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Expression of Human μ or α Class Glutathione S-Transferases in Stably Transfected Human MCF-7 Breast Cancer Cells: Effect on Cellular Sensitivity to Cytotoxic Agents

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SUMMARY

Increased expression of certain glutathione S-transferase (GST) isoenzymes has frequently been associated with the development of resistance to alkylating agents and other classes of antineoplastic drugs in drug-selected cell lines. The question arises whether this phenomenon is causal or is a stress-induced response associated with drug resistance in these cell lines. We have constructed mammalian expression vectors containing the human GSTμ and GSTα2 (Ha2) cDNAs and stably transfected them into the human breast cancer cell line MCF-7. Whereas the parental and pSV2*neo*-transfected cell lines display low GST activity, three individual transfected clones were identified in each group that expressed either GSTμ or GSTα2. The range of GST activities was similar to those observed in cells selected for anticancer drug resistance. The GSTμ specific activities were 56, 150, and 340 mIU/mg, compared with 10 mIU/mg of endogenous GSTμ in control lines. Specific activities in GSTα2-transfected clones were 17, 28, and 52 mIU/mg, compared with no detectable α class GST in control lines. These clonal lines and the parental and pSV2*neo*-transfected control lines were tested for sensitivity to antineoplastic agents and other cytotoxic compounds. The clones with the highest activity in each group were 1.7-fold (GSTα2) to 2.1-fold (GSTμ) resistant to the toxic effects of ethacrynic acid, a known substrate for GSTs. However, the GST-transfected cell lines were not resistant to doxorubicin, L-phenylalanine mustard, bis(2-chloroethyl)-1-nitrosourea, cisplatin, chlorambucil, or the GST substrates 1-chloro-2,4-dinitrobenzene or *tert*-butyl hydroperoxide. Thus, although L-phenylalanine mustard, bis(2-chloroethyl)-1-nitrosourea, chlorambucil, *tert*-butyl hydroperoxide, and 1-chloro-2,4-dinitrobenzene are known to be metabolized by glutathione-dependent GST-catalyzed reactions, there was no protection against any of these agents in MCF-7 cell lines overexpressing GSTμ or GSTα2. We conclude that, at the levels of GST obtained in this transfection model system, overexpression of GSTμ or GSTα2 is not by itself sufficient to confer resistance to these anticancer agents. These studies do not exclude the possibility that GST may be a marker of drug resistance or that other gene products not expressed in MCF-7 cells might cooperate with GST to confer drug resistance.

Cancer chemotherapeutic drugs are identified on the basis of preferential cytotoxicity toward malignant compared with normal host tissues. Decreased sensitivity of tumor cells, or drug resistance, is the principal obstacle to curative chemotherapy for many human cancers. This phenomenon may be inherent in a particular tumor or may be "acquired" after therapy as a result of cytotoxic selection for resistant cells in a heterogeneous population. Chemotherapeutic drug resistance has been associated with several factors, including decreased drug accumulation, enhanced drug metabolism, and alterations in cellular targets (1).

One alteration that has been observed in a number of neoplastic cell lines selected *in vitro* for resistance to anticancer agents is the overexpression, relative to the parental cells, of various GST isoenzymes (2–6). The GST supergene family is composed of three subgroups of soluble or cytosolic isoenzymes (α, μ, and π classes) and a membrane-bound or microsomal type (7). These enzymes have the potential to reduce drug toxicity by several mechanisms, including conjugation of electrophiles to the thiol tripeptide GSH, direct binding of the more lipophilic compounds, and GSH-dependent reduction of peroxides generated by drug metabolism (7–9). In order to test the pharmacologic significance of GST overexpression directly, we have separately transfected human MCF-7 breast cancer cells, which normally have very little GST activity, with two mammalian expression vectors that contain cDNAs encoding the human GSTμ and GSTα2 genes. These cell lines, which express a range of GST activities comparable to those observed in certain cell lines selected for resistance *in vitro*, were com-

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ABBREVIATIONS: GST, glutathione S-transferases; CDNB, 1-chloro-2,4-dinitrobenzene; L-PAM, L-phenylalanine mustard; BCNU, bis(2-chloroethyl)-1-nitrosourea; CDDP, *cis*-diammine-dichloroplatinum(II); *t*-buHP, *tert*-butyl hydroperoxide; kb, kilobases.

pared with parental or control plasmid-transfected cell lines for sensitivity to cytotoxic agents. The cytotoxins selected were either known GST substrates or chemotherapeutic drugs previously used to select resistant cell lines that were subsequently found to overexpress GST isoenzymes. Our results indicate that the two transfected cell lines that express the highest GSTμ and GSTα2 activities were 2.1-fold and 1.7-fold resistant, respectively, to ethacrynic acid, a GST substrate. However, overexpression of GSTμ or GSTα2 did not consistently confer resistance to any of the anticancer agents tested, including doxorubicin, L-PAM, BCNU, chlorambucil, and cisplatin, or to the GST substrates CDNB or *t*-buHP.

