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Effect of Glutathione Depletion on Cisplatin Resistance in Cancer Cells Transfected with the γ-Glutamylcysteine Synthetase Gene

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Overexpression of the human γ-glutamylcysteine synthetase (γ-GCS) gene resulted in cisplatin resistance with an increased glutathione (GSH) content, increased ATP-dependent glutathione *S*-conjugate export pump (GS-X pump) activity and decreased platinum accumulation in human lung cancer cells transfected with a *γ-GCS* cDNA expression vector, as we previously reported. In this study, we examined the effects of buthionine sulfoximine (BSO), a specific inhibitor of γ-GCS, to determine whether GSH depletion alters cisplatin resistance in a *γ-GCS*-transfected cell line, SBC-3/GCS. In the presence of 10 μM BSO for 4 days, SBC-3/GCS still showed resistance to cisplatin, although it was partially reversed. Under these conditions, GS-X pump activity remained up-regulated in spite of low GSH content, and the platinum content was decreased. These data suggest that the GS-X pump itself influences cisplatin resistance, as well as cellular GSH content.

Key words: Cisplatin resistance — γ-Glutamylcysteine synthetase — Glutathione — ATP-dependent glutathione *S*-conjugate export pump

The development of tumor cell resistance to cisplatin is a major therapeutic obstacle. The resistance to platinum compounds, as well as to alkylating agents, is often related to the cellular glutathione (GSH) content.[1–3] Some cisplatin-resistant cell lines have been reported to have increased levels of GSH, but normal glutathione *S*-transferase (GST) activity, and to show enhanced expression or altered regulation of the gene for γ-glutamylcysteine synthetase (γ-GCS), a rate-limiting enzyme in GSH biosynthesis.[4,5] Buthionine sulfoximine (BSO), a specific inhibitor of γ-GCS, has been demonstrated to sensitize cells to the effects of a platinum compound and alkylating agents.[2] BSO treatment also reverses resistance to some drugs, including doxorubicin and vincristine, in cells transfected with the multidrug resistance protein (MRP) gene.[6] Recently, the ATP-dependent glutathione *S*-conjugate export pump (GS-X pump) has been reported to be involved in cisplatin resistance as an efflux pump for the GS-platinum complex.[7–9] Some studies have indicated that MRP mediates drug transport in a similar manner to the GS-X pump.[10,11] We previously demonstrated that human *γ-GCS* gene overexpression produced cisplatin resistance with an increased GSH content and GS-X pump activity in human lung cancer cells transfected with a *γ-GCS* cDNA expression vector.[12] However, it remained unclear whether GS-X pump activity itself confers cisplatin resistance. In this study, therefore, we examined the effects of BSO to determine whether GSH depletion alters cisplatin resistance in a *γ-GCS*-transfected cell line.

We used SBC-3/GCS, a human small-cell lung cancer cell line transfected with the pCR3 plasmid expression vector containing *γ-GCS* heavy subunit cDNA, and SBC-3/neo, containing the plasmid vector without *γ-GCS*. Details of the materials and methods employed have been described previously.[12] To determine the total cellular GSH level, the drug sensitivity and the cellular platinum accumulation, we used an enzyme recycling assay, a tetrazolium dye (MTT) assay and atomic absorption spectrophotometry, respectively. The GS-X pump activity was determined by measuring ATP-dependent [^{3}H]leukotriene C_4 (LTC_4) uptake into membrane vesicles, including inside-out vesicles.[7,9] Differences between the data were analyzed using the unpaired, two-tailed Student's *t* test.

In comparison with SBC-3, SBC-3/GCS cells showed a 2.0-fold higher GSH content, 1.6-fold higher GS-X pump activity, a 30% decrease of intracellular platinum accumulation and 6.7-fold higher resistance to cisplatin, with an IC_{50} value of 1.60 μM. In addition, SBC-3/GCS cells had 14-fold higher resistance to BSO, with an IC_{50} value of 30.2 μM. In each experiment, the data for SBC-3/neo showed no significant difference from those for SBC-3 (Table I).

The BSO treatment reduced the GSH level in SBC-3/GCS cells in a time- and concentration-dependent manner. After 4 days of incubation of SBC-3/GCS with 1 μM BSO, the cellular GSH content was reduced to approxi-

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Table I. Effects of BSO on GSH Content, GS-X Pump Activity and Sensitivity to Cisplatin of Each Cell Line

	SBC-3/GCS			SBC-3		SBC-3/neo	
BSO sensitivity (IC_{50}, μM)	30.2±4.5[a]			2.2±0.1		2.6±0.3	
BSO concentration (μM)	0	1	10	0	1	0	1
GSH content (nmol/mg protein)	8.91±0.98[a]			4.46±0.73		4.90±0.58	
BSO treatment for 1 day		6.32±0.45[e]	2.44±0.26[c]		1.40±0.23		1.80±0.18
4 days		4.25±0.39[d]	0.61±0.12[c]		1.02±0.31		1.29±0.16
Sensitivity to cisplatin (IC_{50}, μM)	1.60±0.07[a]	1.51±0.11	1.16±0.12[d]	0.24±0.02	0.15±0.01[c]	0.27±0.03	0.18±0.03[c]
relative resistance	6.7	6.3	4.8	(1.0)	0.6	1.1	0.8
GS-X pump activity (LTC_4 uptake by membrane vesicles, pmol/mg protein/20 min)	0.22±0.01[a]	0.22±0.01	0.20±0.02	0.14±0.02	0.13±0.01	0.14±0.02	0.13±0.02
Platinum accumulation (nmol/mg protein)	0.40±0.04[b]	0.39±0.04	0.45±0.06	0.56±0.05	0.63±0.05	0.53±0.05	0.57±0.04

