Cancer Chemother Pharmacol (1994) 34: 67–71

Cancer Chemotherapy and Pharmacology

Up-regulation of γ-glutamylcysteine synthetase activity in melphalan-resistant human multiple myeloma cells expressing increased glutathione levels

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Received: 15 October 1993 / Accepted: 30 December 1993

Abstract. Levels of intracellular glutathione (GSH) and the GSH-related enzymes γ-glutamylcysteine synthetase (γ-GCS) and γ-glutamyltranspeptidase (γ-GT) were measured in the melphalan-resistant human multiple myeloma cell line 8226/LR-5 and were compared to those measured in the drug-sensitive 8226/S and doxorubicin-resistant 8226/Dox40 cell lines. Both GSH and γ-GCS activity, the rate-limiting step in the de novo synthesis of GSH, were elevated by a factor of approximately 2 in the melphalan-resistant 8226/LR-5 cells relative to the other two lines. γ-GT activity was not elevated significantly in the /LR-5 cells. Northern analysis with a probe specific for the large subunit of human liver γ-GCS identified two bands (3.2 and 4.0 kb), both of which were increased by a factor of 2–3 in the 8226/LR-5 line. Levels of γ-GCS mRNA expression were comparable in the /S and /Dox40 cell lines. Levels of γ-GT mRNA were similar in the /S and /LR-5 lines but were reduced in the /Dox40 cells. These data suggest that the increased GSH levels associated with resistance to melphalan in the 8226/LR-5 myeloma cells is attributable to up-regulation of γ-GCS. This observation is consistent with recent demonstrations of up-regulation of γ-GCS in melphalan-resistant prostate carcinoma cells and cisplatinum-resistant ovarian carcinoma cells, suggesting that increased expression of γ-GCS may be an important mediator of GSH-associated resistance mechanisms.

Introduction

Glutathione (γ-glutamylcysteinyl glycine; GSH) is a ubiquitous tripeptide involved in multiple aspects of cellular metabolism and functions as an important protective species against oxidative injury and in the detoxification of xenobiotics [19]. This thiol, which comprises greater than 90% of the cellular nonprotein sulfhydryls, has been found to be elevated in a number of drug-resistant tumor cell lines and in tumor cells isolated from patients whose tumors are clinically resistant to drug therapy [18, 25, 27]. The protective properties of GSH and its frequent elevation in association with drug resistance in preclinical and clinical settings have implicated GSH as an important mediator of the drug-resistance phenotype. This possibility is made even more compelling by experiments demonstrating that reduction of intracellular GSH levels can sensitize cells to many chemotherapeutic drugs, including alkylating agents, platinum analogs, and anthracyclines [16, 17, 24].

Although data documenting GSH elevations in refractory tumor cells are common, comparatively little information about the mechanism(s) responsible for the elevations is available. We recently reported that resistance to the alkylating agent melphalan (L-PAM) was associated with an increase in GSH levels and an up-regulation of γ-glutamylcysteine synthetase (γ-GCS) activity, the rate-limiting enzyme in the de novo synthesis of GSH [19], in a series of DU-145 prostate-carcinoma cell lines selected for resistance to L-PAM [3]. Removal of selective pressure resulted in a loss of the drug-resistant phenotype accompanied by a return of GSH and γ-GCS levels to those characteristic of the drug-sensitive parental cell line, implicating up-regulation of γ-GCS in the elevation of GSH and, consequently, resistance to the alkylator. Godwin et al. [12] also documented an up-regulation of γ-GCS mRNA levels in cisplatin-resistant ovarian carcinoma cells expressing elevated GSH levels. Although both studies documented changes in expression of γ-GCS, they differed in that up-regulation of γ-GCS was observed only at very high increases in GSH levels in the ovarian lines, whereas this change occurred early in the progression of the resistant phenotype in the prostate lines and at much more modest GSH elevations (2–3-fold).