Experimental Procedures

Materials. All common reagents were reagent grade or higher (J. T. Baker Chemical Co., Fluka, Aldrich Chemical Co., and Sigma Chemical Co.). The Coomassie blue protein assay reagent was from Pierce Chemical Co. Restriction enzymes, molecular weight markers, guanidine isothiocyanate, and acrylamide were from Life Technologies/BRL. Agarose was from SeaKem. Doxorubicin, L-PAM, chlorambucil, cisplatin, ethacrynic acid, and CDNB were obtained from Sigma. Radioisotopes were obtained from Amersham.

Expression vectors. Mammalian expression vectors were constructed that contained either the human GSTμ or GSTα2 cDNA. Cloning of the human cDNAs for GSTμ (pGTH4; 1138 base pairs) and GSTα2 (pGTH2; 929 base pairs) has been previously reported (10, 11). These cDNAs were subcloned into the *Eco*RI site of the plasmid expression vector pMTP-3H (12), which was the generous gift of Drs. Dwight Kaufman (National Cancer Institute) and Neal Rosen (Georgetown University). This vector utilizes a portion of the human metallothionein IIA promoter (bases −761 to +71 relative to the start of transcription) (13) to drive transcription of the inserted sequence, followed by a downstream SV40 T antigen polyadenylation signal sequence (14). Correct orientation of insertion was verified by restriction analysis of DNA from transformed *Escherichia coli* HB101 colonies, and each vector was amplified, purified by two rounds of CsCl density gradient ultracentrifugation, and dialyzed against 10 mM Tris containing 1 mM EDTA, pH 7.0. The GSTμ and GSTα2 vectors were designated pGSTμ and pGSTα2, respectively.

Cell culture and transfection. Cells were maintained at 37° in a 5% CO_2 atmosphere, in improved minimal essential medium (GIBCO) that contained 4 mM glutamine, 2 mg/liter L-proline, 50 mg/ml gentamycin, and 5% fetal bovine serum (GIBCO). The concentration of zinc in the medium was 0.5 μM, and no additional zinc was added. Logarithmic phase cells were harvested by scraping into ice-cold Tris-buffered saline (50 mM Tris, pH 7.5, 150 mM NaCl) containing 10 mM EDTA for preparation of RNA and DNA and analysis of enzyme activities.

Human MCF-7 breast cancer cells were plated at 4×10^5 cells/15-cm plate and co-transfected the next day by the calcium phosphate precipitation method described previously (12, 15, 16). Cells were transfected with 20 μg of DNA of one of the GST expression vectors or pUC8 control plasmid, along with 2 μg of the selectable marker plasmid DNA pSV2*neo* (16). The next day G418 (1.0 mg/ml) was added, and cells were selected for 3 weeks, with weekly changes of G418 and medium. Surviving colonies (1–20/plate) were cloned, using trypsin/EDTA and 5-mm cloning cylinders (Bellco Glass Co.), and expanded for screening.

Screening and biochemical characterization. Clonal cell lines were screened by the standard GST assay, using CDNB as second substrate (17), and also by Western blotting, using affinity-purified rabbit polyclonal antibodies to GSTμ and GSTα, as previously described (18). The wild-type MCF-7 cell line, one pUC8 (control)-co-transfected clone (obtained from Dr. M. Goldsmith), and three clones in each group with elevated GST in both the activity assay and the Western blot assay were selected for further characterization. Glutathione peroxidase activity was measured in control and GST-positive clones by an NADPH-coupled spectrophotometric assay, as previously described (19). Glutathione levels were assayed by a gluthione reductase-coupled kinetic assay, with bis(dithionitrobenzene) as oxidant (20).

Cytotoxicity studies. Clonogenic survival in the presence of cytotoxic agents was assayed three to five times per drug, as previously described (12, 15), by plating 400 cells/well in duplicate into 12-well Linbro dishes. After 24 hr the indicated concentrations of cytotoxin were added, and cells were allowed to grow for 8–10 days. Surviving colonies were stained with methylene blue and counted using an Artek model 880 digital colony counter. The percentage of survival was calculated relative to the mock-treated control wells, and the LD_{50} values were determined graphically from plots of concentration versus survival.

Northern and Southern blots. Total cellular RNA and DNA were isolated by the guanidinium isothiocyanate and CsCl gradient ultracentrifugation method, and RNA was fractionated by electrophoresis (25 μg/lane) in a 1% agarose gel containing formaldehyde, as previously described (21). DNA was digested overnight with *Eco*RI restriction endonuclease, precipitated, and fractionated by electrophoresis (25 μg/lane) in a 1% agarose gel, as previously described (21). After transfer to nylon membranes (Nytran; Schleicher & Schuell), blots were probed, according to the manufacturer's instructions, with ^{32}P-labeled human pGTH4 (GSTμ) or pGTH2 (GSTα2) cDNA inserts. The final washes were in 0.1X standard saline citrate, 1% sodium dodecyl sulfate, at 55° for 1 hr. The blots were exposed to Kodak XAR-2 film for 2 (pGSTμ) or 4 (pGSTα2) days at −70°.

