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Altered Expression of γ-Glutamylcysteine Synthetase, Metallothionein and Topoisomerase I or II during Acquisition of Drug Resistance to Cisplatin in Human Ovarian Cancer Cells

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This study was designed to elucidate the mechanisms of cisplatin (CDDP) resistance using two human ovarian cancer cell lines, KF and TYK, and two CDDP-resistant lines, KFr and TYK/R, derived from the former lines. KFr and TYK/R showed about 3-fold higher resistance to the cytotoxic effects of CDDP than their parental lines. They also showed a significant increase in sensitivity to not only etoposide, but also (+)-(4*S*)-4, 11-diethyl-4-hydroxy-9-[(4-piperidino-piperidino)carbonyloxy]-1*H*-pyrano[3′,4′:6,7]inodolizino[1,2-*b*]quinoline-3,14(4*H*, 12*H*)-dione hydrochloride trihydrate (CPT-11). Cellular CDDP accumulation levels in KFr and TYK/R were decreased from those of the parental cells. By contrast, the cellular glutathione (GSH) content in KFr cells was 1.7-fold higher than that in KF, whereas TYK/R cells had a 40% lower content than TYK cells. Cellular mRNA levels of drug-resistance-related genes, such as DNA topoisomerase (topo) I and topo II, glutathione *S*-transferase-π (GST-π), γ-glutamylcysteine synthetase (*γ-GCS*), and metallothionein (*hMT*) genes, were compared between drug-sensitive KF or TYK and KFr or TYK/R. KFr cells had 8.5- and 24.7-fold higher mRNA levels of γ-GCS and topo II genes than KF cells while KFr had only a slight increase in GST-π mRNA level as compared with KF. By contrast, TYK/R cells had 2.9- and 1.7-fold higher hMT and topo I mRNA levels than TYK cells. Acquisition of CDDP resistance in human ovarian cancer cells thus appeared to be related mainly to expression of *γ-GCS*, *topo II* and *hMT* genes, and partly to that of *topo I* and *GST-π* genes, in addition to a decrease in CDDP accumulation.

Key words: γ-Glutamylcysteine synthetase — Metallothionein — Topoisomerase — Cisplatin resistance — Human ovarian cancer cell

Resistance to available chemotherapeutic drugs is a major obstacle to effective, potentially curative chemotherapy in ovarian cancer. Although aggressive treatment of advanced-stage ovarian cancer patients with a *cis*-diamminedichloroplatinum (II) (CDDP)-containing combination chemotherapy regimen is associated with a 40% to 60% clinical complete response rate, chemotherapy is rarely curative. Thus, the majority of ovarian cancer patients are destined to die from their disease. Virtually all of these patients die with chemotherapy-refractory cancer. From this perspective, drug resistance may be considered an integral aspect of ovarian cancer biology. Treatment success has been limited by the presence of advanced disease in the majority of patients at the time of diagnosis and by the development of resistance to currently available chemotherapeutic agents.[1] Extensive studies in this area have demonstrated a number of mechanisms which contribute to CDDP-resistance. Among these are reduced drug accumulation, increased levels of detoxifying agents such as glutathione (GSH), and increased repair of the adducts.[2,3] In addition, DNA topoisomerase (topo) II is increased in some CDDP-resistant human ovarian cancer cell lines[4] and in a CDDP-resistant murine leukemia cell line.[5] In contrast, little attention has been paid to the possible role of topo I in CDDP-resistance, except for a recent study.[6]

We have established CDDP-resistant cell lines, KFr and TYK/R, by treating the respective parent cell lines KF and TYK with incrementally increasing concentrations of CDDP.[7–9] In order to elucidate more fully the multiple mechanisms responsible for CDDP-resistance, we attempted to determine the roles of not only drug accumulation, drug detoxification and DNA repair, but also topo I and topo II in the acquired CDDP-resistance.

