Vol. 216, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 2, 1995 Pages 258-264

γ-GLUTAMYLCYSTEINE SYNTHETASE GENE OVEREXPRESSION RESULTS IN INCREASED ACTIVITY OF THE ATP-DEPENDENT GLUTATHIONE S-CONJUGATE EXPORT PUMP AND CISPLATIN RESISTANCE

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Received September 20, 1995

Summary: The ATP-dependent glutathione S-conjugate export pump (GS-X pump) has been suggested to play a role in the mechanism of cisplatin resistance. The purpose of this study was to determine the relationship between intracellular glutathione (GSH) levels and GS-X pump activity and whether GS-X pump overexpression results in cisplatin resistance. We transfected the human γ-glutamylcysteine synthetase (*γ-GCS*) gene into a human small-cell lung cancer cell line, SBC-3, producing SBC-3/GCS. The intracellular GSH content of SBC-3/GCS was twice that of the parental line, its GS-X pump activity was significantly enhanced and cellular cisplatin accumulation decreased. SBC-3/GCS showed higher resistance (relative resistance value of 7.4) to cisplatin than the parental line SBC-3. These data indicate that *γ-GCS* gene overexpression induces cellular cisplatin resistance associated with increases in both the GSH content and GS-X pump activity, resulting in reduced cisplatin accumulation. In conclusion, GS-X pump expression is related to cellular GSH metabolism and involved in cisplatin resistance.

Cisplatin is an effective anticancer agent because of its broad-spectrum antitumor activity. However, the development of tumor cells resistant to cisplatin is a major therapeutic problem. Recently, Ishikawa *et al.* showed that an ATP-dependent glutathione S-conjugate export pump (GS-X pump) was overexpressed in a cisplatin-resistant subline of human leukemia HL-60 cells (1). They indicated the direct formation of a complex between glutathione (GSH) and cisplatin, the GS-platinum complex, and considered that the GS-X pump acted as an efflux pump for glutathione S-conjugates, including the GS-platinum complex (2). Leukotriene C4 (LTC4) has been demonstrated to be a substrate for the GS-X pump (3), and Fujii *et al.* demonstrated increased LTC4 transport in membrane vesicles separated from a cisplatin-resistant cell line of human

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0006-291X/95 $12.00

epidermoid carcinoma KB cells (4). Therefore, we speculated that the GS-X pump is involved in cisplatin resistance.

However, some workers reported that overexpression of the gene encoding the multidrug resistance-associated protein (MRP) resulted in increased GS-X pump-like activity (5,6), and pointed out there was a link between MRP and the GS-X pump. In view of the fact that not all cisplatin-resistant cell lines showed increased GS-X pump activity, they also concluded that the GS-X pump did not always result in cisplatin resistance, but its expression might depend on other factors, such as enhanced GSH metabolism.

The purpose of this study was to determine whether increased intracellular GSH levels result in GS-X pump overexpression and contribute to cisplatin resistance by decreasing cellular cisplatin accumulation. We examined the GS-X pump activity and cisplatin resistance of human small-cell lung cancer cells transfected with the γ-glutamylcysteine synthetase (*γ-GCS*) gene that encodes the rate-limiting enzyme in GSH biosynthesis.