Results

Transfection and screening. The expression vectors containing either GSTμ or GSTα2 were co-transfected along with the selectable marker plasmid pSV2*neo* (16) into human MCF-7 breast cells and selected in the presence of the toxic aminoglycoside antibiotic G418. A total of 130 colonies were isolated, expanded, and assayed for CDNB-conjugating activity (expressed as nmol/min/mg of protein, or mIU/mg). Those clones with GST activity at least 50% greater than the parental MCF-7 cell line were subjected to immunoblot analysis, using immunoaffinity-purified polyclonal rabbit anti-human GST antibodies. From the seven positive GSTμ-expressing and 10 positive GSTα2-expressing clones, three were chosen from each group that spanned the full range of specific activities obtained (designated MCF-7/pGSTμ-1, MCF-7/pGSTμ-2, MCF-7/pGSTμ-3, MCF-7/pGSTα2-1, MCF-7/pGSTα2-2, and MCF-7/pGSTα2-3). One clone (MCF-7/pSV*neo*) transfected with pUC8 and pSV*neo* DNA served as a negative transfected control cell line.

Biochemical characterization. The G418-selected transfectant colonies were cloned and assayed for GST expression by the standard CDNB conjugation assay and by Western blotting, using immunoaffinity-purified antibodies specific for each isoenzyme. The 10 control (pUC8/pSV2*neo*-transfected) clones all had low GST activities. Among the 120 GST clones, 13–15% were positive in both enzymatic and immunoblot assays. The GST activities ranged from 56 to 340 mIU/mg in the GSTμ-transfected clones and from 17 to 52 mIU/mg in the GSTα2-transfected clones. In comparison, the GST activity in the parental (MCF-7) and control pUC8 plus pSV2*neo*-transfected MCF-7 cell lines were 9.8 and 10.7 mIU/mg, respectively (Table 1). The lower specific activity of GST in cells transfected with the GSTα2 expression vector, relative to the values ob-

tained in cells transfected with the GSTμ expression vector, is probably related to the 2.4-fold lower specific activity of this isoenzyme toward CDNB, compared with that of GSTμ (7). The GST activity in the transfectants was stable (<10% variation in GST activity) over the 4–5 months during which the cytotoxicity assays were conducted. Because the α class GSTs are known to exhibit glutathione peroxidase activity toward organic hydroperoxides (but not H_2O_2) (2, 3, 7), the glutathione peroxidase specific activity of the transfectants was assayed using the standard substrate cumene hydroperoxide. As expected, only the GSTα2-transfected clones had increased cumene hydroperoxidase activity (Table 1). GSH levels were also measured and were similar in the transfected and control cell lines (Table 1).

Immunoblot analysis of protein from these cell lines confirmed that the GST expression was specific for the transfected gene product and that the amount of GST protein present was proportional to the specific activity for both GSTμ and GSTα2 (Fig. 1). A very faint band of GSTμ protein was sometimes detectable in control and GSTα2-transfected cell lines, indicating that the low level of GST activity in these cell lines is μ class GST. This is consistent with previous results with MCF-7 cells (18).

Northern blot analysis of total RNA from control and transfected cell lines showed that the proportionality between GST specific activity and protein detected by Western blotting was also reflected in the mRNA content for the respective clones (Fig. 2). The sizes of the mRNAs (1.9 kb for pGSTμ- and 1.6 kb for pGSTα2-transfected cells, respectively) were consistent with transcription from the transfected genes (10–12). The difference in size is due to differences in the 3′ untranslated regions included in each expression vector.

Southern blot analysis of cellular DNA revealed multiple hybridizing fragments, a reflection of the known multiplicity of genes in the human μ class and α class GST subfamilies (7). A single band (1.2 kb in pGSTμ-transfected cells and 0.95 kb in pGSTα2-transfected cells) was detected in each of the GST-transfected cell lines but not in wild-type MCF-7 or control pUC8 plus pSV2*neo*-transfected MCF-7 cell lines (Fig. 3). These bands correspond to the expected restriction fragment size of the respective GST expression vectors. The apparent expression vector DNA copy number was not related to either mRNA or GST protein levels in the GSTμ-transfected clones, and and there was actually an inverse relationship between copy number and expression in the GSTα2-transfected clones. This effect was also observed in previous experiments in which the human GSTα1 cDNA was transfected into MCF-7 cells (22). This is most likely due to proximity to different regulatory elements after random insertion into the genome.

