[CANCER RESEARCH 52, 5115–5118, September 15, 1992]

Advances in Brief

Increase in γ-Glutamylcysteine Synthetase Activity and Steady-State Messenger RNA Levels in Melphalan-resistant DU-145 Human Prostate Carcinoma Cells Expressing Elevated Glutathione Levels[1]

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Abstract

The biochemical and molecular basis for the elevation of glutathione (GSH) levels commonly detected in many drug-resistant cells has not been elucidated. In a series of L-phenylalanine mustard-resistant human prostate carcinoma cell lines (DU-145), resistance was associated with elevated GSH levels, increased activity of γ-glutamylcysteine synthetase (GCS), the rate-limiting enzyme in GSH biosynthesis, and a marked increase in the steady-state levels of GCS-specific transcripts (4.0 and 3.2 kilobases). Loss of the resistant phenotype was accompanied by a reduction in GSH and a return of GCS activity and transcript levels to values comparable to those detected in the drug-sensitive parent cells. These data strongly implicate up-regulation of GCS activity as an important mechanism in the evolution of drug resistance associated with increased levels of intracellular GSH. The results further suggest that the ability to synthesize GSH may be more indicative of resistance than steady-state GSH levels per se.

Introduction

GSH[3] is known to play an important role in the detoxification of various xenobiotics and protection against oxidative stress and radiation injury and has been strongly implicated in the resistance of tumor cells to certain types of chemotherapy (1, 2). Increased intracellular levels of this protective nonprotein thiol are frequently associated with the evolution of drug resistance in preclinical tumor models following selection in the presence of increasing concentrations of certain chemotherapeutic agents, particularly alkylating agents and *cis*-platinum. By comparison to lines derived from drug-responsive counterparts of similar histology, cell lines established from clinically refractory tumors have frequently been found to have higher GSH levels. In certain cases, it has even been possible to document GSH elevations in the resistant line of sensitive/resistant pairs established from individual patients before and after treatment failures (3). Further evidence in support of a role of GSH in drug resistance has also been provided by a wealth of data demonstrating that treatment with buthionine sulfoximine, a specific inhibitor of GCS (4), the rate-limiting step in GSH biosynthesis, results in GSH depletion and increased drug sensitivity *in vitro* and *in vivo* (5, 6). In the case of the L-PAM-resistant DU-145/M5 cells used in the current studies, for example, depletion of GSH by buthionine sulfoximine to levels comparable to those characteristic of the parent cells was accompanied by a return to an L-PAM sensitivity similar to that of the parent cells (7).

Comprehensive investigations of the molecular and biochemical mechanisms responsible for the elevation of GSH certainly seem warranted in light of the frequent association between drug resistance and GSH elevations, yet surprisingly few studies have been conducted. While such efforts have undoubtedly been hampered in the past by the admittedly complex nature of GSH metabolism, much of the relevant biochemistry is now well characterized (8). Importantly, key enzymes involved in the regulation of intracellular GSH levels have been identified, enabling biochemical and molecular characterization of the role they play in regulating GSH levels in drug-resistance tumor cells.

Intracellular GSH is synthesized from its constituent amino acids via two sequential ATP-consuming reactions catalyzed by GCS and GSH synthase, respectively (8). The synthesis of the dipeptide γ-glutamylcysteine by GCS is the rate-limiting step in *de novo* GSH synthesis and conceivably an important control point in the regulation of GSH in drug-resistant tumor cells. In this report, we describe the results of studies designed to evaluate the role of up-regulation of GCS activity in the expression of increased GSH levels in a series of melphalan-resistant DU-145 human prostate carcinoma cell lines.

Materials and Methods

Cell Lines. L-PAM-resistant DU-145 cell lines were developed from parent DU-145 prostate carcinoma cells by growth in progressively increasing concentrations of drug as previously described (7). All cells were maintained in exponential growth in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum and gentamycin. Cultures were maintained at 37°C in a humidified atmosphere of 95% air/5% CO_2. Cells were passaged weekly, and medium was changed at 3–4-day intervals. Twenty-four h after passage, L-PAM was added to the culture medium to a final concentration of 10^{-6}, 10^{-5}, and 5×10^{-5} M for the /M6, /M5, and /M4.5 cell lines, respectively. Cell survival was determined by standard clonogenic assay following a 1-h exposure to L-PAM at 37°C.

