Jpn. J. Cancer Res. 81, 527–535, May 1990

Determinants of Drug Response in a Cisplatin-resistant Human Lung Cancer Cell Line

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To elucidate the mechanism(s) of cisplatin resistance, we have characterized a human non-small cell lung cancer cell line (PC-9/CDDP) selected from the wild type (PC-9) for acquired resistance to cisplatin. PC-9/CDDP demonstrated 28-fold resistance to cisplatin, with cross resistance to other chemotherapeutic drugs including chlorambucil (×6.3), melphalan (×3.7) and 3-[(4-amino-2-methyl-5-pyrimidinyl)]methyl-1-(2-chloroethyl)-1-nitrosourea (ACNU) (×3.9). There was no expression of *mdr*-1 mRNA in either wild-type or resistant cells. The mRNA and protein levels of glutathione *S*-transferase (GST) π were similar in the two lines. A GST-μ isozyme was present in equal amounts and the activities of selenium-dependent and independent glutathione peroxidase and glutathione reductase were unchanged. The mRNA level of human metallothionein IIA and the total intracellular metallothionein levels were reduced in the resistant cells. Significantly increased intracellular glutathione (GSH) levels were found in the resistant cells (20.0 vs. 63.5 nmol/mg protein) and manipulation of these levels with buthionine sulfoximine produced a partial sensitization to either cisplatin or chlorambucil. Increased GSH probably also played a role in determining cadmium chloride resistance of the PC-9/CDDP, even though this cell line had a reduced metallothionein level. Also contributing to the cisplatin resistance phenotype was a reduced intracellular level of platinum in the PC-9/CDDP. Thus, at least two distinct mechanisms have been selected in the resistant cells which confer the phenotype and allow degrees of cross resistance to other electrophilic drugs.

Key words: Cisplatin resistance — Glutathione — Drug accumulation

It has become apparent that a diversity of genotypic adaptations can lead to the expression of acquired drug resistance in tumor cells. The condition of multidrug resistance (MDR[5]) phenotype has been causally linked with increased expression of a membrane glycoprotein.[1] The overexpression of this P-glycoprotein can result from the treatment of tumors with a number of different natural product-derived anticancer drugs. In some instances, concomitant with P-glycoprotein overexpression is an increase in phase I and II detoxification enzymes in drug-resistant cell lines.[2] With electrophilic drugs such as cisplatin [*cis*-diammine-dichloroplatinum(II)] or nitrogen mustards, there are indications that a number of distinct cellular mechanisms may convey resistance. So far, there have been no indications that cisplatin resistance is associated with the classical MDR phenotype. It has been more common to find primary resistance to cisplatin associated with cross-resistance to nitrogen mustards such as melphalan. Such mechanisms as can account for resistance to alkylating agents include decreased intracellular drug levels, increased scavenging nucleophiles and increased repair of nucleic acid damage. A number of cell lines with acquired resistance to cisplatin demonstrate a qualitative inverse relationship between drug sensitivity and total intracellular cisplatin concentration.[3–8] Although ciplatin is believed to enter cells by passive diffusion, an alternative drug accumulation mechanism which may require energy was suggested.[6] The same authors suggested that alterations in drug uptake may be one of the simplest and earliest events leading to cisplatin resistance, although the inheritance of such a trait(s) appears to be recessive in nature, at least in cultured murine L1210 cells.[3] Because the measurement of intracellular platinum levels does not identify the biochemical form of the drug and is affected by a number of conditions, some caution is required when attempting to interpret drug accumulation data.

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[5] The abbreviations used are: MDR, multidrug resistance; BSO, DL-buthionine-*S,R*-sulfoximine; GSH, glutathione; GST, glutathione *S*-transferase; NSCLC, non-small cell lung cancer; CuOOH, cumene hydroperoxide; ACNU, 3-[(4-amino-2-methyl-5-pyrimidinyl)]methyl-1-(2-chloroethyl)-1-nitrosourea; FBS, fetal bovine serum; PBS, calcium-free and magnesium-free Dulbecco's phosphate-buffered saline; CDNB, 1-chloro-2,4-dinitrobenzene; MOPS, 3-(N-morpholino)propanesulfonic acid; BSA, bovine serum albumin fraction V; SDS, sodium dodecyl sulfate; ATCC, American Type Culture Collection; PAGE, polyacrylamide gel electrophoresis.

