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High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis

(γ-glutamylcysteine synthetase/γ-glutamyl transpeptidase/diagnosis/drug resistance/mRNA)

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ABSTRACT **Exposure of human ovarian tumor cell lines to cisplatin led to development of cell lines that exhibited increasing degrees of drug resistance, which were closely correlated with increase of the levels of cellular glutathione. Cell lines were obtained that showed 30- to 1000-fold increases in resistance; these cells also had strikingly increased (13- to 50-fold) levels of glutathione as compared with the drug-sensitive cells of origin. These levels of resistance to cisplatin and the cellular glutathione levels are substantially greater than previously reported. Very high cisplatin resistance was associated with enhanced expression of mRNAs for γ-glutamylcysteine synthetase and γ-glutamyl transpeptidase; immunoblots showed increase of γ-glutamylcysteine synthetase but not of glutathione synthetase. Glutathione *S*-transferase activity was unaffected, as determined with chlorodinitrobenzene as a substrate. These studies suggest the potential value of examining regulation of glutathione synthesis as an indicator of clinical prognosis. The highly resistant cell lines are proving useful for studying the multiple mechanisms by which tumor cells acquire drug- and radiation-resistance.**

Platinum-containing anticancer drugs are effective in treating many solid tumors, including ovarian cancer (1). However, the initial positive response to therapy is limited by development of resistance to these chemotherapeutic agents. Resistance in ovarian cancer is broad; thus, tumors also become refractory to other anticancer drugs and to radiation (1). Currently there is much interest in the mechanisms responsible for development of resistance (see, for example, refs. 1–10). Such resistance is often associated with increased cellular glutathione, consistent with the view that glutathione protects cells against foreign compounds and the effects of radiation (10–13). This is supported by several studies on the effects of alkylating agents and other drugs. For example, resistance to phenylalanine mustard was found to be correlated with the cellular level of glutathione and to be unrelated to uptake or efflux of the drug (14). Recent work suggests that the cellular capacity for glutathione synthesis may also be an important factor in radiation and drug resistance (15, 16).

Glutathione is synthesized intracellularly by the successive actions of γ-glutamylcysteine synthetase and glutathione synthetase (17). Many cells export glutathione, which interacts with membrane-bound γ-glutamyl transpeptidase, leading to formation of products (e.g., γ-glutamylcystine) that are transported into the cell and used for glutathione synthesis (18). γ-Glutamylcysteine synthetase, the enzyme that catalyzes the controlling [and feed back-inhibited (19)] step of glutathione synthesis, and γ-glutamyl transpeptidase, which functions in cellular recovery of cysteine moieties (salvage pathway), are thus key catalysts in glutathione metabolism.

Several studies suggest that glutathione is involved in resistance to radiation and DNA-reactive drugs (platinum analogs and classical alkylating agents) (2–10). Treatment of resistant tumor cells with buthionine sulfoximine, a selective inhibitor of γ-glutamylcysteine synthetase (20–22), partially reverses resistance to the drugs and to radiation (2, 9). These and related findings (5, 6, 14), which indicate that glutathione plays a significant role in drug resistance, have led to clinical trials of buthionine sulfoximine (22, 23).‡ In the present work, highly resistant human ovarian cell lines were developed and used in studies on the mechanism by which glutathione levels are regulated in resistant tumor cell lines.

MATERIALS AND METHODS

Materials. Cisplatin was obtained from Bristol-Meyers Squibb (Syracuse, NY). Chemicals and reagents were obtained from Sigma. Cell culture reagents were obtained from GIBCO.

