Increased Expression of Glutathione S-Transferase Gene in cis-Diamminedichloroplatinum(II)-resistant Variants of a Chinese Hamster Ovary Cell Line

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ABSTRACT

We have isolated cis-diamminedichloroplatinum(II) (CDDP)-resistant variants, C/CDP-1 and C/CDP-2, from a Chinese hamster ovary (CHO) cell line after a stepwise exposure to increasing concentrations of CDDP, and a CDDP-sensitive revertant, R-1, from C/CDP-2 after continuous incubation for 5 months in the absence of CDDP. C/CDP-1 and C/CDP-2 showed 7- and 10-fold higher resistance to CDDP, respectively, compared to CHO cells. C/CDP-2 was cross-resistant to carboplatin, 1-phenylalanine mustard (melphanal), and CdSO4, but not to other anticancer agents. Alkaline elution of DNA showed an increased amount of DNA interstrand cross-linking formation in CHO cells, but not in C/CDP-2 cells, when CHO and C/CDP-2 cells were cultured with CDDP. By contrast, alkaline elution of DNA showed increased formation of DNA cross-links when nuclei of C/CDP-2 cells were treated with CDDP. The activity of glutathione S-transferase (GST) of C/CDP-1 and C/CDP-2 was 4- and 6-fold higher than that of CHO cells, respectively. The cellular level of GST activity of R-1 was almost similar to that of CHO cells. Northern blotting analysis revealed that GST-γ mRNA in both C/CDP-1 and C/CDP-2 cell lines was increased more than 5-fold over that of CHO and R-1 cells. There is no apparent gene amplification of GST-γ gene in CDDP-resistant cell lines. Immunoblot assays showed a specific increase of GST-γ in C/CDP-1 and C/CDP-2, but no increase in GST-M and GST-α. We also compared CDDP-resistant properties of a resistant variant, P/CDP-5, derived from human prostate cancer PC-3 cell line, with those of C/CDP-1 and C/CDP-2 cells and found no increased GST activity in P/CDP-5 cells. Multiple mechanisms might be considered for acquisition of CDDP resistance in various cell lines in culture.

INTRODUCTION

Treatment with CDDP has improved chemotherapeutic effects against various tumors (1). CDDP interacts with its target DNA, causing interstrand cross-links and intrastrand cross-links (2, 3). Acquisition of drug resistance to alkylating agents such as CDDP is developed more slowly and to much lower levels than that to antimetabolite or antibiotic anticancer agents (4). However, the acquisition of CDDP resistance may limit the chemotherapeutic effects of CDDP against various malignant tumors. Establishment of CDDP-resistant cell lines should provide a clue to understanding the underlying mechanisms for the acquired resistance to CDDP in vivo. In this study, we have isolated CDDP-resistant cell lines from CHO cells after stepwise selection in the presence of various concentrations of CDDP and have identified an altered biochemical target in these cell lines. We also compared biochemical properties of a CDDP-resistant cell line which was isolated from a human prostate cancer cell line.

MATERIALS AND METHODS

Isolation of CDDP-resistant Variants of CHO Cells and Cell Culture. CHO cells were grown in monolayer in minimal essential medium (Nissui Seiyaku Co., Tokyo, Japan) containing 10% newborn calf serum (Microbiological Associates, Bethesda, MD), 1 mg/ml Bacto-peptone (Difco Laboratories, Detroit, MI), l-glutamine (0.292 mg/ml), kanamycin (100 μg/ml), and penicillin (100 units/ml) (5, 6).

CDDP-resistant variants of CHO cells were isolated by stepwise selection on exposure to increasing doses of CDDP. CHO cells were exponentially grown at 10^6/100-mm dish, and CDDP was then added to the medium for 3-week intervals at increasing concentrations of 0.2, 0.5, 1.0, 1.5, 2.0, and 4.0 μg/ml CDDP. During the continuous exposure to CDDP, culture medium was replaced with freshly prepared medium containing CDDP at indicated concentrations every 4–5 days.

Colonies selected at each step when exposed to 1.5 and 4.0 μg/ml CDDP were purified, and each purified clone was named C/CDP-1 and C/CDP-2, respectively. CDDP-sensitive revertants were isolated from C/CDP-1 and continuous incubation of C/CDP-2 for 5 months in the absence of CDDP, and a purified revertant was named R-1. We have also isolated a CDDP-resistant cell line, P/CDP-5, from a human prostate cancer PC-3 cell line following the same selection method as that for C/CDP-1 or C/CDP-2.