The role of thiol-containing scavenger molecules in governing resistance to cisplatin has been established primarily by demonstrating increased sensitivity of drug-resistant cell lines pretreated with DL-buthionine-*S,R*-sulfoximine (BSO).[9,10] Frequently, resistance to cisplatin is accompanied by cross-resistance to alkylating agents such as melphalan and similar sensitization to the mustard can be achieved through glutathione (GSH) depletion with BSO.[9,10] Comparison of these two agents is complicated by the role of glutathione *S*-transferase (GST) in determining the rate and extent of drug conjugation with GSH. In the case of mustards, overexpression of the α-GST isozyme has been found in resistant lines[11] and the role of GST's in catalyzing the conjugation of melphalan to GSH has been established.[12] For cisplatin, our earlier work has shown the overexpression of GST π in a human non-small cell lung cancer (NSCLC) cell line, which is inherently resistant to cisplatin,[13] but there is no indication that this isozyme has a role in catalysis of cisplatin conjugation to GSH.

In addition to GSH, another ubiquitous thiol-containing molecule, metallothionein, has also been shown to be involved in cisplatin resistance.[14,15] This class of protein thiol has clusters of thiolates which show varying affinities for heavy metals. Cross-resistance of cells to cadmium salts and cisplatin has been reported in human ovarian and colon carcinoma cell lines.[14] Furthermore, cisplatin, in at least one instance, can cause overexpression of metallothionein mRNA and protein itself.[15] The precise mechanism by which platinum displaces metals already bound to metallothionein and the actual quantitative relationship of metallothionein overexpression to resistance require further clarification.

What is readily apparent from the diversity of existing literature on cisplatin resistance is that multiple mechanisms can account for varying quantitative degrees of resistance. In the present report we have substantiated this indication by showing that no single alteration appears to account quantitatively for resistance to cisplatin or cross resistance to other agents.

MATERIALS AND METHODS

Drugs and chemicals RPMI 1640 medium and PBS were purchased from Nissui Pharmaceutical Co., Tokyo. Chlorambucil, melphalan, BSO, and CuOOH were purchased from Sigma Chemical Co., St. Louis, MO. Chlorambucil was prepared in 100% ethanol just before use. Melphalan was dissolved in serum-free medium heated to 55°C just before use. Cisplatin was obtained from Brystol Myers Co., Tokyo and was prepared in 0.9% NaCl just before use. 3-[(4-Amino-2-methyl-5-pyrimidinyl)]methyl-1-(2-chloroethyl)-1-nitrosourea (ACNU) was purchased from Sankyo Co., Tokyo and was prepared in distilled water just before use. [α-^{32}P]-dCTP was purchased from Amersham Japan, Tokyo.

Cell lines and tissue culture PC-9 and PC-9/CDDP human adenocarcinoma lung cells were grown as half-attached and half-suspension culture in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Immuno-Biological Laboratories, Fujioka), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 at 37°C. PC-9 cell was a generous gift of Dr. Y. Hayata, Tokyo Medical College. An approximately 8-fold cisplatin-resistant cell line, PC-9/0.5 was established as previously described by exposure of PC-9 cells to stepwise increasing cisplatin concentrations.[16] A further 15 months' continuous exposure of PC-9/0.5 cells to 0.5 μg/ml of cisplatin established PC-9/CDDP (28-fold cisplatin-resistant cell line). Doubling times of both cells were the same. The degree of cisplatin resistance in the PC-9/CDDP cell line was stable for at least nine months during subculture in cisplatin-free medium (data not shown). The cisplatin-resistant cell lines were used in this study after 1–9 months of continuous growth in cisplatin-free medium. An adriamycin-resistant human myelogenous leukemia cell line, K562/ADM, was kindly provided by Dr. T. Tsuruo, University of Tokyo. Total RNA of K562/ADM was used as a positive control for *mdr*-1 expression.