Cell Lines. A2780 and A1847 are ovarian cancer cell lines (cisplatin sensitive) derived from patients prior to treatment (24). The A2780/C series of cisplatin-resistance cell lines were developed by intermittent, incremental exposure of the A2780 cells to various concentrations (8–200 μM) of cisplatin (Table 1). A1847/CP6 cells were produced in a manner similar to the A2780/C lines by exposure of A1847 cells to cisplatin. 1A9 is a clonal variant of A2780; 1A9/CP10 and 1A9/4E are cisplatin-resistant variants. Most of the OVCAR ovarian cancer cell lines were obtained from patients who were refractory to platinum-based combination chemotherapy, OVCAR-5 and -7 are cell lines derived from patients prior to treatment (25). All cell lines were maintained in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum and 100 μg of streptomycin, 100 units of penicillin, 0.3 mg of glutamine, and 0.3 unit of insulin (porcine) per ml. Cells were grown at 37°C in 5% CO_2.

Cytotoxicity Assay. Cisplatin sensitivity was determined by the tetrazolium salt assay (26). Cells (1000–32,000) were plated in 150 μl of medium per well in a 96-well plate. After incubation overnight, cisplatin was added in various concentrations (0–300 μM). After 3 days, 40 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 5 mg/ml was added per well. After a 2-hr incubation, the cells were lysed with 100 μl of extraction buffer [20% (wt/vol) sodium dodecyl sulfate (SDS)/50% *N*,*N*-dimethylformamide, pH 4.7) per well. After incubation overnight, the absorbance at 570 nm was measured (Bio-Rad microplate multiscanner); the wells without cells were blanks.

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‡Patients treated with buthionine sulfoximine have been found, in a phase I clinical trial, to have substantial depletion of glutathione in peripheral mononuclear cells.

Table 1. Human tumor cell lines

Cell line	Description	*In vitro* cisplatin resistance*
A1847	Untreated ovarian tumor	S
A1847/CP6	A1847 after intermittent exposure to 6 μM cisplatin	+
A2780	Untreated ovarian tumor	S
A2780/CP8-CP70	A2780 after intermittent exposure to the indicated concentrations of cisplatin	+ to ++
A2780/C30-C300	A2780/CP70 after nearly continuous exposure to the indicated concentrations of cisplatin—i.e., C80 = 80 μM; C200 = 200 μM, etc.	+++ to ++++
1A9	A clonal derivative of A2780	S
1A9/CP10	1A9/CP10 after intermittent exposure to 10 μM cisplatin	+
1A9/4E	1A9/CP10 after nearly continuous exposure to cisplatin increasing to 80 μM	++++
OVCAR-2	Ovarian tumor from a patient refractory to cisplatin	+++
OVCAR-3	Ovarian tumor from a patient refractory to cisplatin	+
OVCAR-4	Ovarian tumor from a patient refractory to cisplatin	+
OVCAR-5	Untreated ovarian tumor (advanced)	+
OVCAR-7	Untreated ovarian tumor	+
OVCAR-8	Ovarian tumor from a high dose (800 mg/m^2) carboplatin-refractory patient	+
OVCAR-10	Ovarian tumor from a cisplatin-refractory (200 mg/m^2) and high-dose carboplatin-refractory (800 mg/m^2) patient	++
PE01	Ovarian tumor, cisplatin sensitive	S
PE04	Ovarian tumor (PE01) after patient became refractory	+
HCT15	Colon carcinoma	S
HCT/CP16	HCT15 after exposure to 16 μM cisplatin	+++

*Approximate fold resistance versus parent: S, parental sensitive; +, low (1–10); ++, moderate (10–50); +++, high (50–300); ++++, very high (300–>1000). These values were obtained by a variety of methods.

Glutathione Determinations. Cells (2–5 × 10^6; about 60% confluent) were lysed by sonication at 4°C in 1 ml of phosphate-buffered saline (PBS). The supernatant was obtained for assay after centrifugation (10,000 × *g* for 10 min at 4°C). The protein was precipitated by adding 12% 5-sulfosalicylic acid (SSA) (1 vol of SSA to 3 vol of sample). After standing on ice for 1–4 hr, the samples were centrifuged (10,000 × *g* for 10 min). The SSA extract was assayed as described (27) with 100 μl of sample and 0.5 unit of glutathione reductase per assay. Protein in the PBS lysate was determined by the Bradford assay (28) (Bio-Rad) with bovine serum albumin as standard.

