, 0.1% SDS) containing 5×10^5 cpm of probe/ml. The filter was then washed at 65° in 2 × standard saline citrate, 0.1% SDS, followed by exposure on X-ray film, with an intensifying screen, at −80° for 3 days or as otherwise indicated. The X-ray film was scanned in a densitometer to estimate the density of the bands. Before blotting, genomic DNAs were digested with the restriction enzymes *Hind*III and *Eco*RI (New England Biolabs) and checked in a 1% agarose gel. For slot blot, amounts of RNA or DNA were loaded according to the manufacturer's instruction (Schleicher & Schuell) and processed for hybridization with probes. The filter was then washed, followed by exposure on X-ray film, with an intensifying screen, for 3 days at −80°.

Results

Characteristics of the sensitive and resistant cells. An ADR human colon cancer cell line (SW620-ADR) that was showing a typical MDR phenotype was maintained as previously described (32). A typical dose-response pattern of Adriamycin sensitivity for SW620 parental and ADR cells is presented in Fig. 1. For example, surviving colonies were barely detected in parental cells treated with 1×10^3 ng/ml Adriamycin, whereas the majority of the ADR cells survived this or even a higher drug concentration. The ID_{50} values (the drug concentration causing 50% inhibition of cell growth) are shown in Table 1. The ID_{50} of Adriamycin for SW620-ADR cells was 45 μg/ml, compared with 0.6 μg/ml for the parental SW620 cells. The calculated fold resistance of ADR cells to Adriamycin was 75. In contrast, ADR cells showed a 1.2-fold resistance to cisplatin, compared with SW620 parental cells, indicating that the SW620-ADR cells were as sensitive to cisplatin as the parental cells. For comparison, the drug sensitivity of a CPR HeLa cell line (HeLa-CPR) (33) was also included. HeLa-CPR cells exhibited an 18-fold resistance to cisplatin, but both HeLa lines showed similar sensitivity to Adriamycin.

Overexpression of *mdr*1 transcripts and ~170-kDa membrane proteins in ADR cells. We have previously shown that SW620-ADR cells have acquired the ability to decrease drug accumulation inside the cell, and this characteristic holds true for a variety of hydrophobic drugs, which is typical of the MDR phenotype (30). It is reasonable to speculate, then, that the ADR cells overexpress P-glycoprotein, a ~170-kDa membrane protein that is believed to be responsible for the MDR phenotype (for review, see Ref. 31). As expected,

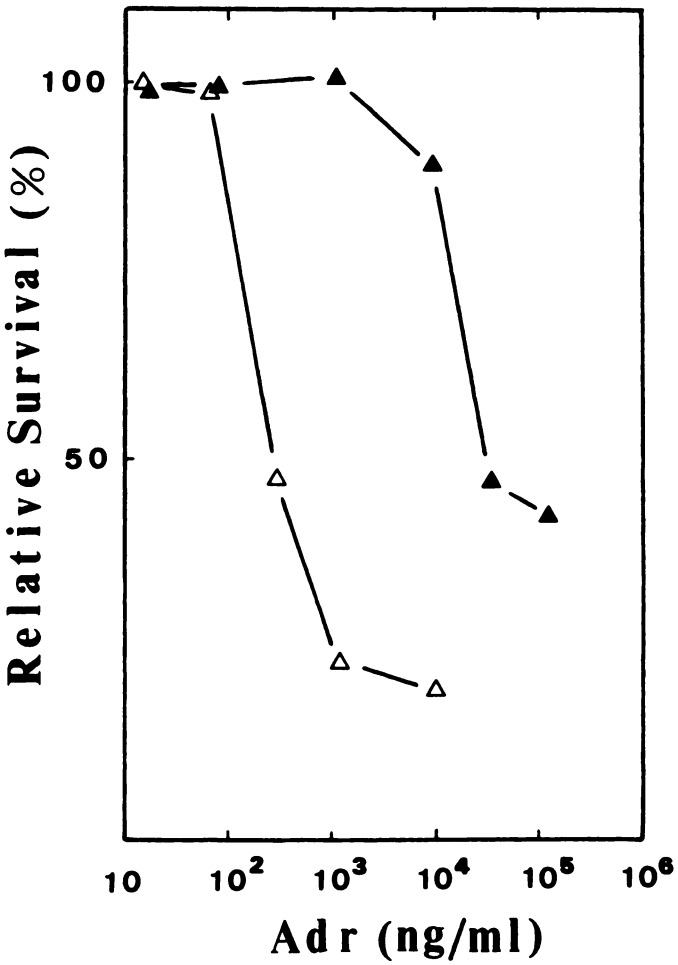

Fig. 1. Relative sensitivity of SW620 parental and ADR cells to Adriamycin (*Adr*). △, SW620 parental cells; ▲, SW620-ADR cells.