Survival studies Clonogenic cell survival was assessed in a double-layer soft agar system, previously described.[17] PC-9 and PC-9/CDDP were plated at a concentration of 10,000 cells/well, yielding a plating efficiency of 18–20%. After plating the upper layer, plates were incubated at 37°C in humidified air with 5% CO_2 for 10 days. Colonies of more than 50 μm in diameter were counted by using an automatic particle counter (CP-2000, Shiraimatsu Co., Tokyo). All clonogenic assays were conducted using continuous drug exposure. Each experiment was performed in triplicate and repeated more than three times. The degree of cytotoxicity was expressed as a % colony survival curve versus drug concentration, and IC_{50} values were defined as drug concentrations inhibiting colony formation by 50% compared to control plates.

BSO treatment To deplete the GSH in the human lung cancer cells, BSO was added to flasks of cells to give a final concentration of 0.1 m*M*. After 24 h, cells were harvested, and used either for clonogenic assay or for GSH measurement. This BSO exposure protocol produced no toxicity in either wild-type or resistant cells (data not shown). The degree of sensitization by BSO treatment was expressed as a dose modification factor, defined as the IC_{50} of the control cell line divided by the IC_{50} of the glutathione-depleted cell line.

Sample preparation Cells in late-log phase were harvested, washed twice with a cold calcium-free and magnesium-free Dulbecco's phosphate-buffered saline (PBS), and centrifuged at 1000 rpm, then the cell pellets were frozen and stored at −80°C until use.

GSH- and glutathione-related enzyme assays All stock cell pellets were sonicated using a Branson Model 450 sonifier for 30 s at a setting output control of 2. Total GSH content was measured by the method of Griffith.[18] Total GST activity was measured by the method of Habig *et al.*[19] with 1 m*M* 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate. Glutathione reductase was assayed spectrophotometrically, by the method of Worthington and Rosemeyer.[20] Glutathione peroxidase activity was assayed using H_2O_2 or CuOOH by the method of Paglia and Valentine.[21]

Metallothionein determination Metallothionein contents were determined by the ^{203}Hg-binding assay as previously described.[22]

Protein determination Protein was measured by the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL).

Cisplatin accumulation For drug accumulation studies, parental and resistant cells in late-log phase were harvested and seeded into 75 cm^2 tissue culture flasks (Corning Glass Works, Corning, NY) at a density of 2×10^6 cells/ml. After a one-hour preincubation period, they were incubated with 5 μg/ml of cisplatin in RPMI 1640 medium supplemented with 10% fetal bovine serum in a 37°C/5% CO_2 incubator for 1, 2, 4, and 8 h. The cisplatin concentration, 5 μg/ml, was chosen in order to obtain measurable platinum levels in this experimental setting. At the end of each time period, cells were collected by centrifugation and washed 3 times with 4°C PBS. The cell pellets were digested in nitric acid at 80°C for 5 h, and then platinum was chelated with sodium diethyldithiocarbamate followed by extraction with chloroform. The cell extracts were analyzed for platinum by atomic absorption spectrophotometry using a Hitachi polarized Zeeman atomic absorption spectrophotometer, Model Z-7000 (Hitachi, Tokyo).