Cytotoxicity studies. Three cloned cell lines each of the pGSTμ and pGSTα2 transfectants, one pUC8/pSV2*neo*-transfected line, and the parental MCF-7 cell line were tested for sensitivity to cytotoxic agents by the clonogenic survival assay. The LD_{50} values were determined for doxorubicin, L-PAM, BCNU, chlorambucil, CDDP, CDNB, *t*-buHP, and ethacrynic acid, as described in Experimental Procedures, and the relative resistance was determined by dividing the results for the trans-

TABLE 1

Biochemical characterization of control and GST-transfected MCF-7 cell lines

Glutathione levels, GST activity, and glutathione peroxidase activity were measured in wild-type MCF cells and in clonal derivatives transfected with either pSV2*neo* only, pSV2*neo* and pGSTμ, or pSV2*neo* and pGSTα2. The results are expressed as the mean ± standard deviation of three separate determinations. Values are mean ± standard deviation.

Cell line	GSH level	GST activity (CDNB)[a]	GSH peroxidase activity (cumene hydroperoxide)[a]
	nmol/mg	*mIU/mg*	*mIU/mg*
MCF-7	ND[b]	9.8 ± 0.4	2.9 ± 0.6
pSV*neo*	121 ± 5	10.7 ± 0.3	2.5 ± 0.6
pGST-μ1	114 ± 6	56 ± 1	2.6 ± 0.4
pGST-μ2	121 ± 3	150 ± 8	2.4 ± 0.5
pGST-μ3	108 ± 4	340 ± 26	2.3 ± 0.5
pGST-α1	105 ± 3	17 ± 1	4.0 ± 0.9
pGST-α2	132 ± 5	28 ± 2	5.1 ± 0.3
pGST-α3	119 ± 4	52 ± 2	9.5 ± 0.6

[a] 1 mIU is defined as 1 nmol/min of activity.
[b] ND, not determined.

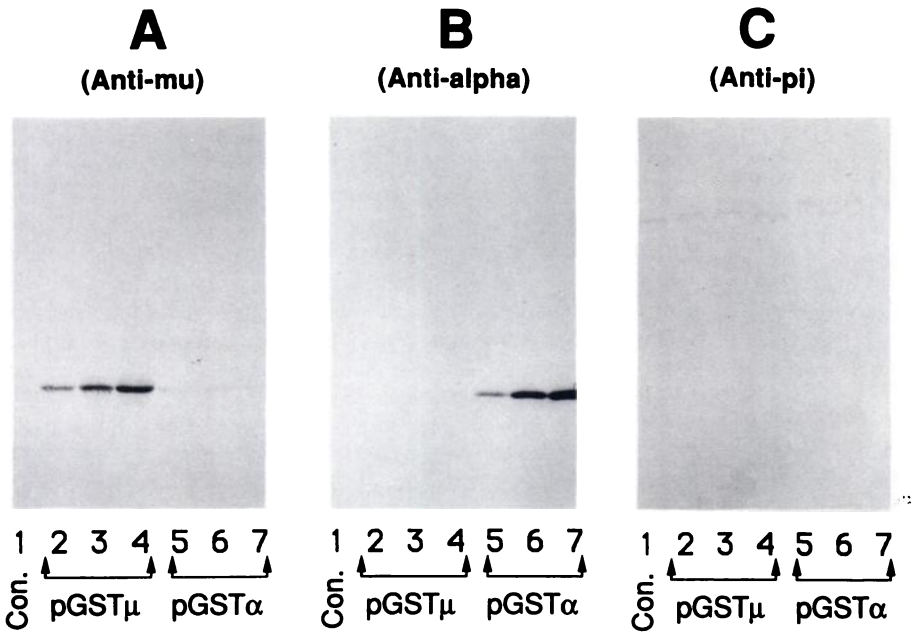

Fig. 1. Immunoblot analysis of control and GST-transfected MCF-7 clonal cell lines. Cytosol (100 μg/lane) was electrophoresed on a 14% sodium dodecyl sulfate-polyacrylamide gel, electrophoretically transferred to a nitrocellulose membrane, and probed with affinity-purified rabbit polyclonal antibodies directed against human μ, α, or π class GSTs, as indicated. *Lane 1*, control MCF-7/pUC8 plus pSV2*neo* transfectant; *lane 2*, MCF-7/pGSTμ-1; *lane 3*, MCF-7/pGSTμ-2; *lane 4*, MCF-7/pGSTμ-3; *lane 5*, MCF-7/pGSTα2-1; *lane 6*, MCF-7/pGSTα2-2; *lane 7*, MCF-7/pGSTα2-3. The faint high molecular weight band in the blot probed with GSTπ antibody is an unidentified nonspecifically reacting protein present in all of the MCF-7 cell lines.