Materials and Methods

Cell lines: The human small-cell lung cancer cell line SBC-3 was established by Prof. I. Kimura and his colleagues at Okayama University (Okayama, Japan). Cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C.
Expression vector for transfection of *γ-GCS* cDNA: The full-length coding region of human *γ-GCS* was obtained by subjecting plasmid DNA from a human thymus cDNA library, pYEUra3 (Clontech, Palo Alto, CA), to the polymerase chain reaction (PCR). The primer sequences were: 5'-GATATCATGGGGCTGCTGTCCCAGGGC-3' and 5'-TCTAGACTAGTTGGATGAGTCAGTTTTAC-3'. The 2.0-kb PCR product was subcloned into the TA cloning site of the eukaryotic expression vector pCR3 (Invitrogen, San Diego, CA), which contains the bacterial neomycin phosphotransferase (*neo*) gene conferring resistance to G418 (Sigma, St. Louis, MO).
Transfection: pCR3 and pCR3-*γ-GCS*, containing pCR3 and the *γ-GCS* PCR product, respectively, were transfected into SBC-3 cells using the Lipofectin reagent (Gibco BRL, Gaithersburg, MD), according to the manufacturer's instructions (7). The cells were cultured in RPMI 1640 medium containing 1 mg/ml G418 for selection. The resulting selected cell lines transfected with pCR3-*γ-GCS* and pCR3 were designated SBC-3/GCS and SBC-3/neo, respectively. The cloned *γ-GCS* PCR product was sequenced using a 7-DEAZA sequencing kit (Takara, Ohtsu, Japan) and T7 and SP6 primers.
Northern blot hybridization analysis: The total RNAs were prepared from the wild-type SBC-3, SBC-3/GCS and SBC-3/neo cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method (8). Approximately 20 μg total RNA was electrophoresed and transferred to a positively charged nylon membrane (Hybond-N+, Amersham Japan., Tokyo, Japan). Restriction enzyme fragments from plasmids carrying human *γ-GCS* and human *β-actin* (Clontech) were used as probes. *γ-GCS* and *β-actin* probes were labeled with [α-^{32}P]dCTP (Du Pont NEN, Boston, MA) to a specific activity of 5 x 10^7 cpm/μg DNA using the *Redi*prime random primer labeling system (Amersham, Buckinghamshire, UK) and used for autoradiography. Hybridization and autoradiography were carried out as described previously (9).
Cellular GSH content: The total cellular GSH levels were measured using the enzyme recycling assay (10), as described previously (11). Briefly, 1-5 x 10^6 cells were suspended in 300 μl phosphate-EDTA solution (125 mM KH_2PO_4, 6.3 mM EDTA, pH 7.5), homogenized and 100 μl 12% (w/v) 5-sulfosalicylic acid was added, protein-free lysates were obtained by centrifugation. The enzyme reaction was carried out using a reaction mixture comprising 125 mM KH_2PO_4, 6.3 mM EDTA, 0.21 mM NADPH, 0.6 mM 5,5'-dithiobis-(2-nitrobenzoic acid) and 0.5 u/ml glutathione reductase at 25°C. The

optical density at 412 nm (A_{412}) was monitored for 3 min and the GSH content was calculated on the basis of the standard calibration curve. The cellular protein concentration was determined by the bicinchoninic acid protein assay (Pierce Chemical., Rockford, IL).

Preparation of membrane vesicles: Membrane vesicles were separated by the method of Ishikawa *et al.* (2). Briefly, 3 x 10^8 cells were suspended in 0.5 mM sodium phosphate buffer, pH 7.0, containing 0.1 mM phenylmethylsulfonyl fluoride and 0.1 mM EGTA, the suspension was centrifuged at 100,000 x g for 40 min, the pellet was suspended in the above buffer, homogenized and layered over 38% (w/v) sucrose solution. After centrifugation at 100,000 x g for 30 min the interface was collected, washed by centrifugation at 100,000 x g for 30 min in a solution (10 mM Tris-HCl, pH 7.4, 250 mM sucrose), the pellet was resuspended in the above solution and passed through a 27-gauge needle to form vesicles, which were stored at -80°C until used.

[^{3}H]LTC$_4$ uptake by membrane vesicles: The standard incubation medium comprised membrane vesicles (30 μg protein), 0.39 nM [14,15,19,20-^{3}H(N)]-LTC$_4$ ([^{3}H]LTC$_4$, Du Pont NEN), 5 mM ATP, 10 mM creatine phosphate, 100 μg/ml creatine kinase, 10 mM $MgCl_2$, 250 mM sucrose and 10 mM Tris-HCl, pH 7.4, in a final volume of 100 μl. The reaction was carried out at 37°C for 20 min, after which the samples were diluted with 1 ml ice-cold stop solution (250 mM sucrose, 100 mM NaCl, 10 mM Tris-HCl, pH 7.4) and applied to Millipore filters (GVWP, 0.22 μm pore size). The filters were washed with 4 ml ice-cold stop solution, dissolved in scintillation fluid and their radioactivity was measured by a liquid scintillation counter. The GS-platinum complex was synthesized according to the procedure of Ishikawa *et al.* (2) to test its inhibitory effect on [^{3}H]LTC$_4$ uptake by membrane vesicles.