MATERIALS AND METHODS

Drugs and chemicals CDDP and etoposide were obtained from Bristol-Myers Squibb Co., Tokyo. (+)-(4*S*)-4, 11-Diethyl-4-hydroxy-9-[(4-piperidino-piperidino)carbonyloxy]-1*H*-pyrano[3′,4′:6,7]inodolizino[1,2-*b*]quinoline-3,14(4*H*, 12*H*)-dione hydrochloride trihydrate

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(CPT-11) was kindly supplied by Daiichi Seiyaku Co., Tokyo. All the other drugs and chemicals not specifically mentioned were purchased from Sigma (St. Louis, MO).

Cell lines and culture The KF cell line was established from tissue of a patient with serous cystadenocarcinoma of the ovary. The CDDP-resistant KFr cell line was obtained after repeated exposure of the parent KF cell line to escalating doses of CDDP.[7] Similarly, TYK/R was an acquired CDDP-resistant cell line generated from the parent TYK cell line established from undifferentiated carcinoma of the ovary.[8] The cells were grown as monolayer cultures in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2m*M* glutamine, 100 units penicillin/ml, and 100 μM streptomycin/ml (Grand Island Biological Co., Grand Island, NY) in a humidified atmosphere of 5% CO_2 at 37°C, and were usually subcultured once a week with 0.25% trypsin. To determine the concentrations of CDDP, etoposide and CPT-11 required for 50% inhibition of cell proliferation *in vitro* (IC_{50}), we seeded 10^4 cells of KF and KFr and 2×10^4 cells of TYK and TYK/R in multidishes (diameter; 1.5 cm) and added each drug at the concentrations indicated. After 4 days of culture without drug replenishment, cells were harvested and counted with a hematocytometer. The degrees of sensitivity to the drugs were compared using the IC_{50} values. Living cells grown in culture dishes were examined with a phase-contrast microscope.

Platinum assay The cultured cells in a preconfluent state were harvested using a rubber policeman after incubation with a small amount of 0.25% trypsin and counted with a hematocytometer. The cell number was adjusted to 10^7 cells/ml with RPMI 1640 medium and CDDP was added to obtain a final concentration of 100 μM in the medium. After 3-h incubation, cells were washed three times with fresh medium, and resuspended in 1 ml of medium. The cell viability was examined by using the trypan blue dye exclusion test. The incubation time at this cell density did not affect the cell viability. The cell suspension was sonicated (50% duty cycle for 3 min) using a W-225 sonicator (Heat Systems-Ultrasonics, Inc., Farmingdale, NY). After centrifugation at 30,000*g* for 1 h, the supernatants were taken for platinum assay. The platinum assay was performed as described previously[10] with a Perkin-Elmer Model 603 atomic absorption spectrometer equipped with a heated graphite atomizer on which the temperature control had been modified to produce a gradual increase in temperature. Protein was determined with a protein microassay kit (Bio-Rad, Richmond, CA).

GSH content Cells ($5–10\times10^6$) at the preconfluent state 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 h, the samples were centrifuged (10,000*g* for 10 min). The SSA extract was assayed by an enzymatic recycling method as reported.[11] Values are the mean of 6 independent experiments. Results were normalized according to the numbers of cells.

Northern blot analysis The total RNA was extracted using the acid guanidium isothiocyanate-phenol-chloroform method[12] from exponentially growing cells. Human DNA topo I cDNA (pHTOPI) was kindly provided by T. Andoh (Sohka University, Tokyo). Human DNA topo II cDNA (pBS-hTOPII) was provided by J. C. Wang (Harvard University, Boston, MA). Human glutathione *S*-transferase (GST)-π was donated by M. Muramatsu (Saitama Medical University, Saitama). Human metallothionein (hMT) cDNA (hMT-II)[13] and human γ-glutamylcystiene synthetase (GCS) cDNA[14] were obtained from the American Type Culture Collection (Rockville, MD). RNA (15 μg) was subjected to electrophoresis in a 1% agarose gel containing 2.2 *M* formaldehyde and then was transferred to a nylon membrane (Hybond N^+; Amersham Japan Tokyo). The filter was hybridized with each ^{32}P-labeled cDNA probe in 50% deionized formamide, 10×Denhardt's buffer, 5×standard saline citrate (SSC) (1×SSC=0.15 *M* NaCl–0.015 *M* sodium citrate, pH 7.0), and 0.1% sodium dodecyl sulfate (SDS) at 30°C for 36 h. The filter was then washed twice in 2×SSC and 0.1% SDS at room temperature.

