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SHORT COMMUNICATIONS

Reversal of melphalan resistance *in vivo* and *in vitro* by modulation of glutathione metabolism

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Alterations in drug uptake and efflux [1], intracellular glutathione (GSH) [2], metallothionein levels [3], activity of drug-metabolizing enzymes [4], and efficiency of DNA repair mechanisms [5] have been proposed to contribute towards melphalan and other alkylating drug resistance. GSH because of its highly nucleophilic nature forms conjugation products with melphalan by a reaction catalyzed by glutathione *S*-transferases (GSTs) [6]. Tumor cells resistant to alkylating agents may exhibit elevated GSH and/or GST levels [2, 7–10]. GSH depletion by a variety of mechanisms has been shown to enhance the cytotoxicity of melphalan in drug-sensitive and -resistant cells *in vitro* [11, 12], suggesting that the inherent cellular GSH level is an important determinant of therapeutic response. However, there are very few reports of reversing alkylating drug resistance by GSH depletion *in vivo* [13, 14].

We have reported recently the development and characterization of melphalan resistance, Mel^R, in the human plasma cell line, HS-Sultan [10]. Tumors formed in nude mice by this Mel^R cell line are resistant to the effects of melphalan *in vivo*. Melphalan resistance in these cells was initially associated with elevated GST activity of the π type without concomitant changes in GSH levels. In this report we have studied the effect of GSH depletion by buthionine sulfoximine (BSO), an inhibitor of γ-glutamyl cysteine synthetase [15], in reversing melphalan resistance *in vitro* and *in vivo*.

Methods

Melphalan-sensitive (Mel^S), -resistant (Mel^R) and -revertant (Mel^{Rev}) HS-Sultan human myeloma cells were maintained in culture and as xenografts in nude mice as described previously [10].

Groups of five mice, each implanted with Mel^R tumors bilaterally treated with and withut BSO, were either killed to determine GSH levels, or served as controls, or received a single dose of melphalan. L-BSO was administered in four i.p. doses of 2.5 mmol/kg 12 hr apart along with a 20 mM oral solution of L-BSO in drinking water during a 36-hr period. Six hours after the final injection of BSO, melphalan treatment groups received a single i.p. dose of 10 mg/kg melphalan. Tumor growth was measured and data were plotted as the mean relative tumor volume (RTV) after the start of melphalan treatment as described previously [19].

Mel^R cells pretreated with 0, 10 or 50 μM D,L-BSO at 5×10^5 cells/mL × 24 hr were incubated with 2 μg/mL melphalan for 1 hr at 37°. After washing the drug out, trypan blue excluding cells were counted for measurement of cell survival after 7 days of incubation. We have determined previously for these cells that cell survival using this method is equivalent to the colony forming assay. GSH, GST and protein estimations on tissue and cell extracts were performed according to the methods of Beutler [16], Habig *et al.* [17], and Bradford [18], respectively. One unit of GST activity is defined as the amount required to catalyze the conjugation of 1 nanomole of GSH to the electrophilic substrate per minute at 25°. Western blotting using antibodies against GST π was performed as described previously [10].

Results and Discussion

Table 1 indicates that, as reported previously [10], Mel^R tumors exhibited 1.8-fold higher GST activity towards both 1-chloro-2,4-dinitrobenzene (CDNB) and ethacrynic acid, compared to the Mel^S tumors. The Mel^{Rev} tumors had intermediate values. Western blot analysis (data not shown) of cytosolic protein from Mel^S, Mel^R and Mel^{Rev} cells using antibodies against GST π was consistent with data in Table 1. With passage of time and repeated treatment with melphalan to maintain drug resistance, the GSH content of Mel^R cells was noted to be significantly higher than that

Table 1. GSH levels and GST activities in Mel^S, Mel^R and Mel^{Rev} cells and tumors

	GSH content (nmol/mg protein)		GST activity (nmol/mg protein)	
	Cells	Tumors	CDNB	Ethacrynic acid
Mel^S	62.23 ± 10.51 (5)	20.20 ± 14.00 (3)	95.90 ± 14.00 (3)	31.25 ± 1.00 (3)
Mel^{Rev}	83.13 ± 4.16 (5)		141.90 ± 6.00 (3)	44.5 ± 0.40 (3)
Mel^R	100.03 ± 5.50 (5)	28.42 ± 8.42 (4)	180.8 ± 1.00 (3)	57.0 ± 3.00 (3)