Nucleic acid analysis Total RNA was isolated from late-log phase cells by acid guanidinium thiocyanate-phenol-chloroform extraction[23] and the RNA was size-fractionated by electrophoresis on 1% agarose gel that contained 6% formaldehyde with 40 m*M* 3-(N-morpholino)propanesulfonic acid (MOPS) buffer containing 5 m*M* sodium acetate and 0.5 m*M* EDTA. The RNA was transferred to positively charged nylon membrane (Hybond-N+, Amersham Japan Co.). The filter was pre-hybridized at 42°C in 50% formamide, 0.65 *M* NaCl, 0.1 *M* Na-PIPES (pH 6.8), 5×Denhardt's solution (1×Denhardt's solution: 0.02% Ficoll, 0.02% polyvinylpyrolidone, 0.02% bovine serum albumin fraction V (BSA)), 0.1% sodium dodecyl sulfate (SDS), 5 m*M* EDTA and 100 μg/ml denatured salmon sperm DNA. Hybridization was performed overnight at 42°C in the same solution containing 10% dextran sulfate and ^{32}P-labeled DNA probe. Probes were labeled with [α-^{32}P]dCTP to a specific activity of 1×10^9 dpm/μg DNA using the multiprime DNA labeling system kit (Amersham Japan Co.). After hybridization, the filter was washed in 2×SSC (1×SSC: 0.15 *M* NaCl, 0.015 *M* sodium citrate) and 0.1% SDS for 10 min with three changes at room temperature, 0.1×SSC and 0.1% SDS for 15 min with three changes at 65°C, and then in 0.1× SSC for several minutes. Autoradiograms were obtained at −80°C on Amersham Hyperfilm-MP with intensifying screens. The probes used were as follows: pGPi2, coding for the human GST-π was kindly provided by Dr. M. Muramatsu, University of Tokyo.[24] A probe coding for the human *mdr*-1, pMDR-1, was a generous gift of Dr. I. B. Roninson, University of Illinois.[25] hMT-IIA, which encodes human metallothionein IIA,[26] was obtained from the American Type Culture Collection (ATCC) (Rockville, MD).

Western blot analysis Cell pellets (5×10^6 cells) stocked at −80°C were suspended in 500 μl of cold PBS, sonicated as described above and then centrifuged at 18,500*g* for 20 min. The supernatant was analyzed on 12.5% polyacrylamide slab gels containing SDS.[27] The samples were applied to the gel at equivalent protein content. Following electrophoresis, the proteins on the gels were electrophoretically transferred to nitrocellulose membranes[28] which were then placed in 50 m*M* Tris/400 m*M* NaCl (pH 7.5) buffer containing 0.05% Tween 20 and rinsed. All rinses were for 45 min with three changes of buffer and all incubations were for 1 h. Membranes were incubated with 50 m*M* Tris/400 m*M* NaCl (pH 7.5) containing 3% BSA followed by the antibody against each GST subunit in the same buffer (diluted 1:1000 for GST-π, 1:500 for GST-α, μ). Membranes were rinsed and incubated with peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories, Richmond, CA) in the buffer containing 3% bovine serum albumin. After rinsing, blots were developed using 1-chloro-4-napthol and H_2O_2 in the buffer containing 11% methanol. The reaction was stopped by rinsing with water. Anti GST-π rabbit polyclonal antibody[29] and anti Ya and Yb1 rabbit polyclonal antibody[30] were generous gifts of Dr. K. Sato, Hirosaki University.

RESULTS

The comparative chemosensitivity to various drugs of the wild-type and resistant cell lines was estimated by a colony-forming assay and is summarized in Table I. The resistant PC-9/CDDP line showed an overall, although

variable, resistance to a range of alkylating drugs. Maximum resistance was expressed to the selecting agent, cisplatin (28.1-fold at the IC_{50} values). Cross resistance to the nitrogen mustards, chlorambucil ($\times 6.3$), melphalan ($\times 3.7$) and ACNU ($\times 3.9$) was less. Because levels of intracellular GSH were elevated in the resistant line (Table III), pretreatment of cells with γ-glutamylcysteine synthetase inhibitor, BSO, was car-

Table I. Comparative Cytotoxicity of Various Agents in the PC-9 and PC-9/CDDP Human Lung Cancer Cell Lines

Drug	IC_{50} (μg/ml)[a]		Resistance ratio[b]
	PC-9	PC-9/CDDP	
Cisplatin	0.22±0.05[c]	6.2±0.4[d]	28.1
Chlorambucil	9.1±0.9	56.9±0.7[d]	6.3
Melphalan	14.0±3.5	52.3±2.7[e]	3.7
ACNU	52.3±2.8	202.7±16.7[e]	3.9
Cadmium chloride	9.8±0.5	19.4±0.5[d]	2.0

a) Drug concentration that inhibits colony formation by 50%.
b) The resistance ratio equals the IC_{50} of the resistant cell line divided by the IC_{50} of the parental cell line.
c) Each value is the mean±SD of the three independent experiments.
d) $P=0.0001$ compared to value for PC-9 (unpaired two-tailed Student's t test).
e) $P=0.0002$ compared to value for PC-9 (unpaired two-tailed Student's t test).