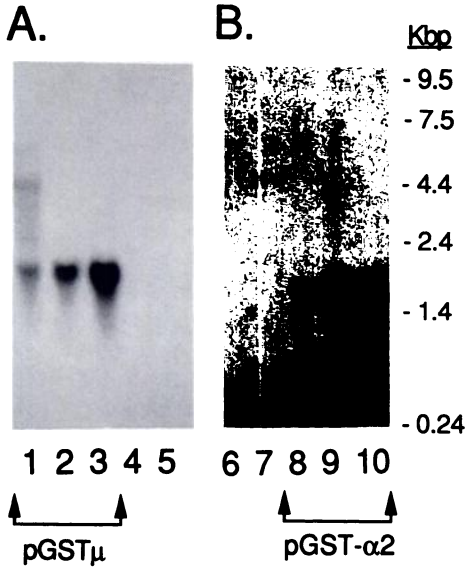

Fig. 2. Northern blot analysis of RNA from control and GST-transfected MCF-7 clonal cell lines. Total cellular RNA (25 μg/lane) was separated on a 1% formaldehyde/agarose gel, transferred to a nylon hybridization membrane, and probed with ^{32}P-labeled human GSTμ (pGTH4) or GSTα2 (pGTH2) cDNA insert, as described in Experimental Procedures. *Lane 1*, MCF-7/pGSTμ-1; *lane 2*, MCF-7/pGSTμ-2; *lane 3*, MCF-7/pGSTμ-3; *lanes 4* and *7*, control MCF-7/pUC8 plus pSV2*neo* transfectant; *lanes 5* and *6*, parental MCF-7; *lane 8*, MCF-7/pGSTα2-1; *lane 9*, MCF-7/pGSTα2-2; *lane 10*, MCF-7/pGSTα2-3.

fected cell lines by the values obtained for the parental MCF-7 cell line.

The relative resistance of the pGSTμ-transfected cell lines to doxorubicin, L-PAM, BCNU, chlorambucil, CDDP, CDNB, and *t*-buHP ranged from 0.7 to 1.3, compared with a range of 0.7 to 1.2 in the control pUC8/pSV2*neo*-transfected control cell line (Table 2). The pGSTμ-3 clone, which expressed the highest GST activity of the GSTμ transfectants, was 2.1-fold resistant to the cytotoxicity of ethacrynic acid ($p = 0.005$), a drug that is both a substrate for and a competitive inhibitor of GSTs (23).

Similar results were obtained with the GSTα2 transfectants. The pGSTα2-3 clone, with the highest GSTα2 expression, exhibited a relative resistance to ethacrynic acid of 1.7 ($p < 0.005$), whereas the relative resistance values for the GSTα2 transfectants ranged from 0.7 to 1.0 for all the remaining chemotherapeutic drugs, as well as the GST substrates CDNB and *t*-buHP. Although small variations were evident in the average LD_{50} and the calculated relative resistance values for individual cell lines with particular cytotoxins, these were not significantly different from the values obtained with either of the control cell lines ($p > 0.05$). Similar results were obtained after exposure of the GST-transfected cell lines to cytotoxin for only 1 hr, followed by replacement with drug-free medium (data not shown). However, the variability and absolute values for LD_{50} were generally higher in these experiments.

Discussion

Previous reports have described the isolation of drug-resistant cell lines that express increased activities of various GST isoenzymes relative to their parental drug-sensitive cell lines. A Chinese hamster ovary cell line (CHO-Chl^r) that was selected for resistance (24-fold) to the alkylating agent chlorambucil and that displayed cross-resistant to L-PAM had a specific activity of 638 mIU/mg, compared with 239 mIU/mg in the parental cell line (24). A chlorambucil-selected Walker 256 rat mammary carcinoma cell line (WR) (25) that was 15-fold resistant to chlorambucil and cross-resistant to cisplatin had 25.4 mIU/mg GST, compared with 12.7 mIU/mg in the parent line. The increased activity in both selected lines was found to be due to elevated activity of α class GST isoenzymes. A rat glioma cell subline (9L-2) that was 3–4-fold resistant to BCNU had lower total GST activity but higher activity of the rat μ class GST isoenzymes 3-3 and 4-4 (26). Interestingly, the purified 4-4 isoenzyme was found to catalyze the inactivation of BCNU by a GSH-dependent denitrosation reaction 6–12 times more effectively than other classes of rat GST (26).

The frequent association between increased α and μ class GST activity and drug resistance has led to the suggestion that GST could be a causative factor in resistance to these cytotoxic agents (2–6). Expression of GST isoenzymes might confer resistance to antineoplastic agents in one of several ways. In addition to the GST-catalyzed inactivation of BCNU, enhancement of drug detoxication by GSTs by conjugation with GSH has been demonstrated for L-PAM, chlorambucil, CDNB, and ethacrynic acid (7, 8, 27, 28). Another potential protective function involves high affinity binding to lipophilic ligands. This is in fact the oldest known function of GSTs, which were originally termed "ligandins" (9). The α class GSTs in particular are characterized by an additional activity, a selenium-independent glutathione peroxidase toward organic hydroperoxides such as *t*-buHP and cumene hydroperoxide (but not H_2O_2) (29). Fatty acid hydroperoxides resulting from lipid peroxidation may also be reduced by GST-catalyzed peroxidase activity (7, 8). Detoxication of lipid hydroperoxides represents a fourth mode of protection that could be mediated by GSTs when cells are exposed to toxic oxygen radicals, for example during the redox cycling of doxorubicin (30).