Autoradiography was performed for 12 h with an intensifying screen at −70°C. The intensity of the autoradiograms obtained was quantified by densitometric scanning. These experiments were repeated three times without synchronizing the cells, and similar results were obtained.

RESULTS

The KFr and TYK/R cells showed about 3.4- and 3.0-fold higher resistance to CDDP than the parental KF and TYK cells in terms of the IC_{50} values. The sensitivity of both CDDP-resistant KFr and TYK/R cells to etoposide (a topo II inhibitor) was significantly ($P<0.01$) higher than that of the parental KF or TYK cells, respectively, while TYK/R and KFr cells also had significantly higher sensitivity to CPT-11 (a topo I inhibitor) than TYK and KF cells (Table I). The intracellular platinum contents of KFr and TYK/R were decreased to 60% and 90% of those of KF and TYK, respectively. Although the intracellular GSH content and the degree of CDDP resistance seemed to correlate between KF and KFr cell lines, the GSH content of TYK/R was significantly ($P<0.01$) lower than that of TYK (Table II).

Expression of γ-GCS mRNA in KFr and TYK/R CDDP-resistant cell lines was 8.5- and 1.2-fold higher

Table I. Sensitivity of Acquired CDDP-resistant Human Ovarian Cancer Cells to Topo I and II Inhibitors

Cells	CDDP IC_{50} (μM)	Etoposide IC_{50} (μM)	CPT-11 IC_{50} (μM)
KF	0.38 ± 0.02[a]	0.62 ± 0.10	2.21 ± 0.52
KFr	1.28 ± 0.10[b] (3.37)[c]	0.40 ± 0.09[b] (0.65)[c]	1.65 ± 0.31 (0.75)[c]
TYK	0.31 ± 0.04	0.84 ± 0.13	1.40 ± 0.22
TYK/R	0.92 ± 0.07[b] (2.97)[c]	0.58 ± 0.07[b] (0.69)[c]	1.04 ± 0.14[b] (0.74)[c]

a) The exposure time to each drug was 4 days and the mean value ($\pm$SD) for each cell line was derived from three separate experiments.
b) $P<0.01$, compared to each parental cell line (unpaired Student's *t* test).
c) The relative resistance of KFr or TYK/R was determined by dividing the IC_{50} value of each cell line by that of the parental KF or TYK cells.

Table II. Cellular CDDP Levels and GSH Contents in Acquired CDDP-resistant Human Ovarian Cancer Cells

Cell line	CDDP level[a]	GSH level[b]
KF	86.8 ± 18.7[c] (1.0)[d]	24.3 ± 5.3 (1.0)
KFr	53.6 ± 8.6[e] (0.6)	41.2 ± 6.9[e] (1.7)
TYK	99.1 ± 20.1 (1.0)	164.0 ± 15.3 (1.0)
TYK/R	89.2 ± 13.2 (0.9)	101.8 ± 10.6[e] (0.6)

a) ng/mg protein.
b) nmol/10^7 cells.
c) Mean$\pm$SD of six independent experiments.
d) The relative level of CDDP and GSH of KFr or TYK/R cells was determined by dividing the value for each cell line by that of the parental KF or TYK cells.
e) $P<0.01$, compared to each paired cell line (unpaired Student's *t* test).