Table II. Potentiation of Cisplatin and Chlorambucil Cytotoxicity by Glutathione Depletion

Cell line	Cisplatin IC_{50} (μg/ml)		Chlorambucil IC_{50} (μg/ml)	
PC-9	0.22±0.05[a]	(1.8)[b]	9.1±0.9	(2.6)
PC-9, BSO(+)	0.12±0.02[c]		3.5±0.7[d]	
PC-9/CDDP	6.2±0.4	(2.4)	56.9±0.7	(3.5)
PC-9/CODP, BSO(+)	2.6±0.3[d]		16.4±2.3[d]	

Drug cytotoxicity was determined by clonogenic assay. Glutathione was depleted with 100 μM BSO.
a) Mean±SD of three independent experiments.
b) Numbers in parentheses, dose modification factor; defined as the IC_{50} of the control cell line divided by the IC_{50} of the glutathione-depleted cell line.
c) $P<0.05$ compared to value for PC-9 (unpaired two-tailed Student's t test).
d) $P<0.005$ compared to value for PC-9/CDDP (unpaired two-tailed Student's t test).

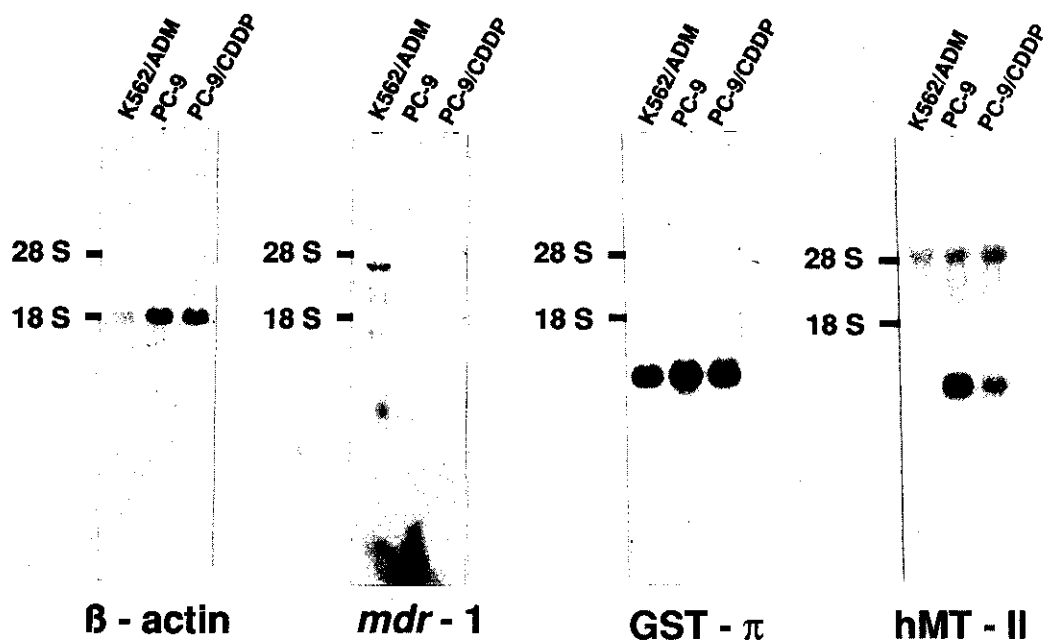

Fig. 1. Expression of *mdr*-1, GST-π, and metallothionein IIA mRNA in the PC-9 and PC-9/CDDP cells. Samples of total RNA (10 μg each) were electrophoresed in a 1% formaldehyde-agarose gel, transferred onto a nylon membrane and hybridized with each ^{32}P-labeled DNA probe. K562/ADM cells were used as a positive control to detect *mdr*-1 gene expression. All hybridization was performed using the same filter and hybridization with β-actin was used to show equivalent loading of total RNA of PC-9 and PC-9/CDDP cells.