Glutathione *S*-Transferase Assay. Activity was measured with 1-chloro-2,4-dinitrobenzene (CDNB) (29). The PBS extract (above) was used after centrifugation. The reaction mixture contained 1 mM glutathione, 1 mM CDNB, and 100 μl of sample in a final volume of 1 ml. The rate of increase in absorbance at 340 nm was measured at 25°C. The results are expressed as nmol/min per 10^6 cells.

Extraction of Cellular RNA and Northern Blots. Total cellular RNA was extracted by a one-step guanidinium isothiocyanate/phenol/chloroform extraction procedure (30). Total RNA (16 μg per lane) was denatured in 50% formamide containing 7.4% formaldehyde and separated by electrophoresis on an agarose (1% agarose/2.2 M formaldehyde) gel. The RNA was blotted by capillary action onto Magna NT membrane filters (Micron Separations, Westborough, MA) in 10× SSC (1.5 M NaCl/0.15 M sodium citrate, pH 7.0) and hybridized (>12 hr) with ^{32}P-labeled probes. A human kidney γ-glutamylcysteine synthetase probe [1.7 kilobases (kb)] was used to screen a human ovarian tumor cDNA library in λZAP II. The synthetase cDNA (≈3.7 kb) and γ-glutamyl transpeptidase cDNA [2.3 kb of *Eco*RI human cDNA (31)] probes were labeled with ^{32}P by nick-translation (about 4 × 10^8 dpm/μg). RNA gels were stained with ethidium bromide and examined to ascertain that equivalent amounts of RNA were analyzed. Filters were washed twice for 30 min each in 2× SSC containing 0.5% SDS and sodium pyrophosphate at 65°C and twice for 30 min each in 0.2× SSC containing 0.5% SDS and sodium pyrophosphate at 55°C. Autoradiography was performed at −70°C for 1–4 days.

Immunoblot (Western Blot) Analysis. Total cellular protein was prepared (32), and the relative protein concentration was estimated fluorometrically (33) with bovine serum albumin as standard. Lysate (100 μg) from cell extracts was mixed with an equal volume of 2× SDS buffer [40% (vol/vol) glycerol/6% SDS/0.25 M Tris·HCl/0.1% bromophenol blue/12% urea, pH 6.8] containing 0.7 M 2-mercaptoethanol and placed at 100°C for 5 min. The samples were separated by electrophoresis on a 10% acrylamide gel (34) using prestained protein molecular weight standards. The separated proteins were transferred electrophoretically to Immobilon-P membrane filters (Millipore) and washed twice (30 min) with 50 mM Tris·HCl, pH 7.5/400 mM NaCl (TBS) containing 0.05% Tween 20 (TBS-T) at 20°C. The filters were preincubated with blocking solution, TBS-A [TBS containing 5% (wt/vol) nonfat dry milk], for 90 min at 20°C with agitation. The filters were incubated with polyclonal antibodies to γ-glutamylcysteine and glutathione synthetases (rabbit anti-rat) (35) diluted 1:250 in TBS-A for at least 12 hr. The filters were then washed three times for 10 min with TBS-T and treated with a secondary antibody (^{125}I-labeled donkey anti-rabbit IgG; diluted 1:300 in TBS-A) for 1 hr. After thorough washing with TBS-T, the filters were air-dried. Autoradiography was performed at −70°C for 1 week.