To evaluate further the role of γ-GCS up-regulation in the expression of GSH-associated drug resistance and to determine whether changes in this key regulatory enzyme

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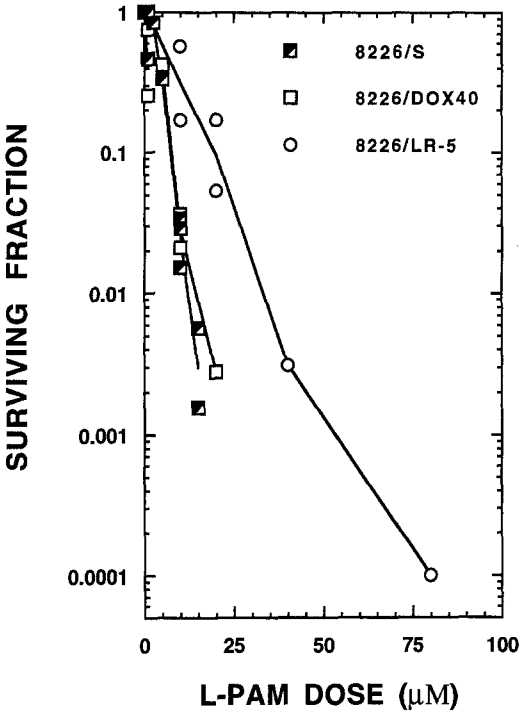

Fig. 1. Dose-response curves generated for 8226/S (◩), 8226/LR-5 (○), and 8226/Dox40 (□) human myeloma cell lines following 1 h exposure to L-PAM at 37° C. Individual data points are shown. *Solid lines* represent the mean response of 2–3 determinations

occur in association with GSH elevations typical of those most commonly detected in resistance models (i.e., 2- to 3-fold resistance), we compared the GSH-related enzyme activities and mRNA levels observed in the L-PAM-resistant 8226/LR-5 human multiple myeloma cell line [6] with those measured in the drug-responsive 8226/S parent cells and another 8226 line, 8226/Dox40, which was selected for resistance to doxorubicin [5]. As was observed in the case of the DU-145 prostate carcinoma cell lines, both GSH and γ-GCS levels were significantly elevated in the L-PAM resistant myeloma cells.

Materials and methods

Cell lines. Parent RPMI 8226/S, L-PAM-resistant 8226/LR-5, and doxorubicin-resistant 8226/Dox40 human multiple myeloma cell lines were obtained from Drs. W. T. Bellamy and W. S. Dalton, Arizona Cancer Center, Tucson, Arizona. Cells were maintained in suspension culture in RPMI 1640 medium supplemented with 10% fetal calf serum, glutamine (1%, v/v), and gentamycin (50 μg/ml). Cultures were incubated at 37° C in a humidified atmosphere consisting of 3% CO_2/97% air. Cultures were fed twice weekly and subcultured at 7-day intervals. The 8226/LR-5 cells were incubated with 5.0×10^{-6} *M* L-PAM at each feeding and subculture. Doxorubicin (4.0×10^{-7} *M*) was added to cultures of the 8226/Dox40 cells once a month. Exponentially growing cultures were used for all studies.

Cell survival following exposure to L-PAM was determined by soft-agar clonogenic assay. Exponentially growing cultures were exposed to L-PAM for 1 h at 37° C and then washed in drug-free medium, and cells were counted. Appropriate cell dilutions were then aliquotted into soft-agar (0.3% Noble agar in complete RPMI 1640 medium) containing 10^4 lethally irradiated (70 Gy) cells/ml. Addition of the lethally irradiated cells was shown to improve plating efficiency significantly (Mulcahy, unpublished data). Following incubation for 3 weeks, colonies consisting of more than 50 cells were enumerated. The surviving fraction was calculated as the ratio of plating efficiencies for treated versus control groups.