ried out to ascertain the importance of GSH in determining drug response. Table II shows that at a BSO concentration which depleted approximately 90% of the intracellular GSH (Table III), sensitization of both PC-9 and PC-9/CDDP to either cisplatin or chlorambucil was achieved. It should be noted that depletion of GSH in the PC-9/CDDP to 6.2 nmol/mg protein was achieved and this level, although lower than that in the wild type (20.0 nmol/mg protein; Table III), did not produce a degree of sensitivity which was equivalent to that of the wild type (Table II). Resistance to chlorambucil was also influenced by BSO pretreatment. In this case, the differential sensitization of the PC-9/CDDP by BSO produced a dose modification factor of 3.5 (Table II). This value was closer to the 6.3-fold resistance, suggesting that the increased GSH was an important contributing factor in determining resistance to chlorambucil.

A Northern blot analysis was undertaken to determine if any genes were overexpressed in the resistant cell line. Fig. 1 compares the mRNA levels for *mdr*-1, GST-π and metallothionein IIA. Equivalent loading of total RNA of both PC-9 and PC-9/CDDP cells was estimated using a β-actin probe. For *mdr*-1, K562/ADM-resistant cells were used as a positive control. For both PC-9 and PC-9/CDDP, negligible expression of the *mdr*-1 gene was found. Southern blot analysis showed that both cells did have the *mdr*-1 gene (data not shown). In terms of GST-π mRNA levels, there was no difference between the wild-type and resistant cells. Using SDS-polyacryl-

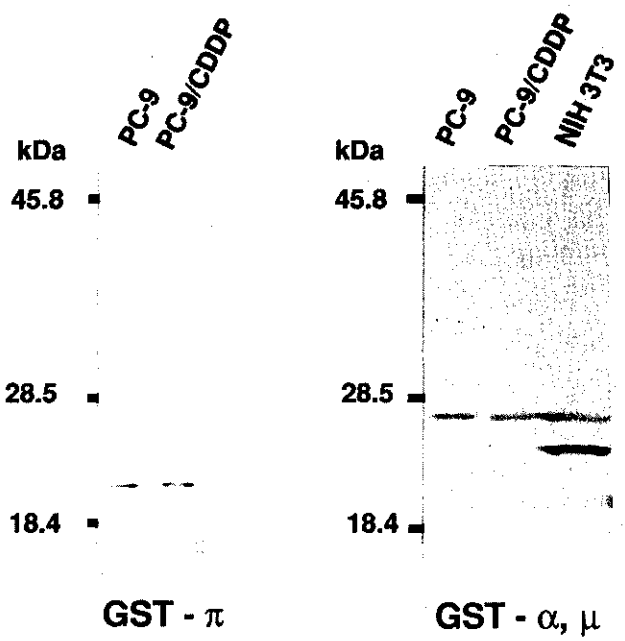

Fig. 2. Western blot analysis of GST isozyme content in the PC-9 and PC-9/CDDP cells. Cell extracts (10 μg protein/lane) were separated on 12.5% SDS-PAGE, transblotted and probed with each polyclonal rabbit anti-GST antibody, as described in the text. NIH3T3 cells were used as a positive control to detect α- and μ-GST subunits. Molecular weight markers are indicated to the left.

Table III. Thiol Content and Glutathione-related Enzyme Activity in the PC-9 and PC-9/CDDP Human Lung Cancer Cell Lines

Thiol content and enzyme activity	PC-9	PC-9/CDDP
Glutathione (nmol/mg protein), n=8	20.0±0.9[a]	63.5±8.5[b]
Glutathione after BSO treatment (nmol/mg protein), n=3	1.8±0.1	6.2±1.0
Metallothionein (pmol/mg protein), n=3	41.5±1.5	12.5±0.9[b]
Glutathione *S*-transferase (nmol/min/mg protein), n=10	190.3±31.6	143.5±21.1[b]
Glutathione reductae (nmol NADPH/min/mg protein), n=3	43.2±1.5	44.1±0.7[c]
Glutathione peroxidase (nmol NADPH/min/mg protein), n=3		
H_2O_2	10.5±0.8	12.8±0.6[c]
CuOOH	11.5±0.1	12.7±0.4[c]

n equals the number of experiments.
a) Mean±SD.
b) P=0.0001 compared to value for PC-9 (unpaired two-tailed Student's t test).
c) Not significant.