The overexpression of GSTs in drug-resistant cell lines suggests that these isoenzymes could mediate drug resistance. However, selection with cytotoxic chemotherapeutic drugs may result in a resistant cell population that differs from the unselected cell line by more than one phenotypic trait, particularly if the selection protocol involves stepwise increases in drug concentration over an extended period (31). Furthermore, multiple genes involved in cellular defense mechanisms may be regulated by common factors and, therefore, may be induced or selected coordinately (32). Thus, it is important to determine whether an observed phenotypic change contributes directly to the observed drug resistance in cell lines that survive cytotoxic selection or, alternatively, whether it is a nonessential "marker" that is merely associated with drug resistance. The objective of this study was to determine whether *de novo* expression of GSTμ or GSTα2 would provide direct protection against cytotoxic anticancer agents by virtue of the detoxication functions described above.

The model system we have chosen to study this question, transfection of MCF-7 cells with GST expression vectors, has several advantages. First, the unselected MCF-7 parental cell line has very low constitutive GST activity (10 mIU/mg) and is sensitive to all the cytotoxic agents tested in the nanomolar to micromolar range. Second, the increased GST expression after transfection allows comparison of cell lines that should

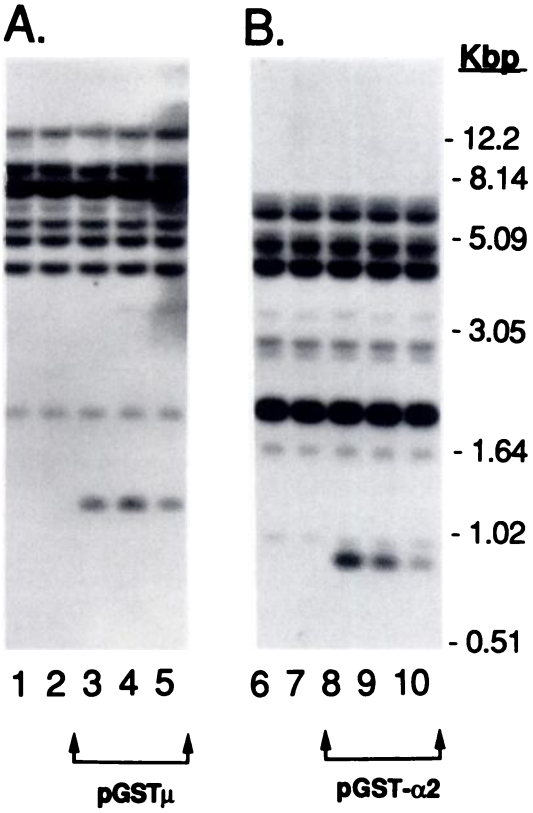

Fig. 3. Southern blot analysis of DNA from control and GST-transfected MCF-7 clonal cell lines. Total cellular DNA was digested with *Eco*RI restriction endonuclease, separated on a 1% agarose gel (25 μg/lane), transferred to a nylon hybridization membrane, and probed with ^{32}P-labeled human GSTμ (pGTH4) or GSTα2 (pGTH2) cDNA insert, as described in Experimental Procedures. *Lanes 1* and *6*, parental MCF-7; *lanes 2* and *7*, control MCF-7/pUC8 plus pSV2*neo* transfectant; *lane 3*, MCF-7/pGSTμ-1; *lane 4*, MCF-7/pGSTμ-2; *lane 5*, MCF-7/pGSTμ-3; *lane 8*, MCF-7/pGSTα2-1; *lane 9*, MCF-7/pGSTα2-2; *lane 10*, MCF-7/pGSTα2-3.

differ only in the level of expression of the transfected gene. Because the LD_{50} values were similar in the parental MCF-7 cell line and MCF-7 cells transfected with pSV2*neo* plus pUC8, the transfected bacterial *neo* gene apparently does not affect cellular sensitivity to the cytotoxic agents tested. Furthermore, in order to eliminate the ambiguity that could arise from clonal heterogeneity, several clones were analyzed in each group to determine whether any differences in sensitivity to cytotoxins were related to the relative level of expression of each isoenzyme.