RESULTS

Relationship Between Resistance and Glutathione Levels. An ovarian cancer cell line derived from an untreated patient (A2780) (24) was exposed to cisplatin (see *Materials and Methods* and Table 1); the cell lines obtained by such selection exhibited a wide range of resistance (Table 2). It is notable that four of the cell lines examined exhibited very marked increases in resistance (>500-fold) to cisplatin. The resistance of cell line C200 was more than 1000-fold higher than that of the parental A2780 cell line. There was good correlation between the degree of resistance and the increase of the cellular level of glutathione; a plot of fold-resistance against cellular glutathione level was essentially linear (plot not shown). The very resistant C200 cell line had about a 50-fold increase in cellular glutathione level. Thus, it appears that cell lines obtained by *in vitro* selection may be useful for studies on the mechanisms of resistance to cisplatin and related drugs.

Table 2. Relationship between cisplatin-resistance and glutathione levels

Cell line	Cisplatin IC_{50}*	Fold resistant	GSH Level†	GSH Fold increase	GST activity‡
A2780	0.19	1	1.75	1	1.3
CP70	5.5	29	22.6	13	1.3
C30	54	280	26.0	15	1.1
C50	100	530	37.8	22	0.97
C80	115	610	52.4	30	1.1
C100	125	660	31.6	18	0.93
C200	205	1080	83.2	48	1.1

*Cytotoxicity was determined by assay with 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT). The IC_{50} is the concentration (μM) that inhibited growth by 50%.
†Total glutathione [GSH + 1/2 glutathione disulfide (GSSG)] is shown as nmol per 10^6 cells.
‡Glutathione *S*-transferase (GST) activity with 1-chloro-2,4-dinitrobenzene is reported as nmol/min per 10^6 cells.

Enzymes Involved in Glutathione Metabolism. The remarkable increase in cellular glutathione observed here might reflect an increase in glutathione synthesis or possibly altered feedback control of synthesis. In an approach to understanding the strikingly increased cellular glutathione levels observed in these tumor cells, we examined the steady-state mRNA levels for γ-glutamylcysteine synthetase. This was facilitated by use of the cDNA for rat kidney γ-glutamylcysteine synthetase heavy subunit (35) to obtain a human kidney clone (S. Jain and A.M., unpublished data). The latter was used to screen an ovarian tumor cDNA library. The full-length clone obtained, which was used for the studies described in Figs. 1–3, recognizes the transcript as a doublet of 3.5 kb and 4.1 kb; this may reflect alternative splicing or aspects of message processing.

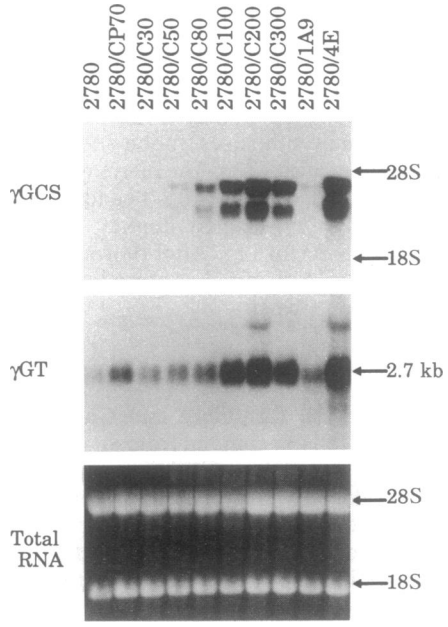

FIG. 1. Northern blot analysis of γ-glutamylcysteine synthetase (γGCS)-specific and γ-glutamyl transpeptidase (γGT)-specific RNA in cisplatin-sensitive and resistant ovarian tumor cell lines. Total cellular RNA was isolated from the cell lines and processed as described in the text. The size (in kb) of the γGT transcript is indicated. RNA gels were routinely stained with ethidium bromide and visualized to ensure that equivalent amounts of total RNA were analyzed in each case (lower panel). The position of the 28S and 18S ribosomal RNA is indicated.