GSH and GCS Activity. Glutathione content was determined using the technique described by Tietze (9) using modifications reported by Bump *et al.* (10). GCS activity was quantitated using a modification of a high-performance liquid chromatography technique described by Nardi *et al.* (11) which measures the rate of γ-glutamylcysteine synthesis following conjugation of the product with monobromobimane. Briefly, following harvesting and rinsing in phosphate-buffered saline, cells were flash frozen in distilled water. After centrifugation, the supernatant was used for protein and enzyme determinations. Aliquots of the supernatant were added to prewarmed (37°C) reaction mixture (100 mM Tris-HCl, 50 mM KCl, 6 mM dithiothreitol, 20 mM $MgCl_2$, 3 mM cysteine, 15 mM glutamic acid, and 6 mM ATP) and maintained at 37°C.

Received 6/29/92; accepted 8/3/92.

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[1] Supported by NIH Grant CA57549 (R. T. M.). H. H. B. is supported by Grant PHS 5T32-CA09614 awarded by the National Cancer Institute, Department of Health and Human Services.

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[3] The abbreviations used are: GSH, glutathione; GCS, γ-glutamylcysteine synthetase; GGT, γ-glutamyltranspeptidase; L-PAM, L-phenylalanine mustard.

Forty-five-μl aliquots were removed at various time intervals and combined with 50 μl 50 mM *N*-ethylmorpholine and 20 μl 50 mM monobromobimane. The mixture was incubated in the dark at room temperature for 15 min, at which time the derivatization reaction was stopped by the addition of 80 μl of 10% sulfosalicylic acid. Following dilution to a final volume of 500 μl, 20 μl was injected onto a Beckman Ultrasphere ODS column (5 μm, 4.6 x 250 mm) running an isocratic mobile phase consisting of 15% methanol and 85% acetic acid (0.25%; pH 3.6). The quantity of γ-glutamylcysteine produced was quantitated by comparison to γ-glutamylcysteine standards derivatized and analyzed as above.

Glutathione content and GCS enzyme activity were normalized on the basis of protein content, determined according to the technique of Bradford (12) and using bovine serum albumin as a standard.

Northern Blotting. Total RNA was isolated from exponentially growing cultures by the guanidinium isothiocyanate-phenol method of Chomczynski and Sacchi (13). RNA samples were size fractionated by formaldehyde/agarose gel electrophoresis and transferred to GeneScreen Plus membranes. Following incubation at 80°C and prehybridization at 42°C, the membranes were hybridized at 42°C with a ^{32}P-labeled probe corresponding to the 764-base pair *Pst*I fragment of the human GCS complementary DNA (14). Membranes were similarly hybridized with ^{32}P-labeled probes for β-actin and GGT. This final probe was a 596-base pair fragment isolated following digestion of the plasmid, phGGT (15), with *Pvu*II and *Bgl*II. phGGT was generously provided by Drs. Goodspeed and Pitot (McArdle Laboratory, Madison, WI).

Message sizes were determined by comparison to the migration of RNA markers (Promega, Madison, WI).

Results

Melphalan-resistant DU-145 prostate carcinoma cell lines were developed by sequential passage over a 2–3-year period in progressively increasing concentrations of L-PAM (7). Three resistant lines, designated DU-145/M6, /M5, and /M4.5, were developed from the parent DU-145 cells and maintained by weekly passage in medium containing 10^{-6}, 10^{-5}, and 5×10^{-5} M L-PAM, respectively. On the bases of 50% inhibition concentration values determined by tetrazolium dye assay, the lines were determined to be ~3-, ~7-, and ~27-fold resistant to L-PAM by comparison to the parent DU-145 cells (Table 1). Similar results were obtained when cell survival was determined by clonogenic assay following a 1-h exposure to L-PAM (Fig. 1).