Table IV. Accumulation of Platinum from Cisplatin in the PC-9 and PC-9/CDDP Human Lung Cancer Cell Lines

Time (h)	Platinum content (ng Pt/mg protein)	
	PC-9	PC-9/CDDP
4	17.0±0.5[a)]	13.8±3.2
8	48.6±2.1	29.1±6.5
12	84.9±3.3	45.0±6.0

a) Each value is the mean±SD of the three independent experiments.

amide gel electrophoretic (PAGE) separation and Western blot analysis against a series of antibodies (Fig. 2), high but equal amounts of the GST-π isozyme were confirmed. Additional analysis of the basic GST isozymes using previously characterized polyclonal antibodies showed the presence of an M1 (lower band, Fig. 2) and M3 (upper band) subunit in the mouse NIH3T3 fibroblast cell line. This cell line served as a positive control. By comparison, the two human lung cancer cell lines expressed only the μ-subunit and at equivalent amounts. The apparent Mr of 27,000 and the migration of the protein between the mouse isozymes is consistent with a human μ-subunit identity. Overall GST activity, using CDNB as the substrate suggested a slightly decreased activity in the resistant cells (Table III). Similarly, metallothionein levels, measured either in terms of the protein (Table III) or as mRNA (Fig. 1), were decreased in the resistant cell line. Both glutathione reductase and glutathione peroxidase (total and selenium dependent) were of similar activity in the wild-type and resistant cell lines (Table III).

Table IV shows the intracellular platinum accumulation versus time. The accumulation of platinum in the resistant cells was significantly lower compared to the PC-9 cells. The platinum accumulation continued to rise for a 12 h period and did not show signs of reaching equilibrium.

DISCUSSION

It is apparent that selection of acquired resistance to cisplatin created a mutant cell line which expresses cross resistance to a broad range of alkylating agents. The maximum extent of resistance was to the selecting drug cisplatin, 28-fold. The resistance ratio to nitrogen mustards, ACNU and cadmium chloride was considerably reduced, suggesting that a plurality of determinants may be responsible for the primary resistance.

In order to assess the multifactorial nature of cisplatin resistance, seven distinct, although potentially overlapping, mechanisms were studied. Initially, the classical MDR phenotype exemplified by increased mRNA for the *mdr*-1 gene was not apparent in the resistant cells. This was not surprising, since there has been little indication, to date, that alkylating agents are linked to the selection of MDR mutants.

Recent reports have, however, suggested that elevations in metallothionein occur as a result of cisplatin selection[15] and can act as a nucleophilic sink for heavy metals such as cadmium, cobalt, bismuth, mercury, nickel, copper, silver and gold.[31] By extrapolation, platinum has been shown to be an adequate substrate for metallothionein[32] and cells which have been made resistant to cadmium, frequently show cross-resistance to cisplatin and other alkylators.[14, 33] In general, no direct qualitative relationship between metallothionein overexpression and degree of resistance has been found. In contradistinction to the previous data, the PC-9/CDDP cell line showed a down-regulation of metallothionein mRNA and subsequent decrease in the overall translated protein product. The greater than 3-fold decrease in metallothionein did not seem to convey any increased sensitivity to cadmium chloride. Indeed, the resistant cells maintained a marked degree of resistance to cadmium chloride, presumably reflecting the potential for the increased GSH to protect against the toxic effects of the metal salts. This possible protective role of GSH against cadmium chloride was also suggested by others.[34] Thus, GSH may obviate or replace the requirement for metallothionein in this cell line.