Growth-inhibition assay: The growth-inhibitory effect of cisplatin was evaluated using the tetrazolium dye (MTT) assay, as reported previously (12). Briefly, 180-μl aliquots of exponentially growing cells (5 x 10^3/ml) were seeded in 96-well microtiter plates, incubated for 12 h at 37°C and 20-μl aliquots of cisplatin (Brystol-Myers Squibb, Tokyo, Japan) at various concentrations were added. After exposure to the drug for 72 h, 20 μl MTT solution (5 mg/ml in PBS) was added to each well, the plates were incubated for a further 4 h, centrifuged at 800 x g for 5 min, the medium was aspirated and 200 μl of dimethyl sulfoxide was added to each well to dissolve the formazan. The optical density of each solution was measured at 562 and 630 nm using Delta Soft II ELISA Analysis for a Bio-Tek Microplate Reader (EL-340, BioMetallics, Princeton, NJ). Wells containing only medium and MTT were used as controls. The IC_{50} was defined as the cisplatin concentration required to reduce the optical density of each test by 50% and the relative resistance was defined as (IC_{50} for transfected subline/IC_{50} for parental cell line).

Cellular cisplatin accumulation: The cellular platinum contents were measured by atomic absorption spectrophotometry as described previously (9). Cells (2 x 10^6/ml) were seeded into 150-x 15-mm tissue culture dishes, pre-incubated for 3 h then incubated with 0.1 or 0.3 mM cisplatin for 3 h at 37°C. These cisplatin concentrations were chosen to obtain measurable platinum levels in this experimental setting. The incubated cells were collected by centrifugation, washed 3 times with PBS, the cell pellets were digested with 60% (v/v) nitric acid at 80°C for 8 h and the extracts were analyzed for platinum using an atomic absorption spectrophotometer, Spectra AA-40 (Varian Instruments, Palo Alto, CA), in the laboratories of Sumitomo Metal Bio-Science (Tokyo, Japan).

Statistical analysis: The data are expressed as means ± SD and differences between them were analyzed using the unpaired, 2-tailed Student's *t*-test. Differences at $p<0.05$ were considered to be significant.

Results

In order to determine whether the *γ-GCS* gene plays a role in cellular membrane GS-X pump overexpression that may result in cisplatin resistance, we transfected cDNA encoding the full-length human *γ-GCS* gene into the human small-cell lung cancer cell line SBC-3 by the lipofection method. The resulting transfected clone was designated SBC-3/GCS. As a control, we also established a cell line, SBC-3/neo, containing the plasmid vector without *γ-GCS*. The expression of *γ-GCS* mRNA was evaluated by

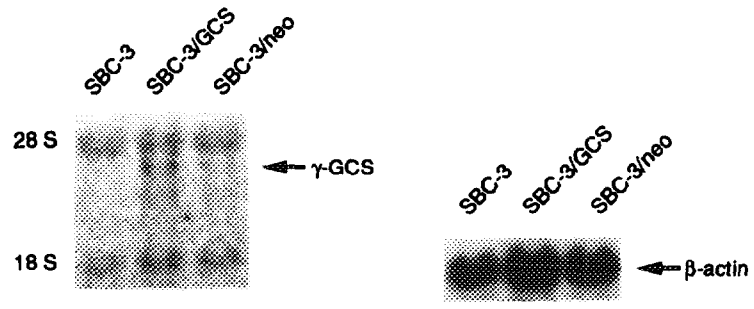

Fig. 1. Expression of *γ-GCS* mRNA in SBC-3, SBC-3/GCS and SBC-3/neo cell lines. Twenty micrograms of each total RNA was analyzed as described in Materials and Methods. *β-actin* probe was used as a control. The mRNA sizes were determined from the mobilities of 18 and 28 S ribosomal RNAs and *β-actin* mRNA.