Resistance to L-PAM in the DU-145/M5 line was previously shown to be associated with elevations of intracellular GSH levels, unaccompanied by significant changes in total glutathione *S*-transferase activity or isozyme profile (7). As shown in Table 1, GSH levels were also significantly increased in the /M6 and /M4.5 cells, although the concentrations were not significantly different in the /M5 and /M6 lines in spite of their difference in L-PAM sensitivity. In an attempt to determine the mechanism(s) responsible for the GSH elevations detected in the resistant lines, the activity of GCS, the rate-limiting enzyme in the *de novo* synthesis of GSH (11), was determined. By comparison to the parent cell line, GCS activity was increased 2- to 3-fold in the /M5 and /M6 cell lines and 15-fold in the /M4.5 line (Table 1).

Table 1 *Relative L-PAM sensitivity and biochemical parameters for DU-145 human prostate carcinoma cell lines*

Cell line	IC_{50} (μM)[a]	GSH[b]	GCS[c]	Relative GCS mRNA level
DU-145	3	117.6 ± 12	0.6 ± 0.1	1
DU-145/M6	10	190.7 ± 10[e]	1.7 ± 0.3[e]	8
	(3.3)[d]	(1.6)	(2.8)	
DU-145/M5	20	186.0 ± 8.2[e]	1.4 ± 0.2[e]	20
	(6.7)	(1.6)	(2.3)	
DU-145/M4.5	80	288.0 ± 7.4[e]	8.8 ± 1.2[e]	50
	(26.7)	(2.4)	(14.7)	
DU-145/M5-out	9	120.5 ± 12	0.6 ± 0.1	1
	(3.0)	(1.0)	(1.0)	

[a] Dose causing 50% inhibition; determined by tetrazolium dye assay; see Ref. 7.
[b] nmol/mg protein (± SEM).
[c] nmol/min/mg protein (± SEM).
[d] Fold increase relative to DU-145 cells.
[e] Significantly different from DU-145 ($P < 0.02$).

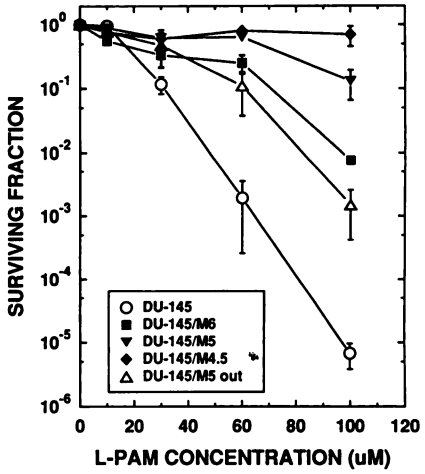

Fig. 1. Survival of DU-145 cell lines following exposure to L-PAM. Cells were exposed to L-PAM for 1 h at 37°C and plated for survival determination by clonogenic assay. *Points*, mean for two determinations; *bars*, SD.

In order to evaluate the stability of the resistant phenotype in the absence of continued selection pressure, /M5 cells were maintained in culture without further exposure to L-PAM for nearly 2 years (designated /M5-out). At the end of this time, these cells were significantly more sensitive to L-PAM than their /M5 counterparts, although still more resistant than the DU-145 parent cells (Table 1; Fig. 1). The GSH-related biochemical parameters were also quantitated in these revertants and compared with the other cell lines in this series. Both the intracellular GSH level and GCS activity returned to the wild-type level (Table 1).

Total RNA was isolated from each of the five DU-145 cell lines and subjected to Northern analysis utilizing a ^{32}P-labeled probe corresponding to a 764-base pair *Pst*I fragment within the coding region of hlGCS-50, a full-length GCS clone isolated from a human liver complementary DNA library (14). The probe hybridized to two transcripts, 4.0 and 3.2 kilobases in length, in each of the five cell lines (Fig. 2). The transcripts were elevated nearly 8-, 20-, and 50-fold in the /M6, /M5, and /M4.5 resistant lines, respectively. The intensities of both the 4.0- and 3.2-kilobase bands were increased to approximately the same extent in each line. As was observed with GSH and GCS activity, the level of GCS-specific transcripts was reduced to wild-type levels in the revertant /M5-out cells.