TABLE 1

ID_{50} values of anticancer agents for the parental and drug-resistant cells

ID_{50} is the drug concentration effective in inhibiting 50% of the cell growth, measured after 4 days of continuous exposure to the drug. Values in parentheses are fold resistance.

Cell line	ID_{50}	
	Adriamycin	Cisplatin
	μg/ml	
SW620	0.6	3.0
SW620-ADR	45.0 (75)	3.5 (1.2)
HeLa	0.8	0.1
HeLa-CPR	0.8 (1)	1.8 (18)

the steady state level of *mdr*1 mRNA was dramatically increased in SW620-ADR cells (Fig. 2, *lane R*, indicated with *arrowhead* at *left*), in contrast to parental cells (Fig. 2, *lane P*). However, the observed intensity of *mdr*1 may be overestimated because of a nonspecific hybridization band around the 18 S rRNA. Membrane proteins of SW620 parental and ADR cells were isolated and separated on 10% SDS-PAGE. A protein band around 170 kDa (indicated with *arrowhead* at *right* in Fig. 2) was consistently overexpressed in ADR cells (Fig. 2, *lane R*) but was not detected in parental cells (Fig. 2, *lane P*). The ~170-kDa membrane protein overexpressed in ADR cells is likely to be P-glycoprotein. Together, the increased drug pumping and cross-resistance to hydrophobic drugs in ADR cells (30) and the results presented here indicate that ADR cells have acquired a typical MDR phenotype.

Increment of GSH levels and the GST enzymatic activity in ADR cells. The GSH level and GST activity were measured in drug-resistant cells. The level of GSH in the cells was normalized to the amount of cellular proteins and is shown in Table 2. SW620-ADR cells contained 250 nmol/mg of protein, compared with 151 nmol/mg of protein in parental cells. There was a 1.7-fold increase, which is significantly different between the two cell lines ($p < 0.01$). On the other hand, the GST enzymatic activity for parental and ADR cells was 128 and 321 nmol/min/mg of protein, respectively. The difference is also statistically significant ($p < 0.01$). In contrast, HeLa and HeLa-CPR cells showed similar levels of GSH. The GST enzymatic activity for HeLa and CPR cells was 43 and 23 nmol/min/mg of protein. The difference is significant ($p < 0.02$). The biological significance of the reduced total GST activity in the CPR cells, compared with the parental cells, is not clear. The results of the measurements of GSH and GST by biochemical means in ADR cells were supported by a functional analysis, i.e., BSO inhibition (see below). GST-α enzymatic activity was also included for reference. As shown in Table 3, the parental and the ADR cells showed no difference in GST-α activity. In contrast, HeLa-CPR and parental cells contained 3.99 and 0.68 nmol/min/mg of protein of the enzymatic activity, respectively. There was an approximately 6-fold enhancement in GST-α activity in HeLa-CPR cells, compared with their parental cells.

Drug sensitization by BSO in ADR cells and in parental cells to a lesser extent. The role of GSH in drug sensitivity was tested in the cells by BSO inhibition. A sublethal concentration of BSO (50 μM) was used to test its cytotoxic effect. The ID_{50} of Adriamycin, with or without BSO, is shown in Table 4. The ID_{50} of Adriamycin for SW620 and ADR cells was 0.56 and 40, respectively. When BSO was added, the ID_{50} for the two cell lines was 0.4 and 22 μg/ml, respectively. There was a 1.4- and 1.8-fold increase in drug sensitivity for the parental and ADR cells, respectively. The data indicate that inhibition of GSH synthesis by BSO renders both the MDR and the parental cells sensitive to the drug, affecting the former more than the latter. However, the same BSO effects were not seen with drugs such as puromycin and actinomycin D (which also produce MDR) (data not shown), suggesting that the BSO effects are probably only seen in drugs that form free radicals.