Northern blot analysis and was significantly higher in SBC-3/GCS than parental SBC-3 and SBC-3/neo cells (Fig. 1). Very low *γ-GCS* expression was detected in the latter 2 cell lines. No significant differences between *MRP* mRNA expression in SBC-3, SBC-3/GCS and SBC-3/neo cells were detected by reverse transcription -PCR analysis (data not shown).

As γ-GCS is a rate-limiting enzyme in GSH biosynthesis, the intracellular GSH contents were measured. The GSH contents of SBC-3, SBC-3/GCS and SBC-3/neo cells were 3.04, 6.59 and 3.05 nmol/mg protein, respectively (Table 1).

We examined whether the GS-X pump was overexpressed in SBC-3/GCS cells using [^{3}H]LTC$_4$ as a substrate. The GS-X pump activity was determined by measuring [^{3}H]LTC$_4$ uptake into membrane vesicles, including inside-out vesicles. The [^{3}H]LTC$_4$ uptake values for the membrane vesicles from SBC-3, SBC-3/GCS and SBC-3/neo cells were 0.134, 0.207 and 0.140 pmol/mg protein/20 min, respectively (Table 2). [^{3}H]LTC$_4$ uptake by membrane vesicles from SBC-3/GCS cells was significantly higher compared with SBC-3 and SBC-3/neo cells ($p<0.0001$), indicating that the GS-X pump activity in SBC-3/GCS cells had increased. [^{3}H]LTC$_4$ uptake by the membrane vesicles was inhibited when the reaction was carried out in the presence of 100 μM GS-platinum:

Table 1. Intracellular GSH content

	SBC-3	SBC-3/GCS	SBC-3/neo
GSH content (*nmol/mg protein*)	3.04± 0.21 [a]	6.59± 0.67 [b]	3.05± 0.41

The GSH contents were measured using the enzyme recycling assay, as described in Materials and Methods. a) Each value is the mean±SD of 6 independent experiments. b) $p<0.0001$ compared with the value for SBC-3 and SBC-3/neo (unpaired, 2-tailed Student's *t*-test).

Table 2. ATP-dependent [^{3}H]LTC$_4$ uptake by membrane vesicles

Condition	LTC$_4$ uptake for 20 min (*pmol/mg protein*)					
	SBC-3	(%)	SBC-3/GCS	(%)	SBC-3/neo	(%)
ATP (+) [a]	0.134 ± 0.0208 [d]	(100)	0.207 ± 0.0118 [e]	(100)	0.140 ± 0.0186	(100)
+ GS-platinum (100 μM) [b]	0.021 ± 0.0018	(16.0)	0.034 ± 0.0069	(16.4)	0.022 ± 0.0039	(15.5)
ATP (−) [c]	0.011 ± 0.0023	(8.2)	0.016 ± 0.0018	(7.5)	0.011 ± 0.0031	(7.7)

Membrane vesicles (30 μg protein) were incubated with 0.39 nM [^{3}H]LTC$_4$ at 37°C for 20 min in 100 μl incubation medium, as described in Materials and Methods. a) The reaction was carried out with 5 mM ATP. b) The reaction was carried out with 5 mM ATP and 100 μM GS-platinum. c) The reaction was carried out without ATP. d) Each value is the mean ± SD of 5 independent experiments. e) $p < 0.0001$ compared with the value for SBC-3 and SBC-3/neo (unpaired, 2-tailed Student's *t*-test).

uptake by each cell line was about 16% of the value without GS-platinum. Furthermore, in the absence of ATP, uptake was about 8% of that in its presence.