Values are means ± SD; the number of determinations is given in parentheses. Statistics were performed using Student's one-tailed *t*-test. For GSH levels in cells, the differences between Mel^S vs Mel^{Rev}, Mel^S vs Mel^R, and Mel^{Rev} vs Mel^R were significant at $P < 0.0025$, $P < 0.00005$ and $P < 0.005$, respectively. For GSH levels of tumors, the differences were not significant, $P = 0.1$. The P values of the GST activity with respect to CDNB between Mel^S vs Mel^{Rev}, Mel^S vs Mel^R, and Mel^{Rev} vs Mel^R were $P < 0.025$, $P < 0.01$ and $P < 0.01$, respectively. For ethacrynic acid, all P values were significant at $P < 0.005$.

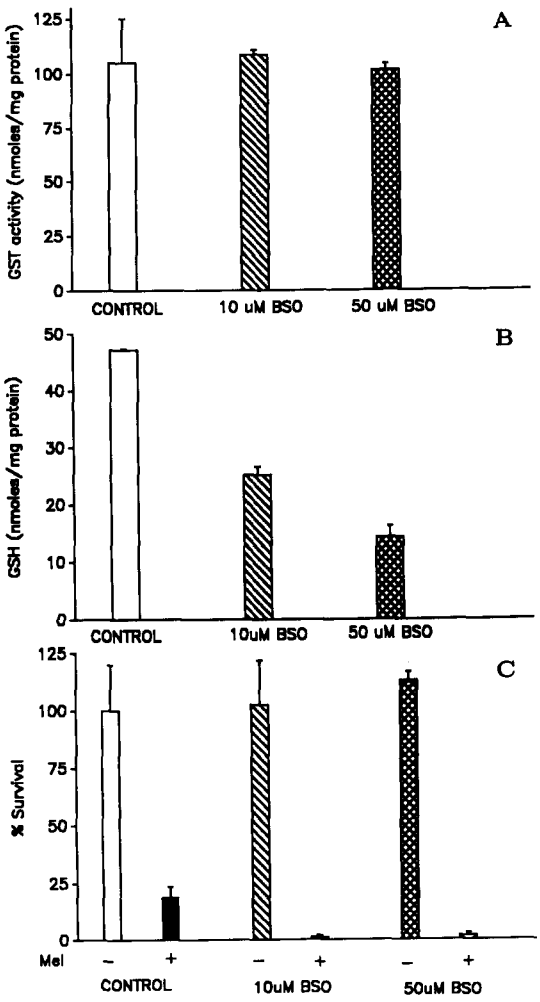

Fig. 1. Effect of BSO on GST activity, GSH levels and melphalan cytotoxicity *in vitro*. Cytosolic extracts of control and BSO treated Mel^R cells were used for measurement of GST activity towards CDNB (panel A) and GSH levels (panel B). Values are means ± SD, N = 3. The decrease in GSH was significant at $P < 0.005$ using Student's *t*-test for both 10 and 50 μM BSO treated compared to control cells and $P < 0.025$ between 10 and 50 μM BSO. The level of cell killing between melphalan alone (2 μg/mL) and melphalan + BSO was significant, $P < 0.0001$ (panel C).

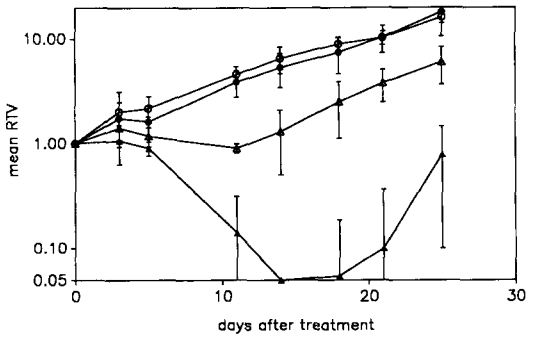

Fig. 2. Effect of BSO treatment on melphalan-induced tumor regression *in vivo*. Mean relative tumor volume (RTV) as a function of days after melphalan treatment is shown in control (○), BSO treated (●), melphalan treated (△), and BSO + melphalan treated (▲) mice. L-BSO was administered in four i.p. doses of 2.5 mmol/kg 12 hr apart along with a 20 mM oral solution of L-BSO in drinking water during a 36-hr period. Six hours after the final injection of BSO, melphalan treatment groups received a single i.p. dose of 10 mg/kg melphalan. Values are means ± SD, N = 8–10 tumors. The differences at day 11 between control mice and melphalan only and melphalan + BSO treated mice were statistically significant at $P < 0.005$ using Student's *t*-test. In addition, the differences between melphalan and melphalan + BSO treated mice were significant at $P < 0.005$.