The magnitude of the increases in GSTμ and GSTα2 activities and enzyme protein in the transfected clones appeared to be equivalent to or higher than the increases in most of the GST-overexpressing drug-resistant cell lines that have been reported (25, 26). The two clones with the highest GSTμ and GSTα2 expression (the MCF7/pGSTμ-3 and MCF7/pGSTα2-3 transfectants) had 2.1-fold and 1.7-fold resistance, respectively, to ethacrynic acid cytotoxicity (Table 1). However, increased GST expression did not confer protection against cytotoxicity of any of the anticancer agents tested or against the cytotoxicity of the GST substrates CDNB or *t*-buHP (Table 2). We conclude, therefore, that expression of GSTμ or GSTα2 in the range of activities obtained in these transfectants is not by itself sufficient to confer protection against the cytotoxic effects of these chemotherapeutic agents and GST substrates.

The gene transfer approach has been used previously in this laboratory and by other investigators to study the relationship between GST expression and anticancer drug resistance. Studies to date have focused primarily on the π class GST, because this isoenzyme has been most frequently associated with drug resistance (1–6) and also with neoplastic transformation (33–35). Increased resistance (2–4-fold) to CDDP was observed after transfection of Chinese hamster ovary cells with the human GSTπ gene (36). Our laboratory has previously transfected MCF-7 cells with a GSTπ expression vector and observed resistance to ethacrynic acid (3–4-fold) in three separate clones, but no consistent protection was observed against the cytotoxicity of doxorubicin, L-PAM, or CDDP (12). Another group reported that transfection of the GSTπ gene into NIH-3T3 cells resulted in 4.6-fold resistance to ethacrynic acid and a modest change in sensitivity to doxorubicin (1.7–3-fold in LD_{37}, with less apparent difference at the LD_{50}) (37). Furthermore, GSTπ-transfected 3T3 cells were not resistant to CDDP, chlorambucil, L-PAM, or radiation (37). In contrast, high levels of GSTπ activity were obtained after transfection of the human GSTπ gene into yeast cells, and this was associated with increased resistance to doxorubicin and chlorambucil (38).

The observation that GST-mediated detoxication of BCNU is catalyzed preferentially by μ class GSTs (26) and the finding that resistance to alkylating agents has been associated with the α class GSTs have prompted the study of the protective effects of these isoenzymes as well. Transient expression of GSTπ or the rat 1-1 (α class) or 3-3 (μ class) GSTs in monkey COS cells, which were transfected with GST expression vectors and subsequently enriched for the GST-expressing cells by fluorescence-activated cell sorting, resulted in low level (1.1–1.5-fold) resistance to chlorambucil, L-PAM, and CDDP (39). Expression in yeast of the human GSTα1 gene, which resulted in high intracellular enzyme levels, was associated with increased resistance to doxorubicin and chlorambucil (38). In contrast, recent experiments in our laboratory showed that human MCF-7 cells stably transfected with the human GSTα1 gene were resistant to ethacrynic acid but not resistant to doxorubicin, chlorambucil, L-PAM, CDDP, or CDNB (22). Thus, studies in MCF-7 cells transfected with the human GSTπ, GSTμ, GSTα1, or GSTα2 genes have shown that increased expression of all three classes of human cytosolic GST isoenzymes (α, μ, and π) resulted in increased resistance to ethacrynic acid and in some instances to benzo(*a*)pyrene diol-epoxide, another known substrate for GST isoenzymes. However, in MCF-7 cells, overexpression of any of the three classes of GST isoenzymes failed to result in any consistent alteration in sensitivity to a variety of anticancer agents.

Several possible reasons could account for the failure of GST to afford protection against the cytotoxic substrates tested in transfected MCF-7 cells. First, the levels of GST activity attained in the transfected cell lines may be too low to confer protection. Although the levels of activity observed in the GSTS gene-transfected MCF-7 cells are somewhat lower than those achieved in the yeast cell system, the levels achieved are comparable to those reported in animal and human cell lines selected for resistance to anticancer agents (25, 26). Furthermore, although GST has been shown to enhance the conjugation of drugs such as chlorambucil, BCNU, and L-PAM with

TABLE 2

LD_{50} values and relative resistance of GSTμ- and GSTα2-transfected MCF-7 human breast cancer cell lines

Clonogenic survival assays were performed as described in Experimental Procedures. Values are mean ± standard error of three to five determinations. The figures in parentheses are the relative resistance, defined as the ratio of the LD_{50} of each transfectant clone with each drug to the LD_{50} of the wild-type MCF-7 cells with the same drug.