The membranes were also hybridized with a GGT-specific probe. No differences in the level of GGT message were detected, however (data not shown).

Discussion

In light of the extensive literature suggesting an association between GSH elevations and alkylating agent resistance, we initiated studies designed to examine the mechanism(s) responsible for such increases. While many factors can contribute to an increased steady-state level of GSH, we elected to focus initially on GCS. This enzyme, which is feedback inhibited by

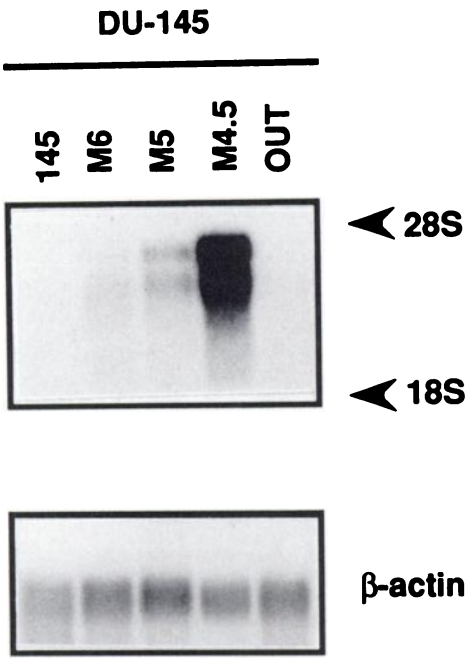

Fig. 2. Northern blot analysis of GCS expression. Total RNA (8 μg/well) was size fractionated on a 1.2% agarose/formaldehyde gel transferred to membranes and hybridized with a GCS-specified probe as described in "Materials and Methods."

GSH, catalyzes the first step in GSH biosynthesis and represents an important regulatory point in GSH homeostasis (8). Precedent for increased GSH levels secondary to elevated GCS activity has been provided in studies examining the effects of heavy metal (16) or prostaglandin A_2 (17) exposure on GSH.

In all three resistant DU-145 cell lines GSH levels, GCS activity, and the levels of GCS-specific transcripts were significantly elevated. Growth of /M5 cells in the absence of L-PAM exposure resulted in a loss of resistance accompanied by a progressive decrease in each of these biochemical parameters with time, culminating in a return to levels not significantly different from that of the parent DU-145 cells. No differences were detected in message levels for GGT, an enzyme which contributes to GSH regulation by functioning as a salvage pathway for cysteine moieties and which has been reported to be elevated in some drug-resistant cell lines (3, 18). These data therefore support the hypothesis that selection in the presence of L-PAM results in an increase in GCS mRNA and enzyme activity contributing to a net increase in GSH. Whether the increased transcript levels reflect amplification, up-regulation of GCS gene transcription, or alterations in message half-life is currently being investigated.

In previous studies, Northern analysis of total RNA isolated from rat kidney, rat liver, and human kidney using the same GCS-specific probe utilized in this study identified a single transcript (14). In the case of human kidney the message was 4.0 kilobases in length, corresponding to the larger message detected in RNA from the DU-145 cell lines. The significance of the smaller band detected by Northern blots of RNA from the tumor cells is currently unknown and may represent tissue-specific differences in processing. Both bands have been detected in RNA isolated from all cultured human tumor cell lines we have thus far examined, including myeloma, colon carcinoma, and ovarian carcinoma lines (data not shown).