of Mel^S cells (Table 1). The Mel^{Rev} cells exhibited intermediate values. However, the GSH content between Mel^S and Mel^R tumors (Table 1) showed much variability and the differences were not significant. In addition, the GSH content of the tumor cells was lower than that of the cells. These differences may be due to tumor cell heterogeneity *in vivo*. The Mel^R cells *in vitro* exhibited a concentration-dependent depletion of GSH in response to BSO (Fig 1B), with a 50% depletion in cells treated with 10 μM BSO and a 75% depletion in cells treated with 50 μM BSO. BSO treatment alone of Mel^R cells had no significant effect on cell survival (Fig. 1C) or GST activity (Fig. 1A).

The survival of Mel^R cells treated with 2 μg/mL melphalan varied between 20 and 40% in different experiments (Fig. 1C) and was lower than the previously reported value of 80% [10]. This is related to the instability of melphalan resistance requiring periodic melphalan treatment *in vivo* and *in vitro*. Mel^R cells that were pretreated with 10 or 50 μM BSO showed only 2% survival in the presence of melphalan (Fig. 1C). There was no increase in the level of cell killing by melphalan between 10 and 50 μM BSO treated cells even though 50 μM BSO treated cells exhibited a greater reduction of GSH levels suggesting a potential limitation of the effects of GSH depletion.

L-BSO pretreatment of nude mice bearing Mel^R tumors resulted in a 63 and 72% depletion of GSH in the liver and Mel^R tumors, respectively. GSH-depleted Mel^R tumors showed a rapid and almost complete regression by day 15 to melphalan in comparison to tumors whose GSH was not depleted (Fig. 2). This tumor regression was followed by a rapid relapse, suggesting incomplete sensitization to melphalan by GSH depletion in all tumor cells, or existence of a subpopulation of cells with a GSH/GST-independent mechanism of melphalan resistance.

These and other studies from the literature demonstrate that both GSH and GST levels may be important in the modulation of melphalan cytotoxicity [8–14]. Our earlier studies using the HS-Sultan myeloma cells have shown that the development of melphalan resistance in these cells is associated initially with the induction of a π class GST isoenzyme [10]. In the present report, we have demonstrated that Mel^R cells which have reverted to a melphalan-sensitive state, Mel^{Rev}, are associated with a decrease in the levels of the induced GST π to levels between those in resistant and sensitive cells (Table 1). This supports our hypothesis that the increased GST π levels may contribute towards Mel^R in these cells by increasing the efficiency of its

conjugation to GSH [6]. However, as discussed below, it is difficult to separate the effects of GST from GSH since the two processes are intimately linked in drug detoxification.

During the course of the present studies we reinvestigated the levels of GSH in Mel^S, Mel^R, and Mel^{Rev} cells and tumors (Table 1). In contrast to our earlier observations [10], a significant increase in GSH levels was observed in Mel^R cells *in vitro*, with Mel^{Rev} cells exhibiting intermediate values. The elevated GSH values may be in response to the periodic treatment with melphalan which was necessary to maintain drug resistance *in vitro*, suggesting that alterations in GSH/GST in response to drugs is a dynamic process. However, the increase in GSH levels was not associated with higher levels of Mel^R than previously observed by us [10].