The drug sensitivities were evaluated after one day of preincubation followed by exposure to each drug for three days. To evaluate the effect of BSO on cisplatin sensitivity, cells were incubated with BSO for four days with exposure to cisplatin for the last three days. The IC_{50} value was defined as the drug concentration that inhibited cell growth by 50%. Relative resistance values were defined as (IC_{50} for transfected subline or BSO-treated cells/IC_{50} for SBC-3 without BSO).
The cellular GSH levels were measured after incubation with or without BSO for one day or four days.
To evaluate the GS-X pump activity, membrane vesicles were prepared from cells incubated with or without BSO for four days. The vesicles (30 μg protein) were incubated with 0.39 nM [^{3}H]LTC_4 and 5 mM ATP at 37°C for 20 min.
For measurement of the cellular platinum content, cells were preincubated with or without BSO for four days, then incubated in the presence of 0.1 mM cisplatin for three hours.
Each value is the mean±SD of three independent experiments.
a) $P<0.001$, *b*) $P<0.01$ compared with the value for SBC-3 and SBC-3/neo (unpaired, two-tailed Student's t test).
c) $P<0.001$, *d*) $P<0.005$, *e*) $P<0.05$ compared with the value without BSO treatment (unpaired, two-tailed Student's t test).

mately the levels in SBC-3 and SBC-3/neo without BSO treatment. BSO did not significantly alter the GS-X pump activity, platinum content or sensitivity to cisplatin of SBC-3/GCS cells. When SBC-3/GCS cells were treated with 10 μM BSO, the IC_{50} value for cisplatin was significantly reduced to 1.16 μM ($P<0.005$), but the cells still showed 4.8-fold higher resistance than SBC-3. Under these conditions, over 90% of GS-X pump activity remained in the SBC-3/GCS cells although the GSH content was depleted to less than 1 nmol/mg protein, and the platinum content did not change significantly. In relation to the platinum content, the GSH level in depleted cells might be sufficient for conjugation to occur. These data suggest that the GS-X pump function is maintained under the GSH-depleted condition (Table I).

We can explain the cisplatin resistance of the *γ-GCS*-transfected cell line SBC-3/GCS in terms of two mechanisms, increased intracellular cisplatin detoxification due to the high GSH level, and increased GS-platinum efflux due to elevated GS-X pump activity. These intracellular processes cause a decrease in the amount of platinum that binds to DNA. In the present study, GSH depletion with BSO for 4 days did not completely reverse the cisplatin resistance of SBC-3/GCS cells, in which GS-X pump activity remained increased and the platinum content was still decreased. Cisplatin itself did not inhibit LTC_4 uptake by the membrane vesicles (data not shown). Therefore, decreased platinum accumulation in SBC-3/GCS cells was a result of increased efflux of GS-platinum complex. We speculate that the formation of a complex of platinum with GSH might occur more efficiently owing to the increase in the GS-platinum efflux by the GS-X pump, resulting in a reduced content of non-GSH-binding platinum. We conclude that *γ-GCS* gene overexpression was responsible for the cisplatin resistance of SBC-3/GCS cells by inducing GS-X pump activity.

It is unclear how the GS-X pump activity was increased in SBC-3/GCS. In the previous report, we speculated that increased cellular GSH level up-regulated GSH conjugate formation, which might induce the GS-X pump activity. In the present study, however, we found that GSH depletion did not change GS-X pump activity. These results indicate that other pathways may exist between *γ-GCS* gene expression and GS-X pump induction, independent of GSH elevation. As one possible mechanism, we considered the role of GST-π, which catalyzes the formation of GSH conjugates. However, there was no significant difference in *GST-π* mRNA expression between SBC-3/GCS and parental cells with or without BSO (data not shown).This is consequent on the fact that GS-platinum complex can be formed without enzymatic catalysis.[7]

Finally, *γ-GCS* gene transfection did not affect *MRP* expression, at least at the mRNA level, in SBC-3/GCS as

judged from northern blot hybridization analysis (data not shown). We have no evidence as to whether *γ-GCS*-transfection influences post-transcriptional changes in mRNA and post-translational changes in MRP protein. Although there is insufficient evidence for MRP being directly involved in cisplatin resistance,[13] a recent study has indicated cisplatin-inducible *MRP* overexpression in a cisplatin-resistant cell line.[14] In addition, some homologs of MRP, such as the canalicular multispecific organic anion transporter of rat[15] and human,[16] have been reported. These transporters are thought to confer drug resistance and to be relevant to the GS-X pump. Further experiments are required to explain the relationship of MRP and its homologs with the GS-X pump activity.

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