In order to elucidate whether *γ-GCS* transfection induced resistance to cisplatin, we examined the sensitivity of these cell lines to cisplatin using the MTT assay. The IC$_{50}$ values of cisplatin for SBC-3, SBC-3/GCS and SBC-3/neo cells were 0.16, 1.18 and 0.18 μM, respectively (Table 3). The SBC-3/GCS cell line showed resistance to cisplatin with a relative resistance value of 7.4 compared with SBC-3 cells. SBC-3/neo did not show resistance to cisplatin, suggesting that *γ-GCS* gene expression selectively influences the cytotoxic effect of cisplatin on the SBC-3/GCS cell line.

The intracellular platinum contents were determined by atomic absorption spectrophotometry, and those of SBC-3, SBC-3/GCS and SBC-3/neo cells were 81.7, 50.0 and 76.1 ng/mg protein, respectively, when these cells were incubated with 0.1 mM cisplatin for 3 h, and 204.5, 145.1 and 196.8 ng/mg protein, respectively, with 0.3 mM cisplatin (Table 4). The accumulation of platinum by SBC-3/GCS cells was significantly lower than that by SBC-3 and SBC-3/neo cells ($p < 0.005$).

Table 3. Sensitivity to cisplatin

	SBC-3	SBC-3/GCS	SBC-3/neo
IC$_{50}$ (*μM*) [a]	0.16 ± 0.04 [b]	1.18 ± 0.14 [c] (7.4) [d]	0.18 ± 0.05 (1.1)

Each cell line was cultured for 3 days in the continuous presence of cisplatin. The cytotoxic effect of cisplatin was measured by the tetrazolium dye (MTT) assay, as described in Materials and Methods. a) IC$_{50}$ values: drug concentrations that inhibited cell growth by 50%. b) Each value is the mean ± SD of 3 independent experiments. c) $p < 0.0005$ compared with the value for SBC-3 and SBC-3/neo (unpaired, 2-tailed Student's *t*-test). d) Values in parentheses: relative resistance values.

Table 4. Intracellular Pt content

Cisplatin concentration (*mM*)	Pt content (*ng/mg protein*)		
	SBC-3	SBC-3/GCS	SBC-3/neo
0.1	81.7 ± 5.3 [a]	50.0 ± 4.0 [b]	76.1 ± 5.6
0.3	204.5 ± 10.6	145.1 ± 4.7 [b]	196.8 ± 6.5

Cells were incubated in the presence of cisplatin for 3 h. The cell extracts were analyzed for platinum by atomic absorption spectrophotometry as described in Materials and Methods. a) Each value is the mean ± SD of 3 independent experiments. b) $p<0.005$ compared with the value for SBC-3 and SBC-3/neo (unpaired, 2-tailed Student's *t*-test).

Discussion

Although the mechanism of cisplatin resistance is thought to be multifactorial (13), the major resistance factor differs according to the cell line. In some cisplatin-resistant cell lines, decreased cellular accumulation of cisplatin was a consistent finding (14), whereas in others, the cellular detoxification systems were increased by thiol-containing scavenger molecules, such as GSH (9) and metallothionein (15). Furthermore, functional overexpression of the GS-X pump was reported in some cell lines, including cisplatin-resistant sublines, and GS-X pump activity was suggested to be related to cellular GSH metabolism and/or cisplatin accumulation (1,2). Therefore, we considered that further investigation into the function of the GS-X pump was required to elucidate the mechanism of cisplatin resistance. In this context, it is important to elucidate the relationship between intracellular GSH levels and GS-X pump expression, and to explicate whether GS-X pump overexpression results in cisplatin resistance by decreasing cellular cisplatin accumulation.