Overexpression of the GST-π gene in ADR cells. Mammalian cells have evolved a family of GST enzymes used for coupling with GSH in the process of quenching free radicals. Among these, overexpression of GST-π, as a consequence of transfection of the gene, is correlated with the development of resistance of the cells to Adriamycin (32). Here, we are reporting that human cells with the acquired MDR phenotype also expressed a high level of GST-π. As shown in Fig. 3, both HeLa parental and HeLa-CPR cells expressed a relatively low level of GST-π, as opposed to SW620 parental and SW620-ADR cells. Quantitation by scanning densitometry showed a 2-fold increase of GST-π mRNA in ADR cells. For comparison, mRNA levels of *mdr*1 are also shown (see Fig. 3, *MDR*). However, the intensity of *mdr* mRNA is probably overpresented, because of the relatively high background of the nonspecific hybridization. The gene probe was retrieved, and the

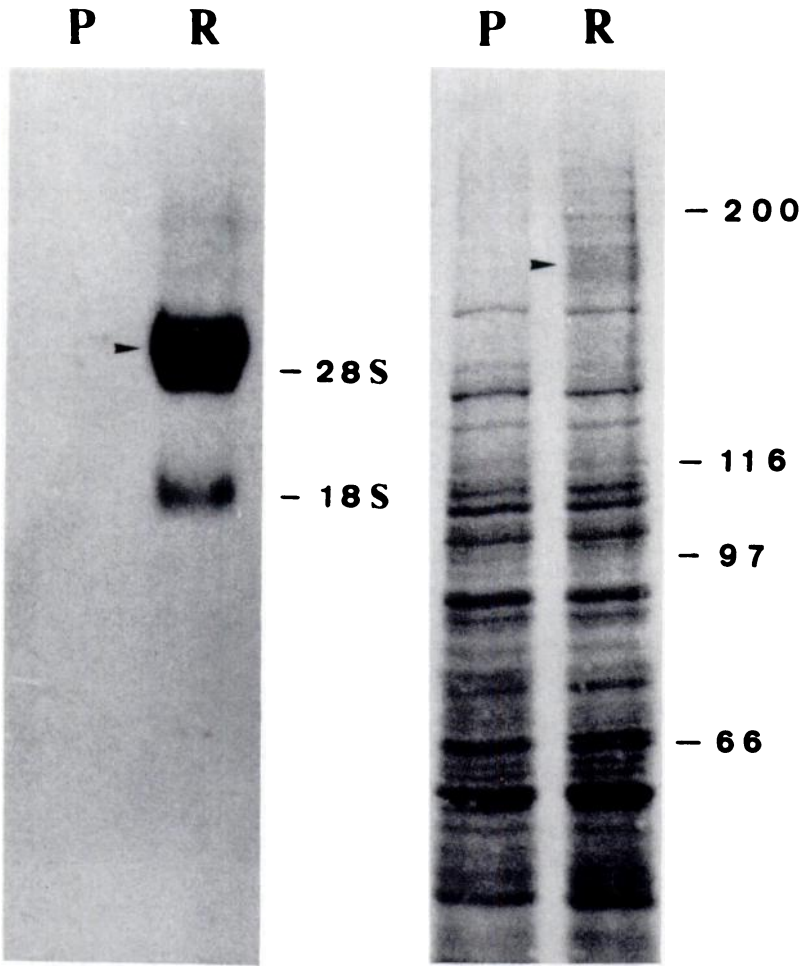

Fig. 2. Northern blot hybridization of *mdr*1 gene and SDS-PAGE of membrane proteins in SW620 parental and ADR cells. *Left*, Northern hybridization. Ten micrograms of total cellular RNA were loaded. *Right*, SDS-PAGE of cell membrane proteins. Ten micrograms of proteins were loaded, after which the gel was separated and visualized by silver-staining. *P*, SW620 parental cells; *R*, SW620-ADR cells. The location of 28 S and 18 S rRNA is indicated for Northern hybridization. Protein size markers are also shown at the *right*.

TABLE 2

Intracellular GSH levels and GST activities in the parental and drug-resistant cell lines

Values are mean ± standard deviation of three or four determinations.