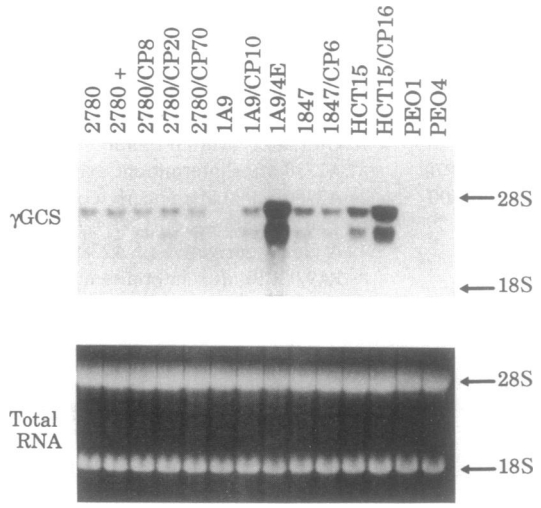

FIG. 2. Northern blot analysis of γ-glutamylcysteine synthetase (γGCS) mRNA in ovarian and other tumor cell lines (see Table 1). Lane "2780 +" indicates a study in which A2780 cells were treated with 100 μM cisplatin for 1 hr prior to harvest.

As indicated in Fig. 1, dramatic increases in message levels for γ-glutamylcysteine synthetase were seen in cell lines C50, C80, C100, C200, and C300; comparably lower levels were observed in cell lines C30, CP70, and parental A2780, which had substantially lower levels of glutathione and of cisplatin resistance. Examination of the highly resistant cell lines for the amplification of the gene for γ-glutamylcysteine synthetase by Southern blot analysis revealed no change in gene copy number (data not included). A clonal derivative of A2780 (1A9) expressed low levels of mRNA, whereas cell line 1A9/4E, a very highly resistant variant, exhibited high levels of mRNA for γ-glutamylcysteine synthetase, comparable to the level observed in C200 cells.

To evaluate an alternative pathway involved in regulation of cellular glutathione levels, we examined the cell lines listed in Table 2 for altered expression of the γ-glutamyl transpeptidase. Fig. 1 reveals that γ-glutamyl transpeptidase mRNA levels are elevated (2- to 3-fold) in CP70 cells with moderate (≈30-fold) cisplatin resistance as compared with the parental cell line A2780. Examination of very highly resistant variants (C100, C200, C300, and 4E) showed a dramatic increase (15- to 40-fold) in γ-glutamyl transpeptidase steady-state mRNA levels.

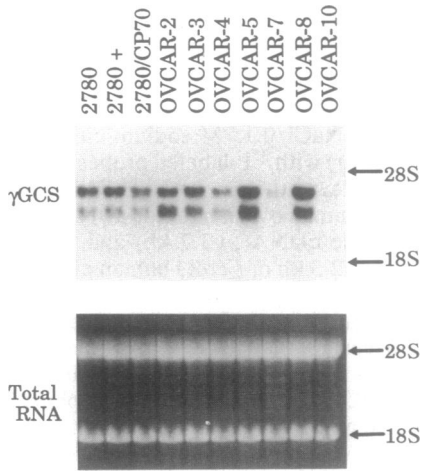

FIG. 3. Northern blot analysis of ovarian tumor cell lines (see Figs. 1 and 2 and Table 1). γGCS, γ-glutamylcysteine synthetase.