Chemicals. CDDP and VP-16 were obtained from Nihon Kayaku Company, Tokyo, Japan; carboplatin and teniposide were from Bristol Meyer Co., Kanagawa, Japan; Adriamycin and CdSO4 were from Sigma Chemical Co., St. Louis, MO; mitomycin C and melphalan were from Kyowa Hakko Co., Tokyo, Japan. l-Chloro-2,4-dinitrochlorobenzene was purchased from Nakarai Chemicals, Ltd., Kyoto, Japan. 1^4C]-Thymidine (53.2 μCi/mmol) and 1^H]-thymidine (20 Ci/mmol) were purchased from New England Nuclear, Boston, MA.

Colony Formation and Growth Curves. To assay colony formation, we first seeded 300 cells of CHO, R-1, and 3000 cells of C/CDP-1, C/CDP-2, and P/CDP-5 in a 35-mm dish in the absence of drugs at 37°C for 18 h. Cells were incubated for an additional 7 days with various drugs. Colonies appeared with a plating efficiency of 70–80% (CHO, R-1, and PC-3) and 7–8% (C/CDP-1, C/CDP-2, and P/CDP-5). Colony number was counted after Giemsa staining (6, 7). Drugs were freshly prepared in dimethyl sulfoxide or ethanol and all control experiments were done by adding the same amount of the solvent. To calculate relative resistance to various anticancer agents, the dose required to reduce the surviving fraction to 10% of the initial fraction (LD50) of each cell line was compared with that of each parental cell line. To assay growth curves, 10^5 cells were plated and the next day the cells were exposed to 0.5, 1.0, or 3.0 μg/ml of CDDP for an additional 4 days. Medium was changed with freshly prepared CDDP every 2 days and the number of surviving cells was counted by trypan dye exclusion.

Alkaline Elution Assay. The alkaline elution procedure was done according to the published method (8, 9). Cells (3 × 10^6) were seeded in 35-mm dishes and incubated at 37°C for 24 h in 1 ml of minimal essential medium containing 0.02 μCi [1^H]-thymidine. The sample cells exposed to [1^H]-thymidine were then exposed to various concentrations of CDDP for 2 h and irradiated on ice prior to elution. Control cells which were not treated with CDDP were incubated with 0.2 μCi [1^H]-thymidine for 2 h and irradiated on ice prior to elution. Cells were incubated for an additional 3 days. Cells were collected at 24 h following irradiation and elution was started by the addition of 0.5 ml of 0.3 M NaOH and 2% SDS. The sample was incubated at 65°C for 2 h, and alkali-labile DNA was isolated on a CsCl gradient.
thymidine and irradiated. An equal number (3 x 10^4 cells) of the sample cells and the control cells were applied onto polyvinyl chloride filter (2 µm pore size), lysed with SDS-NaCl-EDTA solution (40 mM EDTA-2 m NaCl-0.2% sodium dodecyl sarcosine at pH 10.0) and washed with the SDS-NaCl-EDTA solution containing 0.5 mg/ml proteinase K. The DNA was eluted from the filters at a rate of 0.07 ml/min using a solution of 20 mM EDTA (free acid) at pH 12 with tetrappropylammonium hydroxide. Fractions were collected at 1-h intervals over 6 h. The radioactivity of each eluted fraction and the remaining radioactivity on the filter were counted.

Alkaline elution with isolated nuclei was also followed. CHO or C/CDP-1 cells labeled with radioactive thymidine were treated for 10 s with 5 ml detergent-buffer containing 1% Nonidet P-40, 30 mM HEPES, 200 mM sucrose, 40 mM NaCl, 5 mM MgCl2, and 5 mM ethylene glycol bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid, pH 8.0. Adhering nuclei were washed once with phosphate-buffered saline and scraped into 50 mM HEPES, pH 7.5, containing 10% sucrose. Nuclei were obtained after centrifugation at 3000 rpm for 10 min at 4°C and resuspended in 50 mM HEPES containing 10% sucrose at 10^4 nuclei/µl. Nuclei of [1^4]Cthymidine-labeled cells were incubated with various concentrations of CDDP for 2 h at 37°C and then irradiated with γ-rays. Control samples of [3H]thymidine-labeled nuclei were not treated with CDDP but were irradiated. Equal numbers of the control and sample nuclei from 1 x 10^6 cells, respectively, were assayed for alkaline elutions as described above.

GST Assay. GST activity was measured by the method of Habig et al. (10). Cells were sonicated and centrifuged at 15,000 x g at 4°C for 30 min and the supernatant was assayed for GST activity when 1-chloro-2,4-dinitrochlorobenzene was used as substrate.