Cell line	GSH level	GST activity
	nmol/mg of protein	*nmol/min/mg of protein*
SW620	151 ± 22.5	128 ± 9.1
SW620-ADR	250 ± 39.9 (< 0.01)[a]	321 ± 31.9 (< 0.01)[a]
HeLa	89 ± 13.5	43 ± 6.5
HeLa-CPR	94 ± 37.7 (NS)[b]	23 ± 4.8 (<0.02)[a]

[a] These probability values (indicated in parentheses), as determined by the two-tailed *t* test, show the significance of the difference in GSH and GST between SW620 and SW620-ADR or between HeLa and HeLa-CPR cells.

[b] NS, not significant ($p > 0.2$); there is no significant difference in GSH and GST between HeLa and HeLa-CPR cells.

TABLE 3

Intracellular GST-α activities in the parental and drug-resistant cell lines

Values are mean ± standard deviation of three determinations.

Cell line	GST-α activity
	nmol/min/mg of protein
SW620	3.64 ± 0.56
SW620-ADR	3.03 ± 0.47 (NS)[a]
HeLa	0.68 ± 0.13
HeLa-CPR	3.99 ± 0.56 (<0.01)[b]

[a] NA, not significant ($p > 0.2$); there is no significant difference in GST-α between SW620 and SW620-ADR cells.

[b] This probability value (indicated in parenthesis), as determined by the two-tailed *t* test, shows the significance of the difference in GST-α between HeLa and HeLa-CPR cells.

same blot was used to hybridize with the β-actin probe (Fig. 3, *ACT*). The actin mRNA was similar in the SW620 and ADR cells. In fact, a slightly greater amount of actin mRNA was detected in HeLa parental and CPR cells, indicating that the overexpression of GST-π found in ADR cells is not an artifact of sample loading.

Normal GST-π gene copy in ADR cells. The gene copy and the mRNA level of GST-π in the cells were further quantitated using slot blotting (Fig. 4). Indicated amounts of DNA or RNA of parental or resistant SW620 cells (Fig. 4A) or HeLa cells (Fig. 4B) were used for the assay. The same DNA level of GST-π gene was found in SW620-ADR and parental cells, indicating that there was no gene amplification of GST-π in ADR cells. There was 2-fold amplification of this gene in HeLa-CPR cells, compared with the HeLa parental cells. By quantitation of the mRNA level of GST-π, a 2-fold increase was detected in ADR cells, whereas in HeLa cells there was a much lower GST-π mRNA level. Prolonged exposure of the X-ray film did not show GST-π overexpression (data not shown). The slot blotting results are consistent with the Northern blotting.

Discussion

As previously reported, the resistance of the ADR colon cancer cells used in this study is associated with a decrease in drug accumulation, relative to the parental line (32). Although

TABLE 4

Effect of BSO on Adriamycin toxicity

Cells were incubated in medium containing Adriamycin, in the presence or absence of 50 μM BSO, for 4 days and were measured for inhibition of cell growth. Values are mean ± standard deviation of three or four determinations. Numbers in parentheses are fold sensitization, as calculated from the ratio of ID_{50} of cells without BSO to that of cells treated with BSO.

Cell line	ID_{50}		
	−BSO	+BSO	p^a
	μg/ml		
SW620	0.56 ± 0.07	0.4 ± 0.05 (1.4)	<0.005
SW620-ADR	40 ± 3.5	22 ± 2.0 (1.8)	<0.01

[a] These probability values, as determined by the two-tailed *t* test, show the significance of the difference between cells with and without BSO treatment.

selected by exposure only to Adriamycin, the ADR cells have developed a phenotype that is characteristic of MDR. Because the magnitude of this alteration (2–3-fold) is insufficient to account for the overall level of drug resistance in these cells (75-fold), we have investigated other potential mechanisms that might be associated with MDR. In this report, we have observed a 2-fold overexpression of GSH level and GST enzymatic activity in the ADR cells. In addition, BSO increased the cellular sensitivity to Adriamycin in both the ADR and the parental cells, affecting the former more than the latter. The results indicate that the level of GSH/GSTs in these cells affects anthracycline resistance. Because the effects seen with BSO are not observed with puromycin and actinomycin D, to which the ADR cells are cross-resistant, the effects are probably specific for anthracycline resistance *per se* and not for MDR in general. Because our ADR cells possess features typical of the MDR phenotype, the overexpression of P-glycoprotein in MDR cells, as frequently found, may not account for all of the MDR mechanism. Other investigations have also shown that BSO treatment can sensitize MDR MCF-7 breast cancer cells and P388 leukemia cells to doxorubicin toxicity (42–43). In contrast, a HeLa cell line resistant to cisplatin, the resistance of which was not mediated by the MDR mechanism, did not show any detectable increase in the level of GSH.