GSH, γ-GCS, and γ-glutamyltranspeptidase activities. Total GSH content was determined using the technique described by Tietze [26] using modifications reported by Bump et al. [8]. γ-GCS activity was quantitated using a modification of the high-performance liquid chromatographic (HPLC) technique of Nardi et al. [21] as described elsewhere [3]. Briefly, the technique measures the rate of synthesis of γ-glutamylcysteine following conjugation of the product with monobromobimane, separation on a Beckman Ultrasphere ODS column (5 μm, 4.6 × 250 mm) with an isocratic mobile phase consisting of methanol:water (14:86) containing 0.25% acetic acid (pH 3.6), and fluorescence detection at excitation and emission wavelengths of 370 and 485 nm, respectively. γ-Glutamyltranspeptidase (γ-GT) activity was measured on the particulate fraction according to the technique described by Meister et al. [20]. GSH content and enzyme activities were normalized on the basis of the protein content determined by the Bradford technique [7] utilizing bovine serum albumin as a standard.

Northern blotting. Total RNA was isolated from exponentially growing cells by the guanidinium isothiocyanate-phenol method of Chomczynski and Sacchi [9]. RNA samples were size-fractionated by formaldehyde/agarose gel electrophoresis and transferred to Gene-Screen Plus membranes. The membranes were hybridized at 42° C with a ^{32}P-labeled probe corresponding to the 764-bp *Pst*I fragment of human GCS cDNA [11]. Membranes were similarly hybridized with probes for β-actin and human γ-GT. The latter probe was a 596-bp fragment isolated following digestion of the plasmid phGGT with *Pvu*II and *Bgl*II. phGGT was generously provided by Drs. D. C. Goodspeed and H. C. Pitot [13] of the McArdle Laboratory (Madison, Wis.). Labeled membranes were visualized using the PhosphorImager system (Molecular Dynamics). Relative levels of mRNA were determined utilizing PhosphorImager software after normalization to β-actin expression.

Results

As shown in Fig. 1, the 8226/LR-5 myeloma cells were more resistant to L-PAM exposure than were either the wild-type 8226/S cells or the doxorubicin-resistant 8226/Dox40 line following the exposure of exponentially

Table 1. Biochemical characteristics of RPMI 8226 human multiple myeloma cell lines

Cell line	GSH (nmol/mg)	GCS (nmol min^{-1} mg^{-1})	γ-GT (nmol min^{-1} mg^{-1})	GCS mRNA (*x*-fold expression)[a]
8226/S	64.6± 8.4	1.12±0.06	99.4±14.6	–
8226/LR-5	132.3±17.6*	2.77±0.64*	125.2± 8.2	2–3
8226/Dox40	64.9± 6.6	1.38±0.26	92.3± 5.0	1

Data shown for GSH, GCS, and GT represent mean values ± SD
[a] relative to 8226/s
* $P < 0.05$

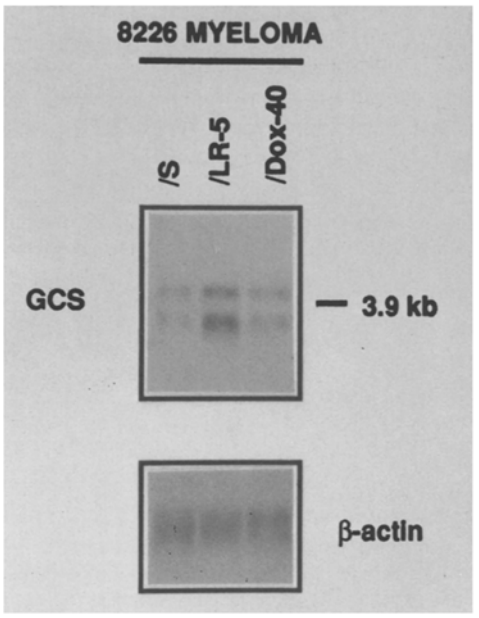

Fig. 2. Northern blot of total RNA isolated from 8226 cells and hybridized with a ^{32}P-labeled γ-GCS-specific probe (for specific details pertaining to probes, see Materials and methods). The *lower panel* represents the membrane rehybridized with a probe specific for β-actin following removal of the γ-GCS probe by boiling in distilled water