DNA and RNA Analysis with GST-α cDNA Probe. High molecular weight DNA was isolated from each cell line by the method of Maniatis et al. (11), and cytoplasmic RNA was isolated according to standard procedures (12). Rat GST-α cDNA (pGP-5) (13) was used as probe. DNA was digested with a restriction enzyme, EcoRI, electrophoresed on 0.7% agarose, and transferred to nitrocellulose filters by the method of Southern (14) for Southern blot analysis. To assay Northern blot hybridization, 10-µg RNA samples were electrophoresed in 1% agarose 2.2 M formaldehyde gels and transferred to nitrocellulose filters (15). A 32P-labeled cDNA fragment with a specific activity of 10^3 cpm/µg DNA was prepared by a random primed method. Hybridization was carried out in 50% deionized formamide, 10 x Denhardt's buffer, 5 x standard saline-citrate (SSC; 1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and 0.1% SDS at 30°C for 36 h (15). The filter was washed twice in 2 x standard saline-citrate and 0.1% SDS at room temperature.

Western Blot Analysis of GSTs. Cytosol protein fractions extracted from 10^7 cells were run on 10% SDS-PAGE (80 µg protein/lane). Protein fractions on SDS-PAGE were electrophoretically transferred onto nitrocellulose filters in 25 mM HEPES, 92 mM glycine, and 20% methanol for 2 h at 20 V. Nitrocellulose membranes were further incubated with antibodies against human GST-α, GST-μ, and GST-α (1:2000) for 1 h at room temperature. The membranes were rinsed with Tris-buffered saline, treated with biotinylated secondary antibody, and then developed according to the manufacturer's specification (Vecstain ABC-Go kit; Vector Laboratories, Inc., California). Antibodies raised against GST-α, GST-μ, and GST-α were kindly donated to us by Drs. S. Tsuchida, K. Satoh, and K. Sato (Hirosaki University School of Medicine) (16, 17).

RESULTS

Two CDDP-resistant variants, C/CDP-1 and C/CDP-2, were derived from CHO cells. We compared the effect of CDDP on the growth curve of C/CDP-1 and C/CDP-2 with that of CHO cells. Fig. 1 shows growth curves of CHO, C/CDP-1, and C/CDP-2 cells in the absence or presence of 1.5 and 3.0 µg/ml CDDP. Both C/CDP-1 and C/CDP-2 cells grew with a doubling time of 30 h while CHO cells grew with one of 20 h. Addition of 1.5 and 3.0 µg/ml CDDP to culture medium of CHO cells inhibited growth immediately. By contrast, there appeared to be only a slight inhibition of growth of C/CDP-1 and C/CDP-2 cells in the presence of 3.0 µg/ml CDDP (Fig. 1). Dose-response curves of CHO, C/CDP-1, C/CDP-2, and R-1 cells to CDDP. Three hundred cells of CHO (O) and R-1 (■) types and 3000 cells of C/CDP-1 (▲) and C/CDP-2 (△) were respectively seeded in the absence of CDDP for 18 h. Then the cells were exposed to various concentrations of CDDP and incubated for 7 days. The number of colonies was counted after Giemsa staining. Values are mean ± SD (bars) of triplicate dishes.
creased formation of the cross-links as a function of CDDP. Alkaline elution patterns of DNAs of CHO cells showed increased formation of DNA cross-links as a function of CDDP. In particular, interstrand DNA cross-linking formation was assayed alkaline elutions of CHO and C/CDP-2 cells with various doses of CDDP. As seen in Fig. 4, alkaline elution difference in the formation of DNA-protein cross-links between CHO cells and their CDDP-resistant counterpart. The amount of DNA interstrand cross-linking formation was determined by alkaline elution as described in Fig. 3. The sample (PC-3 or P/CDDP-5) and control (CHO) cells were respectively incubated with [3H]thymidine and [14C]thymidine, and sample [14C]thymidine-labeled cells of PC-3 (A) and P/CDDP-5 (B) were exposed to various doses of CDDP for 2 h. CDDP: 0 µg/ml (C), 10 µg/ml (Φ), 50 µg/ml (Δ), and 100 µg/ml (A).

CDDP than was PC-3 and showed cross-resistance to various anticancer agents including mitomycin C, melphalan, carboplatin, and VP-16 (Table 1).