Table III. Expressions of Drug-resistance-related Genes in CDDP-resistant Human Ovarian Cancer Cells

Cell line	mRNA levels				
	topo I	topo II	γ-GCS	GST-π	MT
KF	1.0[a]	1.0	1.0	1.0	0[b]
KFr	0.8	24.7[c]	8.5[c]	1.4	0
TYK	1.0	1.0	1.0	1.0	1.0
TYK/R	1.7[c]	0.8	1.2	1.0	2.9[c]

a) mRNA levels of drug-resistance-related genes were normalized based on densitometric scanning of northern blots for the parental KF and TYK cells (in three separate experiments).
b) Expression of its mRNA was not detectable in northern blots.
c) $P<0.01$, compared to each parental cell line (unpaired Student's *t* test).

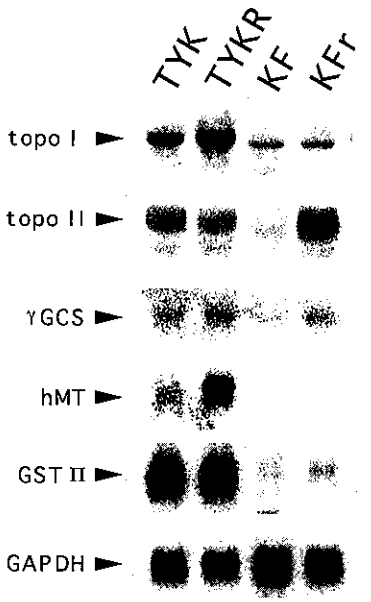

Fig. 1. Northern blot analysis of human γ-GCS, GST-π, MT, and DNA topo I and II mRNAs. Samples of total RNA (15 μg) extracted from various ovarian cancer cell lines were subjected to electrophoresis in 1% agarose gel containing 2.2 *M* formaldehyde, transferred to a nylon membrane, and hybridized with ^{32}P-labeled cDNA probe. Labeled GAPDH probe was used as the control.

than that by the paired CDDP-sensitive cell lines, respectively. However, the levels of expression of GST-π mRNA in TYK and TYK/R were similar, while that in KFr was 1.4 times that in KF. Expression of hMT mRNA was not detectable in KF and KFr cell lines, while its expression in TYK/R was 2.9-fold higher than that in TYK (Table III and Fig. 1).

Regarding the expression of topo I and topo II mRNAs, the CDDP-resistant KFr cell line was characterized by 24.7-fold higher expression of topo II (but not topo I) mRNA than the KF cell line, while the CDDP-resistant TYK/R cell line showed 1.7-fold higher expression of topo I (but not topo II) mRNA than the TYK cell line (Table III and Fig. 1).

DISCUSSION

Various mechanisms have been proposed for the development of CDDP resistance, including an altered influx or efflux of the drug, improved drug detoxification via elevated hMT or GSH levels, enhanced DNA repair capacity or decreased DNA accessibility.[1, 15] It has also been proposed that changes in oncogene expression may mediate repair of CDDP-induced DNA damage.[16] Of these various mechanisms, a reduced accumulation of CDDP has seemed to be the most common in CDDP-resistant cancer cells. We have also demonstrated that the mechanism of acquired CDDP-resistance in human ovarian cancer cells involves a decrease of CDDP accumulation in the tumor cells.[17] In the present study, although the intracellular platinum accumulation in the CDDP-resistant cell lines (KFr and TYK/R) was decreased, the extent of the decrease can account only partly for the resistance of these cell lines (especially TYK/R), suggesting the involvement of other mechanisms (Tables I and II).