The results of the present studies are similar in many respects to those recently reported by Godwin *et al.* (19). These investigators reported that very high levels of resistance to cisplatin in human ovarian carcinoma cells expressing increased GSH levels were associated with the enhanced expression of GCS mRNA. Northern analysis identified two GCS transcripts comparable in size to those we detected in our cell lines. While our results are consistent with theirs, the two studies differ in many notable respects. First, Godwin *et al.* report that high GCS mRNA levels and protein levels were only detected in cell lines expressing extremely high levels of platinum resistance (>~300-fold) and/or GSH content (>20-fold); relative GCS enzyme activities for the various cell lines examined were not reported by these investigators. In contrast, elevations in enzyme activity and message levels were detected even in the DU-145/M6 line, which was only 3-fold more resistant to L-PAM than the parent cell line, while an estimated 50-fold increase in GCS mRNA levels was detected in the /M4.5 cells, approximately 30-fold more resistant than the DU-145 cells. These lower levels of resistance, particularly of the DU-145/M6 and /M5 lines, are likely to be considerably more consistent with levels detected in clinical settings and therefore suggest that up-regulation of GCS activity may be an important mechanism involved in the development of clinical resistance to alkylating agents. As a final distinction between the two studies, elevations in the prevalence of GGT mRNA were found in cells expressing relatively low levels of platinum resistance, whereas no differences were observed in this study.

Neither GSH level nor GCS activity alone was representative of the degree of L-PAM resistance. This is apparent in a comparison of the /M5 and /M6 cells which had comparable GSH and GCS activities despite a consistent difference in L-PAM sensitivity, which was particularly evident by clonogenic assay (Fig. 2). Interestingly, the GCS mRNA levels (Fig. 2) were elevated in the more resistant /M5 cells. In fact, rank-ordering of the cells on the basis of the amount of mRNA detected by Northern blotting was most representative of their relative L-PAM sensitivities. The apparent discrepancies between relative GSH levels, GCS activities, and mRNA levels suggest that posttranscriptional and/or posttranslational events may also serve regulatory functions in GSH synthesis. A similar lack of correlation between message levels and the amount of enzyme detected by Western blotting was also observed by Godwin *et al.* What then is the appropriate parameter or parameters to evaluate when examining the relationship between GSH and drug resistance? Moore *et al.* (20) recently hypothesized that GSH content per se may be less indicative of the resistance level than overall GSH synthetic capacity. These investigators found that the radioresistance of *Escherichia coli* transformed with genes encoding the two bacterial enzymes responsible for GSH synthesis was correlated with their improved ability to synthesize GSH rather than the resultant elevation in GSH itself. The results of the current study and that conducted by Godwin *et al.* are both consistent with this concept and emphasize the need to determine the cellular capacity for GSH synthesis as well as GSH content in studies examining the role of GSH in protection or drug resistance.

Additional studies are required to definitively establish a role for increased GCS mRNA levels and enzyme activity in GSH-related drug resistance. To this end, experiments in which the full-length complementary DNA encoding human GCS has been introduced into mammalian cells are currently in progress. Nevertheless, the current studies clearly indicate that increases in GCS activity accompanied by changes in steady-state mRNA levels can be induced by exposure to L-PAM. In addition to

providing mechanistic information, the present studies may have practical implications for the use of GSH parameters as correlates of clinical prognosis, suggesting the need to assess factors other than static GSH content in assessing possible clinical prognosis.