The cellular GSH content of our SBC-3/GCS cells overexpressing the *γ-GCS* gene was about twice that of the parental cell line and the GS-X pump activity, evaluated by measuring membrane vesicle LTC_4 transport, was also increased in SBC-3/GCS cells. These data suggest that a high intracellular GSH content can increase the GS-X pump activity. There were no significant difference between *MRP* mRNA expression in SBC-3, SBC-3/GCS and SBC-3/neo cells. Therefore, the increased GS-X pump activity in SBC-3/GCS cells was independent of *MRP* expression. Furthermore, the IC_{50} value of cisplatin for SBC-3/GCS cells was 7.4 times higher than the parental SBC-3 cell value. We hypothesize there are two reasons for the cisplatin resistance of SBC-3/GCS cells: increased intracellular cisplatin detoxification due to the high GSH level and increased GS-platinum efflux by the GS-X pump. In fact, cellular platinum accumulation decreased in SBC-3/GCS compared with SBC-3 cells. These intracellular sequential processes, the formation of the GS-platinum complex and elimination by the efflux pump, reduce the amount of cisplatin that reaches the nucleus and links with DNA .

We conclude that *γ-GCS* gene expression caused the cisplatin resistance of SBC-3/GCS cells by increasing the intracellular GSH content, inducing GS-X pump overexpression and decreasing cellular cisplatin accumulation. These findings suggest that the GS-X pump activity depends, at least in part, on cellular GSH metabolism and is related to cisplatin resistance.

Acknowledgments

This work was supported, in part, by Grants-in-Aid from the Ministry of Health and Welfare for the 2nd-Term Comprehensive 10-Year Strategy for Cancer Control, the Ministry of Education, Science and Culture, Japan and the Bristol-Myers Squibb Foundation.

References

1. Ishikawa, T., Wright, C.D., and Ishizuka, H. (1994) J. Biol. Chem. 269, 29085-29093.
2. Ishikawa, T., and Ali-Osman, F. (1993) J. Biol. Chem. 268, 20116-20125.
3. Schaub, T., Ishikawa, T., and Keppler, D. (1991) FEBS Lett. 279, 83-86.
4. Fujii, R., Mutoh, M., Sumizawa, T., Chen, Z., Yoshimura, A., and Akiyama, S. (1994) J. Natl. Cancer Inst. 86, 1781-1784.
5. Leier, I., Jedlitschky, G., Buchholz, U., Cole, S.P.C., Deeley, R. G., and Keppler, D. (1994) J. Biol. Chem. 269, 27807-27810.
6. Müller, M., Meujer, C., Zaman, G.J.R., Borst, P., Scheper, R.J., Mulder, N.H., de Vries, E.G.E., and Jansen, P.L.M. (1994) Proc. Natl. Acad. Sci. USA 91, 13033-13037.
7. Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J.P., Ringold, G.M., and Danielsen, M. (1987) Proc. Natl. Acad. Sci. USA 84, 7413-7417.
8. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.
9. Fujiwara, Y., Sugimoto, Y., Kasahara, K., Bungo, M., Yamakido, M., Tew, K.D., and Saijo, N. (1990) Jpn. J. Cancer Res. 81, 527-535.
10. Griffith, O.W. (1980) Anal. Biochem. 106, 207-212.
11. Nakagawa, K., Saijo, N., Tsuchida, S., Sakai, M., Tsunokawa, Y., Yokota, J., Muramatsu, M., Sato, K., Terada, M., and Tew, K.D. (1990) J. Biol. Chem. 265, 4296-4301.
12. Horiuchi, N., Nakagawa, K., Sasaki, Y., Minato, K., Fujiwara, Y., Nezu, K., Ohe, Y., and Saijo, N. (1988) Cancer Chemother. Pharmacol. 22, 246-250.
13. Timmer-Bosscha, H., Mulder, N.H., and de Vries, E.G.E. (1992) Br. J. Cancer 66, 227-238.
14. Morikage, T., Ohmori, T., Nishio, K., Fujiwara, Y., Takeda, Y., and Saijo, N. (1993) Cancer Res. 53, 3302-3307.
15. Kasahara, K., Fujiwara, Y., Nishio, K., Ohmori, T., Sugimoto, Y., Komiya, K., Matsuda, T., and Saijo, N. (1991) Cancer Res. 51, 3237-3242.