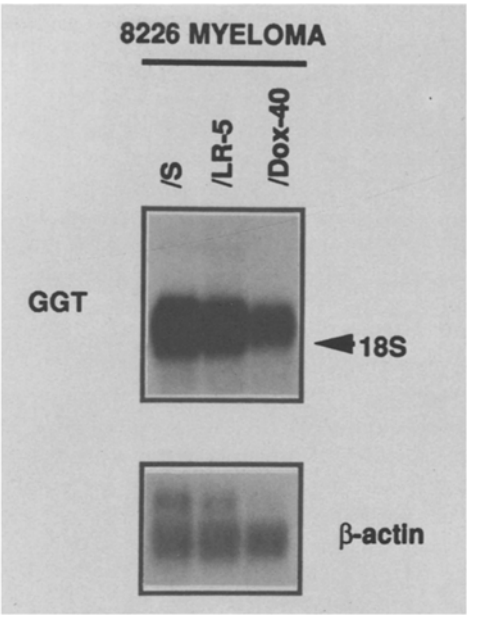

Fig. 3. Total RNA from each cell line was hybridized with a ^{32}P-labeled probe specific for γ-GT. For the blot shown in the *lower panel*, the membrane was prepared as described in Fig. 2

growing cells to L-PAM for 1 h. Using a ratio-of-slopes method to compare sensitivity, we found that the /LR5 cells were 3 times more resistant than the 8226/S cells; this observation is in good agreement with previously reported data for these cells.

The GSH level was elevated by a factor of approximately 2 in the 8226/LR-5 line maintained in our laboratory as compared with the 8226/S parent line (Table 1). This significant ($P < 0.05$) increase in GSH content was accompanied by a significant ($P < 0.005$) increase in γ-GCS enzyme activity. The ~2.5-fold increase in enzyme activity was comparable with the difference between the GSH levels measured in the two cell lines. In contrast to the L-PAM-resistant line, no difference in GSH or γ-GCS activity was detected between the doxorubicin-resistant 8226/Dox40 cells and the sensitive parent cells. The levels of γ-GT, an enzyme that can function as a salvage pathway for GSH synthesis [15] and has been implicated in resistance to L-PAM [1, 2], were also compared among the three cell lines. However, no significant difference in γ-GT activity was detected among the three cell lines.

Total RNA isolated from each of the three cell lines was subjected to Northern analysis using probes for both human γ-GCS and human γ-GT. As shown in Fig. 2, the γ-GCS probe hybridized to two RNA species, approximately 3.2 and 4.0 kb in length, respectively, in each of the cell lines. Both RNA bands were expressed at levels ~2–3 times higher in the /LR-5 cells than in either the /S parent cells or the /Dox40 cells, both of which expressed the RNAs at similar levels. RNA hybridizing with the γ-GT probe was abundant in all three 8226 cell lines (Fig. 3) γ-GT mRNA levels were comparable in the parent and L-PAM-resistant lines but were reduced in the doxorubicin-resistant cells.

Discussion

In their original characterization of the 8226/LR-5 cell line, Bellamy et al. [6] reported a 1.7-fold increase in GSH content relative to 8226/S cells and implicated this elevation in the mechanism of L-PAM resistance. As has been the case for the majority of cell lines in which increased GSH levels have been implicated in drug-resistant phenotypes, mechanisms contributing to an increase in the steady-state levels of GSH in 8226/LR-5 cells have not been identified. Intracellular GSH is synthesized from its constituent amino acids via sequential adenosine triphosphate (ATP)-consuming reactions catalyzed by γ-GCS and GSH synthase, respectively. The first step in the de novo synthesis of GSH, catalyzed by γ-GCS, is the rate-limiting step and also the site of negative feedback inhibition mediated by GSH [19]. Recent evidence indicates that up-regulation of this key regulatory enzyme may be involved in GSH elevations evoked by certain toxic insults [10, 23] and also in the expression of GSH-associated innate and/or acquired resistance to alkylating agents [3] and cisplatin [12]. To evaluate further the relevance of γ-GCS up-regulation in the response to chemotherapy, we compared γ-GCS activity and mRNA levels in 8226/LR-5 cells and their drug-sensitive counterparts.