In the course of these studies, it was evident that only very highly resistant variants of A2780 possess constitutively high steady-state levels of γ-glutamylcysteine synthetase mRNA. Therefore, we evaluated a variety of additional cell lines with various degrees of cisplatin resistance (from different origins) for expression of γ-glutamylcysteine synthetase mRNA. Figs. 2 and 3 show analysis of γ-glutamylcysteine synthetase mRNA levels for the cell lines listed in Table 1. Notably, many of the cell lines with low resistance to cisplatin (A2780, A2780/CP8, CP20, 1A9, 1A9/CP10, A1847, and A1847/CP6) expressed low mRNA levels. A human colon carcinoma cell line (HCT15) exhibited a significant level as compared with A2780 cells (3-fold), whereas a cell line (HCT15/CP16) selected after exposure of HCT15 to cisplatin exhibited much more mRNA (>10-fold) (and increased resistance). Treatment of sensitive or resistant cell lines with cisplatin for up to 24 hr did not lead to a noticeable increase in mRNA (Fig. 2 and data not shown). Little or no increase of mRNA levels was observed in lines PE01 and PE04 which are, respectively, cisplatin sensitive and resistant cell lines derived from the same patient (36). In a series of cell lines obtained from human ovarian tumors (OVCAR), it was found that the OVCAR-7 cell line (from an untreated cisplatin-responsive patient), which was highly sensitive to cisplatin *in vitro*, showed very low mRNA levels for γ-glutamylcysteine synthetase (Fig. 3); this patient responded to intensive cisplatin-based chemotherapy. Cell lines OVCAR-3 and -4, which exhibited some resistance, had levels of mRNA comparable to A2780. Of the tumor cell lines from refractory patients, OVCAR-2 exhibited levels of mRNA similar to A2780, whereas one (OVCAR-10) showed very low levels. This is in contrast to OVCAR-8, which was derived from a patient that failed high-dose carboplatin therapy and showed low-level resistance *in vitro*. This cell line exhibited a 2- to 3-fold increase in γ-glutamylcysteine synthetase mRNA levels as compared with the cell lines OVCAR-2, OVCAR-3, and A2780.

Western blots were carried out on the cell lines given in Table 2 by using antibodies to rat kidney γ-glutamylcysteine synthetase (Fig. 4). These studies showed that the amount of γ-glutamylcysteine synthetase protein is significantly increased in cell lines that are highly (>300-fold) resistant to cisplatin. Under denaturing conditions, γ-glutamylcysteine synthetase is dissociated to yield two subunits: a light subunit and a heavy subunit at $M_r \approx 31,000$ and 70,000, respectively. It has been demonstrated previously that the heavy subunit of the enzyme exhibits all of the catalytic activity (37) and its activity is inhibited by glutathione.[§] We found significant increase in the levels of both subunits of γ-glutamylcysteine synthetase, which seems to parallel the degree of resistance in certain cell lines (Fig. 4). The band at M_r 91,000 is observed in all of the lanes analyzed in Fig. 4; the level of this protein does not correlate well with the degree of resistance, and the nature of this band will require further study. Western blot analysis using antibody to glutathione synthetase did not show an increase in the level of this protein in any of the cell lines (data not included).

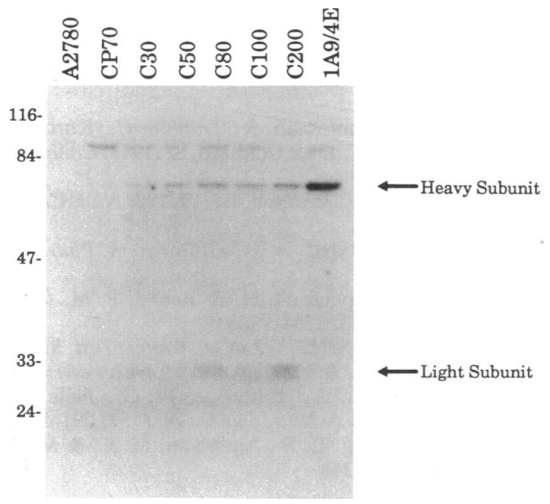

FIG. 4. Western blot analysis of γ-glutamylcysteine synthetase in parental A2780 cells and platinum-resistant variants. Total cellular proteins were isolated, separated, and blotted as described in the text. All lanes contained 100 μg of total protein. Rat polyclonal antibody was used. Arrows represent the position of the protein subunits (M_r 70,000 and 31,000) as determined by SDS/PAGE using molecular weight standards (shown × 10^{-3}).