Resistant cells selected by prolonged drug exposure often exhibit multiple genetic and biochemical differences from the parental cell lines. These changes may affect the expression of many gene products associated with drug resistance, such as P-glycoprotein, GSTs, and metallothionein. Our data reveal the coordinated overexpression of P-glycoprotein and GST-π genes in the ADR cells. A 2-fold increase of GST-π mRNA was measured in ADR cells. This may reflect, although it does not prove, the overexpressed total GST activity. The results are consistent with the observations of Nakagawa *et al.* (32), that transfection of c-H-*ras*-transformed NIH-3T3 cells with human GST-π cDNA conferred a 2–3-fold rise in Adriamycin

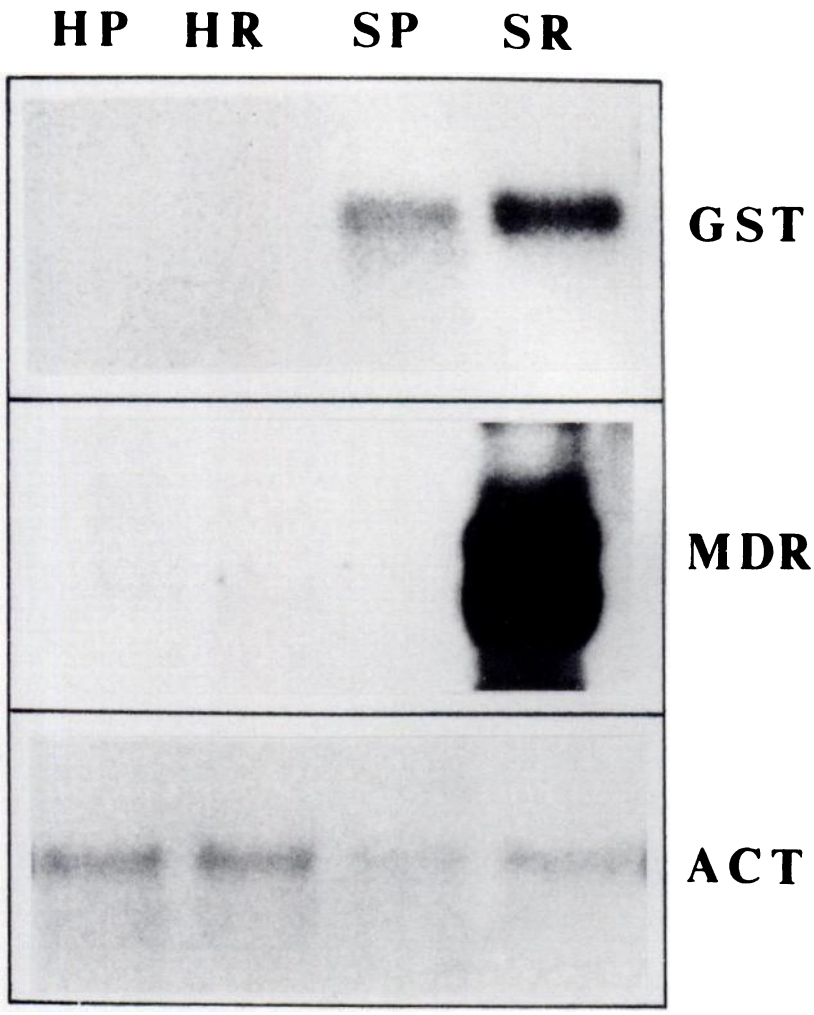

Fig. 3. Northern blot hybridization of GST-π gene in SW620 parental, SW620-ADR, HeLa parental, and HeLa-CPR cells. Ten micrograms of total cellular RNA were loaded in each lane. The same blot was probed with gene-specific probe (indicated at the *right*), retrieved, and reprobed with the second probe. *HP*, HeLa parental; *HR*, HeLa-CPR; *SP*, SW620 parental; *SR*, SW620-ADR cells. *GST*, GST-π; *MDR*, *mdr*1; *ACT*, β-actin.