Cell line	LD_{50}							
	Doxorubicin	L-PAM	BNCU	Chlorambucil	Cisplatin	Ethacrinic acid	CDNB	*t*-buHP
	nM				μM			
MCF-7 (wild-type)	4.3 ± 0.9 (1.0)	1.2 ± 0.2 (1.0)	39 ± 5 (1.0)	9.8 ± 1.8 (1.0)	0.59 ± 0.03 (1.0)	3.4 ± 0.6 (1.0)	0.67 ± 0.07 (1.0)	15.5 ± 3.6 (1.0)
MCF-7/pSV*neo*	3.0 ± 0.5 (0.7)	1.4 ± 0.2 (1.2)	34 ± 1 (0.9)	11.4 ± 2.7 (1.2)	0.63 ± 0.09 (1.1)	3.6 ± 0.8 (1.1)	0.59 ± 0.14 (0.9)	13.4 ± 3.4 (0.9)
MCF-7/pGSTμ-1	3.4 ± 0.4 (0.8)	0.9 ± 0.2 (0.8)	33 ± 5 (0.9)	7.8 ± 1.6 (0.8)	0.49 ± 0.05 (0.8)	3.5 ± 0.8 (1.0)	0.70 ± 0.10 (1.0)	12.0 ± 0.8 (0.8)
MCF-7/pGSTμ-2	3.2 ± 0.4 (0.7)	1.0 ± 0.2 (0.8)	32 ± 3 (0.8)	8.1 ± 1.9 (0.8)	0.51 ± 0.06 (0.9)	3.1 ± 0.5 (0.9)	0.70 ± 0.01 (1.0)	13.3 ± 2.6 (0.9)
MCF-7/pGSTμ-3	4.4 ± 1.1 (1.0)	1.6 ± 0.3 (1.3)[a]	38 ± 3 (1.0)	9.4 ± 2.4 (1.0)	0.60 ± 0.02 (1.0)	7.2 ± 1.6 (2.1)[b]	0.61 ± 0.16 (0.9)	16.3 ± 3.7 (1.1)
MCF-7/pGSTα2-1	3.2 ± 0.6 (0.7)	1.2 ± 0.2 (1.0)	29 ± 3 (0.7)	8.4 ± 2.2 (0.9)	0.52 ± 0.07 (0.9)	3.4 ± 0.6 (1.0)	0.58 ± 0.13 (0.9)	12.0 ± 0.8 (0.8)
MCF-7/pGSTα2-2	3.2 ± 0.6 (0.7)	1.1 ± 0.1 (0.9)	28 ± 2 (0.7)	7.7 ± 1.5 (0.8)	0.57 ± 0.03 (1.0)	3.1 ± 0.7 (0.9)	0.58 ± 0.13 (0.9)	10.3 ± 2.1 (0.7)
MCF-7/pGSTα2-3	3.3 ± 0.3 (0.7)	0.9 ± 0.1 (1.0)	29 ± 3 (0.7)	9.2 ± 1.9 (0.9)	0.57 ± 0.03 (0.9)	5.7 ± 0.6 (1.7)[b]	0.59 ± 0.06 (0.9)	12.3 ± 3.3 (0.8)

[a] $0.05 < p < 1.0$.
[b] $p < 0.05$ (Student's *t* test).

GSH, these drugs are relatively poor substrates for GSTs. For example, the conjugation of chlorambucil with GSH was reported to be enhanced by GST by a factor of only 2–3-fold over the nonenzymatic rate (28). Similarly, the reported value for the V_{max} of purified rat μ class GST (4-4) in the BCNU denitrosation reaction is <0.1 IU/mg, and the apparent K_m for these substrates ranges from 0.3 to 1.9 mM (26). Thus high intracellular enzyme and substrate concentrations may be required to see any effect. This is the most likely explanation for the lack of protection against CDNB cytotoxicity in these cell lines. Although CDNB is a relatively good GST substrate at high concentrations, the K_m for CDNB ranges from 70 to 2000 μM for various GSTs.[2] Thus, it is not surprising that protection is not conferred against CDNB, because the LD_{50} concentration (around 1 μM) is 2–3 orders of magnitude lower than the K_m values of these isoenzymes (around 500 μM). However, it should be noted that the specific activity for the substrate ethacrynic acid is also relatively low (7), yet transfection of three different cytosolic GST isoenzymes nevertheless confers 1.7–4-fold resistance to this agent (Table 2) (12, 22).

It is also possible that other factors are required in order for GST to confer any protective effect. Hence, overexpression of GST in transfected MCF-7 cells may not by itself be sufficient to cause resistance to any particular anticancer drug. For example, post-translational modification of GST may affect its substrate specificity, as shown by experiments in which reaction of purified rat α class GST with protein kinase C resulted in an altered affinity of GST for bilirubin (40). Although other factors may be induced in resistant cells during chronic drug exposure, the isolated increase in GST expression may be insufficient by itself to result in drug resistance.

Thus, although this report and previous transfection studies in human MCF-7 human breast cancer cells have failed to demonstrate a consistent effect of any of the three classes of cytosolic GST isoenzymes with respect to anticancer agents, these studies do not eliminate the possibility that enhanced expression of these enzymes might contribute to resistance under other circumstances. The question of whether increased GST expression might modulate sensitivity to cytotoxic agents by an indirect effect or in concert with other factors will be of interest in future studies.

[2] A. Townsend, unpublished observations.

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