DISCUSSION

Several mechanisms may be involved in the development of drug- and radiation-resistant tumor cells. Treatment with alkylating agents and platinum-containing drugs is often associated with an increase in tumor cell glutathione levels, and this may also be accompanied by resistance to radiation. Radiation resistance is not typically associated with the type of resistance found in conjunction with overexpression of a specific membrane glycoprotein (9); nevertheless, it is reasonable to postulate that many types of tumor-cell resistance observed clinically are associated with more than a single biochemical mechanism. Glutathione may protect cells by binding to or reacting with drugs, by interacting with reactive oxygen moieties or with other radicals produced by radiation, by preventing damage to proteins or DNA, or by participating in repair processes.

The studies reported in Table 2 show a close correlation between glutathione levels and resistance to cisplatin; the cellular levels of glutathione and the degrees of cisplatin resistance found are striking as compared with those reported previously. Since γ-glutamylcysteine synthetase and γ-glutamyl transpeptidase are important catalysts for the synthesis (17) and salvage (18) pathways, respectively, of glutathione metabolism, it would be expected that these enzymes would be increased in resistant cells. Since glutathione synthetase does not catalyze the rate-limiting step of glutathione synthesis, increased levels of this enzyme would not be expected nor were they found. In contrast, the steady-state mRNA levels of γ-glutamylcysteine synthetase and γ-glutamyl transpeptidase were increased in highly resistant cells. Increased activity of γ-glutamyl transpeptidase has previously been found in a number of tumor cells (see, for example, refs. 38–47) and may reflect an increased requirement for glutathione by certain tumor cells. The regulation of cellular glutathione levels involves not only the activity of γ-glutamylcysteine synthetase but also depends upon the availability of cysteine moieties whose derivation from extracellular glutathione and transport into the cell involves the activity of γ-glutamyl transpeptidase (18). Thus, it is possible that both γ-glutamylcysteine synthetase and γ-glutamyl transpeptidase contribute to the regulation of intracellular glutathione levels and acquired cisplatin resistance.

The steady-state levels of γ-glutamylcysteine synthetase mRNA and the enzyme protein levels do not always correlate well. For example, A2780/C200 and 1A9/4E have similar mRNA levels, but the latter cell line has more γ-glutamyl-

[§]The cDNA for the heavy subunit of rat kidney γ-glutamylcysteine synthetase (35) has recently been expressed in *Escherichia coli*; the recombinant heavy subunit, like the heavy subunit obtained by dissociation of the holoenzyme, exhibits a much higher apparent K_m for glutamate than does the isolated holoenzyme. These studies (48) and subsequent work support the conclusion that the light subunit functions in the regulation of enzyme activity.

cysteine synthetase heavy subunit protein (and little if any detectable light subunit). On the other hand, A2780/C200 and A2780/C80 have similar protein levels, but the former cell line has more mRNA than the latter. This suggests that enzyme stability or translational control may be involved; further studies of these phenomena are needed. Both may be affected by cellular signals that increase the capacity for glutathione synthesis, which may be an essential feature of resistance (15, 16).

These studies have shown that it is possible to produce very high levels of cisplatin resistance (>1000-fold) in cultured human tumor cells. Cisplatin resistance is accompanied by an increase in cellular glutathione which is essentially linear with resistance. The increase in glutathione may initially be accomplished by increases in γ-glutamyl transpeptidase steady-state mRNA, but as resistance and glutathione levels increase, the expression of γ-glutamylcysteine synthetase is elevated. These changes occur in the absence of gene amplification. Our results support a direct causal relationship between glutathione and resistance produced by classical alkylating agents, platinum drugs and irradiation, but further work is needed. Studies on the effect of overexpression and antisense expression of γ-glutamylcysteine synthetase are necessary to confirm the relationships between glutathione synthesis and drug-resistance.

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