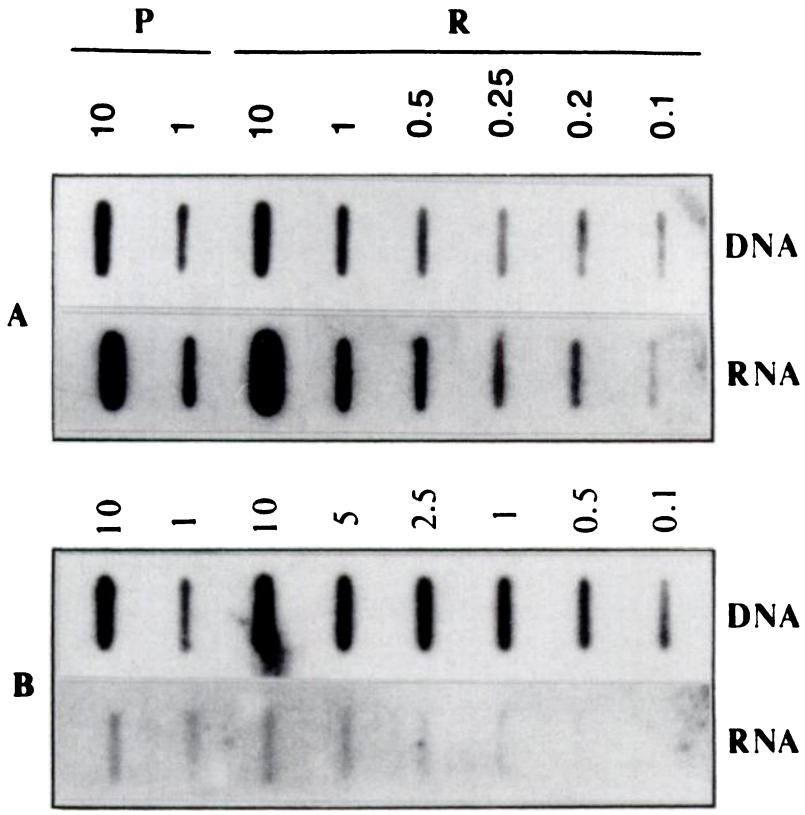

Fig. 4. Slot blot hybridization of GST-π in DNA or RNA of SW620 parental, SW620-ADR, HeLa parental, and HeLa-CPR cells. The amounts of DNA or total RNA loaded on each slot are indicated at the *top of each panel*. *P*, parental cells; *R*, resistant cells. A, SW620 cells; B, HeLa cells.

resistance at the IC_{37} drug concentration. Interestingly, protection was observed only at low drug concentrations; at IC_{50} concentrations and above, the resistant patterns of the transfected and parental cells were identical. However, other gene transfer experiments yielded conflicting results. As reported by Moscow *et al.* (44), expression of various levels of GST-π in different transfected MCF-7 clones was associated with resistance to known substrates of GST-π (i.e., ethracrynic acid, BPDE). No consistent alteration in the IC_{50} of doxorubicin was observed when these transfected clones were compared with parental cells. Their subsequent experiments suggest that overexpression of GST-π, either alone or in concert with P-glycoprotein, does not appear to influence MDR significantly. Therefore, the level of GSTs in the cells is not necessarily associated with anthracycline resistance. Because MCF-7 cells contain minimal GST activity and no detectable GST-π, the conflict between these studies may be due to cell specificity. In addition, we showed an increase in GST-α activity in CPR cells but not in ADR cells, compared with their parental lines. These findings confirm the idea that GST-α is important for cellular resistance to alkylating agents. We also found that the overexpression of GST-π in our ADR cells is not because of gene amplification, suggesting that the mechanism of GST-π overexpression is different from that of *mdr* P-glycoprotein, whose overexpression in the ADR cells is dictated by gene amplification (30). The influence of GSH/GSTs on Adriamycin cytotoxicity indicates a potential role of GSH metabolism in the development of anthracycline resistance *per se* and not MDR. In any case, the ADR cells are extremely resistant to Adriamycin. Given the marginal level of GSH-related resistance in these cells, it seems doubtful that such a mechanism would be elicited at the levels of resistance shown in patients. Because the BSO effect on drug resistance is often seen at low drug concentrations, the GSH-related resistance might be relevant in the early stages of the development of drug resistance in clinical practice.

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