Resistance to anticancer agents is often attributed to changes in cytosolic thiol levels. Several investigators have demonstrated that the intracellular GSH content increased with increasing CDDP-resistance.[18] However, it has been reported that the GSH content in CDDP-resistant cell lines derived from Chinese hamster ovary cells[19] or bladder cancer cells[6] was not changed. The role of GSH in the CDDP-resistance remains unclear. In the present study, the intracellular content of GSH was significantly increased in the CDDP-resistant KFr cell line. The GSH content was about 1.7-fold higher in KFr cells, being consistent with the increased γ-GCS mRNA level (γ-GCS is a key enzyme for GSH synthesis). Increased level of GSH might be one factor which is involved in acquired CDDP-resistance in KFr cells. However, the GSH levels of the CDDP-resistant TYK/R were significantly lower than those of the CDDP-sensitive TYK (Tables II and III). Thus, there seemed to be no consistent correlation between the GSH content and the extent of CDDP-resistance in our paired cell lines. Since we determined only the GSH contents in this study, we should explore whether the total sulfhydryl contents may be involved in the CDDP-resistance of our cell lines. KFr showed an about 8.5-fold increase in γ-GCS mRNA expression, and an about 24.7-fold increase in topo II mRNA as compared with its parental KF cells, but expression of other drug-resistance-related genes was not markedly changed (Table III and Fig. 1).

In addition, we examined the expression of hMT mRNA to evaluate the relationship between CDDP-resistance and hMT expression in our established ovarian cancer cell lines. The KFr cell line showed no expression of hMT mRNA at our limit of detection. In the other CDDP-resistant cell line, TYK/R, expression of the *hMT* gene, but not other genes, was significantly increased (about 2.9-fold higher) (Table III). Enhanced expression of *hMT* gene is often correlated with acquisition of CDDP-resistance in various cancer cell lines.[20] Schilder *et al.*[21] reported that only two of five cell lines with *in vitro*-induced resistance to CDDP had detectable hMT mRNA, while the other CDDP-resistant cell lines, as well as the parental A2780 ovarian cancer cell line, showed no expression of hMT mRNA, and further, there was variable expression of hMT mRNA among the OVCAR cell lines. These observations are consistent with our present observations, suggesting that CDDP-resistance can be acquired with or without induction of hMT gene expression by CDDP. Enhanced expression of the *hMT* gene might be involved in acquired CDDP-resistance in TYK/R cells, but involvement of other undetermined factors must also be considered.

The cellular levels of topo I and topo II are closely correlated with drug sensitivity to topo I or II-targeting agents, such as camptothecin, etoposide, teniposide, amsacrine and adriamycin.[22, 23] In our present study, KFr cells had 24.7-fold higher topo II mRNA levels, while TYK/R cells had 1.7-fold higher topo I mRNA levels than the parental counterpart (Table III and Fig. 1). Our previous study demonstrated that a CDDP-resistant cell line from human bladder cancer shows an increased topo I level, resulting in acquisition of collateral sensitivity to camptothecin.[6] Similarly, KFr cells with higher topo II mRNA levels showed a significant collateral sensitivity to etoposide (a topo II inhibitor), while collateral sensitivity to CPT-11 (a topo I inhibitor) was observed only in TYK/R cells with higher topo I mRNA levels. Although TYK/R cells had slightly decreased topo II levels compared to TYK cells, the levels in KFr cells were extremely high when compared to those in KF cells (Fig. 1). Such alterations in topo II expression were cell cycle-independent (data not shown). Alterations of topo II or topo I levels are expected to change DNA structure or conformation, subsequently resulting in modulation of availability to the DNA-targeting anticancer agent, CDDP. The repair of cisplatin-damaged DNA is enhanced in cisplatin-resistant cell lines and repair-deficient mutants are hypersensitive to cisplatin. The enhanced expression of topo I or topo II might promote the repair of cisplatin-damaged DNA, which would result in the loss of drug sensitivity to cisplatin in cisplatin-resistant KFr and TYK/R cell lines. Pleiotropic changes involving GSH levels, decreased CDDP accumulation and enhanced topo I or topo II level are induced during the acquisition of CDDP-resistant phenotype in KFr and TYK/R cells. Lack of a clear pattern of change in gene expression emphasizes the underlying biologic complexity of the CDDP-resistance mechanisms.

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