References

1. Arrick, B. A., and Nathan, C. F. Glutathione metabolism as a determinant of therapeutic efficacy: a review. Cancer Res., *44:* 4224–4232, 1984.
2. Wolf, C. R., Lewis, A. D., Carmichael, J., Adams, D. J., Allan, S. G., and Ansell, D. J. The role of glutathione in determining the response of normal and tumour cells to anticancer drugs. Biochem. Soc. Trans., *15:* 728–730, 1987.
3. Lewis, A. D., Hayes, J. D., and Wolf, C. R. Glutathione and glutathione-dependent enzymes in ovarian adenocarcinoma cell lines derived from a patient before and after the onset of drug resistance: intrinsic differences and cell cycle effects. Carcinogenesis (Lond.), *9:* 1283–1287, 1988.
4. Griffith, O. W. Mechanism of action, metabolism, and toxicity of buthionine sulfoximine and its higher homologs, potent inhibitors of glutathione synthesis. J. Biol. Chem., *257:* 13704–13712, 1982.
5. Ozols, R. F., Louie, K. G., and Plowman, J. Enhanced melphalan cytotoxicity in human ovarian cancer *in vitro* and in tumor-bearing nude mice by buthionine sulfoximine depletion of glutathione. Biochem. Pharmacol., *36:* 147–153, 1987.
6. Ono, K., Komuro, C., Tsutsui, K., Shibamoto, Y., Nishidai, T., Takahashi, M., Abe, M., and Shrieve, D. C. Combined effect of buthionine sulfoximine and cyclophosphamide upon murine tumours and bone marrow. Br. J. Cancer, *54:* 749–754, 1986.
7. O'Leary, M., Hickson, K., Mulcahy, R. T., and Wilding, G. Changes in glutathione (GSH)/glutathione-*S*-transferase (GST) detoxification system in melphalan (M) resistant human prostate cancer cells. Proc. Am. Assoc. Cancer Res., *31:* 375, 1990.
8. Meister, A. Metabolism and function of glutathione. *In:* D. Dolphin, R. Poulson, and O. Avramovic (eds.), Coenzymes and Cofactors. Glutathione: Chemical, Biochemical and Medical Aspects, pp. 367–474. New York: John Wiley and Sons, 1989.
9. Tietze, F. Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione. Application to mammalian blood and other tissues. Anal. Biochem., *27:* 502–522, 1969.
10. Bump, E. A., Taylor, Y. C., and Brown, J. M. Role of glutathione in the hypoxic cell cytotoxicity of misonidazole. Cancer Res., *43:* 997–1002, 1983.
11. Nardi, G., Cipollaro, M., and Loguercio, C. Assay of γ-glutamylcysteine synthetase and glutathione synthetase in erythrocytes by high-performance liquid chromatography with fluorimetric detection. J. Chromatogr., *530:* 122–128, 1990.
12. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., *72:* 248–254, 1976.
13. Chomczynski, P., and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., *162:* 156–159, 1987.
14. Gipp, J. J., Chang, C., and Mulcahy, R. T. Cloning and nucleotide sequence of a full-length cDNA for human liver γ-glutamylcysteine synthetase. Biochem. Biophys. Res. Commun., *185:* 29–35, 1992.
15. Goodspeed, D. C., Dunn, T. J., Miller, C. D., and Pitot, H. C. Human γ-glutamyl transpeptidase cDNA: comparison of hepatoma and kidney mRNA in the human and rat. Gene (Amst.), *76:* 1–9, 1989.
16. Chung, A-S., and Maines, M. D. Effect of selenium on glutathione metabolism. Induction of γ-glutamylcysteine synthetase and glutathione reductase in the rat liver. Biochem. Pharmacol., *30:* 3217–3223, 1981.
17. Ohno, K., and Hirato, M. Induction of γ-glutamylcysteine synthetase by prostaglandin A2 in L-1210 cells. Biochem. Biophys. Res. Commun., *168:* 551–557, 1990.
18. Ahmad, S., Okine, L., Wood, R., Aljian, J., and Vistica, D. T. γ-Glutamyl transpeptidase (γ-GT) and maintenance of thiol pools in tumor cells resistant to alkylating agents. J. Cell. Physiol., *131:* 240–246, 1987.
19. Godwin, A. K., Meister, A., O'Dwyer, P. J., Huang, C. S., Hamilton, T. C., and Anderson, M. E. High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. Proc. Natl. Acad. Sci. USA, *89:* 3070–3074, 1992.
20. Moore, W. R., Anderson, M. E., Meister, A., Murata, K., and Kimura, A. Increased capacity for glutathione synthesis enhances resistance to radiation in *Escherichia coli*: a possible model for mammalian cell protection. Proc. Natl. Acad. Sci. USA, *86:* 1461–1464, 1989.