Enzyme systems associated with GSH have also been implicated in a number of instances of drug resistance. Earlier studies have associated GST-α overexpression with resistance to nitrogen mustards,[11] GST-μ with nitrosoureas[35] and GST-π with adriamycin.[2] An increased expression of GST-π has also been found in NSCLC cell lines which are intrinsically resistant to cisplatin,[13] although there is no theoretical or experimental indication that cisplatin acts as a substrate for the isozyme. Indeed, the transitional intermediate aziridinium ion may well be critical to the substrate specificity of GST-α subunits for nitrogen mustards; such a metabolite would not be expected as a cisplatin metabolite. Thus, it would seem that the presence of GST-π in the NSCLC lines[13] may be an effect rather than a cause of resistance. Certainly, in the PC-9/CDDP cells there is no indication of increased transcription or translation of the GST-π gene. Similarly, both the resistant and sensitive cells express a GST-μ subunit to an equal degree. The overall GST activity, together with the glutathione reductase and glutathione peroxidase activities, remains statistically similar between the wild-type and resistant cells. This would be consistent with the fact that cisplatin exposure did not create oxidative conditions which could contribute towards an overt cellular stress response, such

as has been identified previously,[36] although the inducing agent was adriamycin not cisplatin.

The two most important determinants of resistance in the PC-9/CDDP cell line appear to be increased level of intracellular GSH and decreased cisplatin accumulation. Although the exact mechanism by which GSH modulates cisplatin cytotoxicity is unknown, a previous study has shown that GSH binds to cisplatin.[37] Thus, an increased level of intracellular GSH may form more GSH-cisplatin conjugates and consequently obviate the binding of cisplatin to DNA, a critical target of cisplatin cytotoxicity. A recent report has suggested that GSH has some role in DNA repair,[38] so it is possible that elevated GSH may contribute to increased DNA repair activity in the resistant cells. On the other hand, BSO depleted the GSH of the resistant line to a level approximately equal to that of the wild type. The degree of sensitization achieved was not commensurate with the degree of resistance (Table II), implicating the involvement of at least one additional mechanism. This would appear to be at least partly fulfilled by the decreased intracellular platinum concentrations found in the resistant cells. The atomic absorption spectroscopy technique estimates total platinum and can not distinguish either the chemical form of the drug or whether it is bound or unbound. Nonetheless, it is apparent that the uptake and accumulation of platinum with time is significantly less in the resistant cell line compared to the wild type. This has been reported in other cisplatin-resistant human tumor cell lines,[3-8] although the precise mechanism(s) of the reduction has yet to be elucidated. A number of factors can confuse the interpretation of the intracellular platinum data. These include the balance between influx and efflux, reversible protein binding, intracellular compartmentalization, binding of the ultimate electrophile to a cellular target, and repair and removal of the ultimate damage. Whatever the precise reason(s) for reduced intracellular platinum, it is reasonable to conclude that this will have an impact upon overall cell survival and thus contribute to the resistant phenotype.

Recently, there have been several reports that suggest a role for increased DNA repair activity as a factor in cisplatin resistance.[38,39] Using an alkaline elution method, we have shown that the ability to repair cisplatin-induced interstrand cross-links was identical in both cisplatin-sensitive and -resistant cells.[40] The activity for repair of intrastrand cross-links, major cisplatin-DNA adducts, has yet to be elucidated and experiments analyzing more precisely the repair mechanism of cisplatin-induced DNA damage are under way in our laboratory.

In summary, cisplatin has selected a resistant mutant which has at least two distinct alterations from the wild type. Although many common factors associated with other types of drug resistance remain unchanged, modified drug accumulation and increased protective thiols contribute significantly to the resistant phenotype. Some of the same properties which allow for cisplatin resistance also convey a cross resistance to other electrophilic agents, suggesting that the therapeutic utility of alkylating agents may be impaired by prior exposure to selective pressures provided by cisplatin treatment.

ACKNOWLEDGMENTS

This research was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare for the Comprehensive 10-Year Strategy for Cancer Control, a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan and funds from the Adult Disease Memorial Foundation. We thank Dr. Nobumasa Imura, Dr. Masahiko Sato, and Dr. Sei-ichiro Himeno (Kitasato University) for their assistance with the measurements of metallothionein, glutathione reductase and glutathione peroxidase.

(Received December 8, 1989/Accepted February 24, 1990)

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