In conclusion, GSH depletion by a variety of mechanisms has been shown to enhance the cytotoxicity of melphalan in drug-sensitive and -resistant cells *in vitro* [11–14], suggesting that inherent cellular GSH levels are an important determinant of therapeutic response. The present data indicate that it is possible to overcome melphalan resistance in human myeloma cells by GSH depletion associated with elevated GST π acivity and GSH levels. This may indicate that the unavailability of GSH for enzymatic/non-enzymatic conjugation of melphalan renders the cell incapable of efficiently detoxifying melphalan. Although experimental evidence indicates that GSH depletion does not result in enhanced melphalan host toxicity in mice [13], the utility of such approaches should be determined by clinical trials. We believe our data support the initiation of clinical trials to overcome melphalan resistance by manipulating GSH metabolism in patients with multiple myeloma as well as other malignancies.

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REFERENCES

1. Redwood WR and Colvin M, Transport of melphalan by sensitive and resistant L-1210 cells. *Cancer Res* **40**: 1144–1149, 1980.
2. Calcutt G and Connors TA, Tumour sulphydryl levels and sensitivity to the nitrogen mustard merophan. *Biochem Pharmacol* **12**: 839–845, 1963.
3. Kelley SL, Basu A, Teicher BA, Hacker MP, Hamer DH and Lazo JS, Overexpression of metallothionein confers resistance to anticancer drugs. *Science* **241**: 1813–1815, 1988.
4. Colvin M, Russo JE, Milton J, Dulik DM and Fenselau D, Enzymatic mechanisms of resistance to alkylating agents in tumor and normal tissues. *Adv Enzyme Regul* **27**: 211–221, 1988.
5. Robson CN, Lewis AD, Wolf CR, Hayes JD, Hall A, Proctor SJ, Harris AL and Hickson ID, Reduced levels of drug-induced DNA cross-linking in nitrogen mustard resistant Chinese hamster ovary cells expressing elevated glutathione *S*-transferase activity. *Cancer Res* **47**: 6022–6027, 1987.
6. Dulik DM, Fenselave C and Hilton J, Characterization of melphalan–glutathione adducts whose formation is catalyzed by glutathione transferase. *Biochem Pharmacol* **35**: 3405–3409, 1986.
7. Ahmad S, Okine L, Le B, Najarian P and Vistica DT, Elevation of glutathione in phenylalanine mustard resistant murine L-1210 leukemia cells. *J Biol Chem* **262**: 15048–15053, 1987.
8. Wan AL and Tew KD, Increased glutathione *S*-transferase activity in a cell line with acquired resistance to nitrogen mustards. *Cancer Treat Rep* **69**: 677–682, 1985.
9. Buller AL, Clapper ML and Tew KD, Glutathione *S*-transferase in nitrogen mustard-resistant and -sensitive cell lines. *Mol Pharmacol* **31**: 575–578, 1987.
10. Gupta V, Singh SV, Ahmad M, Medh RD and Awasthi YC, Glutathione and glutathione *S*-transferases in a human plasma cell line resistant to melphalan. *Biochem Pharmacol* **38**: 1993–2000, 1989.
11. Suzukake K, Petro BJ and Vistica DT, Reduction in glutathione content of L-PAM resistant L1210 cells confers drug sensitivity. *Biochem Pharmacol* **31**: 121–124, 1982.
12. Green JA, Vistica DT, Young RC, Hamilton TC, Rogan AM and Ozols RF, Potentiation of melphalan cytotoxicity in human ovarian cancer cell lines by glutathione depletion. *Cancer Res* **44**: 5427–5431, 1984.
13. Ozols RF, Louis KG, Plowman J, Behrens BC, Fine RL, Dykes D and Hamilton TC, 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.
14. Skapek SX, Colvin OM, Griffith OW, Elion GB, Bigner DD and Friedman HS, Enhanced melphalan cytotoxicity following buthionine sulfoximine-mediated glutathione depletion in a human medulloblastoma xenograft in athymic mice. *Cancer Res* **48**: 2764–2767, 1988.
15. Griffith OW and Meister A, Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (*S-n*-butyl homocysteine sulfoximine). *J Biol Chem* **254**: 7558–7560, 1979.
16. Beutler E, Reduced glutathione. In: *Red Cell Metabolism, a Manual for Biochemical Methods* (Ed. Beutler E), pp. 131–132. Grune & Stratton, New York, 1984.
17. Habig WH, Pabst MJ and Jakoby WB, Glutathione *S*-transferase: The first enzymatic step in mercapturic acid formation. *J Biol Chem* **249**: 7130–7139, 1974.
18. Bradford MM, 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.

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