As was recently observed for cisplatin-resistant ovarian carcinoma cell lines [12] and L-PAM-resistant prostate carcinoma cells [3], the increase in GSH levels measured in 8226/LR-5 cells was associated with an increase in γ-GCS activity and γ-GCS-specific mRNA levels. However, in the case of the 8226/LR-5 cells, the magnitude of the increase in GSH, γ-GCS-specific mRNA levels, and γ-GCS enzyme activity were comparable, in contrast to the previous studies in which these parameters were not well correlated. In these latter cases, other changes, including posttranslational

modifications, were proposed to account for discrepancies between the parameters. The doxorubicin-resistant 8226/Dox40 cells developed by Bellamy et al. [5] by selection in progressively increasing concentrations of drug were also reported to express elevated GSH levels. However, the line used in our investigation did not express this phenotype. The GSH levels and γ-GCS activity measured in the 8226/Dox40 cells were not significantly different from those typical of the parent myeloma cells. The L-PAM dose-response curve generated for these cells was also similar to that constructed for 8226/S. The data presented for the L-PAM-resistant 8226/LR-5 cells, however, support the hypothesis that induction of γ-GCS activity may represent an important component of drug-resistant phenotypes characterized by elevations in intracellular GSH levels. Furthermore, confirmation of this phenotypic change in another resistant cell line suggests that it may represent a fairly common component of GSH-associated drug resistance and may not necessarily be restricted to a single histological tumor type.

Regulation of intracellular GSH levels is admittedly complex, and it is conceivable that other factors, acting alone or in concert with changes in levels of γ-GCS, might contribute to the establishment of higher steady-state GSH levels in drug-resistant cells. One potential candidate is γ-GT, a membrane-bound enzyme that can function as a salvage pathway for GSH synthesis by virtue of its ability to provide precursors for the GSH synthetic pool following hydrolysis of γ-glutamyl-containing compounds, including extracellular GSH [15]. In addition to providing a source of cysteine, γ-GT can provide γ-glutamylcysteine, the substrate for GSH synthase, thereby effectively circumventing feedback inhibition of γ-GCS by GSH. Elevations in γ-GT activity have long been implicated in increased GSH levels in tumor cells resistant to certain chemotherapeutic agents, including L-PAM.

Ahmad et al. [1] reported that the elevated content and increased Vmax of γ-GT contributed to the maintenance of increased GSH pools in L-PAM-resistant L1210 cells by providing cysteine for γ-GCS, which was also found to be elevated in the resistant population. Godwin et al. [12] documented an increase in γ-GT mRNA levels in cisplatin-resistant ovarian carcinoma cells with elevated levels of GSH and suggested that up-regulation of this enzyme precedes up-regulation of γ-GCS in the evolution of the drug-resistant phenotype. Lewis et al. [18] found elevated GSH levels and increased γ-GT activity but no detectable change in γ-GCS levels in a population of resistant cells isolated from lung tumors following the development of clinical resistance. In light of this precedence, the role of γ-GT in the L-PAM resistance of the 8226/LR-5 cells was examined by comparing the enzyme activity and mRNA levels detected in these cells with those measured in the 8226/S parent cells. In contrast to the findings described above, however, there was no evidence of increased γ-GT levels or mRNA levels in the resistant myeloma cells.

Evidence of up-regulation of the GSH synthetic enzyme, γ-GCS, in another resistant cell line expressing elevated GSH levels further emphasizes the role this key regulatory enzyme may play in the expression of this relatively common resistance phenotype. Although the data available do not rule out the possible involvement of other factors, they do establish γ-GCS as a potential target for therapeutic interventions designed to circumvent GSH-associated drug resistance. To this end, phase I clinical trials of buthionine sulfoximine (BSO), a specific inhibitor of γ-GCS [14] have been initiated [4, 22].

Acknowledgements. We thank Mary Beth Wartman for excellent technical assistance and the National Institutes of Health for grant CA57549.

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