The primary lesion responsible for the cytotoxicity of CDDP is likely a result of its reaction with DNA (18), and interstrand DNA-DNA linking and DNA-protein linking as well as intrastrand cross-links are identified in CDDP-treated tumor cells (2, 19). In particular, interstrand DNA cross-linking formation is often correlated with the cytotoxicity of CDDP. We examined whether the formation of DNA cross-links differed between CHO cells and their CDDP-resistant counterpart. The alkaline elution patterns of DNAs of CHO cells showed increased formation of the cross-links as a function of CDDP from 0 to 100 µg/ml (Fig. 3). By contrast, treatment of C/CDDP-2 cells with various doses of CDDP caused only a slight effect if any on the formation of DNA-DNA cross-links. Assay for alkaline elutions without proteinase K showed no significant difference in the formation of DNA-protein cross-links between CHO and C/CDDP-2 cells. As seen in Fig. 4, alkaline elution patterns of DNAs of PC-3 showed also increased formation of DNA cross-links as a function of CDDP, while there appeared to be no apparent DNA cross-links in P/CDDP-5 cells. We then assayed alkaline elutions of CHO and C/CDDP-2 cells with isolated nuclei. Nuclei were isolated from 10^6 cells of [14C]-thymidine-labeled CHO cells and [14C]thymidine-labeled CDDP-2 cells, respectively, and the nuclei of [14C]thymidine-labeled C/CDDP-2 cells were then incubated in the presence of various doses of CDDP. An equal number of nuclei of the both cell lines was examined for DNA-linking formation by alkaline elution. Treatment of isolated nuclei with CDDP caused formation of DNA links in C/CDDP-2 cells as well as CHO cells (Fig. 5).

Resistance to CDDP as well as zinc or cadmium in cultured...
cells is correlated with the overproduction of metallothionein (20). Dot blot analysis of metallothionein gene mRNA showed similar levels of the metallothionein gene expression between CHO and C/CDP-2 cells, and there appeared to be no amplification of the gene in C/CDP-2 cells. On the other hand, cellular levels of thiols like GSH have been inferred to influence drug resistance to melphalan and CDDP in ovarian cancer cell lines (21, 22). We, however, could not observe any apparent difference in the GSH levels in C/CDP-2 and CHO cells; GSH levels (nmol/10^6 cells) were found to be 2.6 ± 0.4 (SE) in CHO cells and 2.8 ± 0.2 in C/CDP-2 cells. A GSH-dependent enzyme, GST, which catalyzes the conjugation of xenobiotics, carcinogens, and anticancer agents with GSH, is often higher in tumor cells resistant to chlorambucil, cyclophosphamide, Adriamycin, mitomycin C, and CDDP (23, 24). We thus measured GST activity of CHO, R-1, C/CDP-1, and C/CDP-2 cells. The cellular activity of GST of C/CDP-1 and C/CDP-2 was found to be about 4- and 6-fold higher, respectively, than CHO cells (Table 2). A revertant R-1 derived from C/CDP-2 showed a slightly higher GST activity than CHO cells. Continuous culturing for 2 weeks with CDDP before the assay of GST activity did not further significantly increase GST activity of C/CDP-1 and C/CDP-2 cells. Table 2 shows no significant difference in the GST activity between PC-3 and PC/CDP-5.

GST species in rats, humans, and mice are grouped into three classes, GST-α, GST-μ, and GST-γ (17, 25). Polyclonal antibodies raised against human GST-α, GST-μ, and GST-γ cross-react with GSTs in hamsters or rats as well as humans. To test which GST is increased in our CDDP-resistant cell lines of CHO cells, protein fractions of CHO, C/CDP-1, C/CDP-2, and R-1 cells were separated by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with antibodies against GST-α, GST-μ, and GST-γ. Western blot analysis showed an apparent two bands of GST-α subunits with molecular weights of 26,000 to 29,000 (25) which were increased in C/CDP-1 and C/CDP-2 cells (Fig. 6). However, there were no apparent bands for GST-γ in CHO and R-1 cells. No significant expression of GST-μ and GST-α was observed in C/CDP-1 and C/CDP-2 cells. Since GST-α was the only isoenzyme detected by Western analysis using antibodies directed against the GST-α, GST-γ, and GST-μ forms of the enzyme, our Western blot analysis appeared to show poor quality. Both CDDP-resistant CHO cell lines specifically expressed GST-α, but not GST-μ and GST-γ.

To examine whether the increased level of GST-α is due to enhanced expression of the GST-α gene, Northern blotting analysis was done with rat GST-α cDNA (13). Enhanced expression of GST-α mRNA was observed in C/CDP-1 and C/CDP-2 cells in comparison with either their parental CHO or R-1 cells (Fig. 7). Densitometric analysis shows more than 5-fold higher expression of GST-α mRNA in C/CDP-1 and C/CDP-2 cells than that of CHO cells. C/CDP-2 cells expressed rather higher levels of GST-α mRNA than C/CDP-1 cells, which is consistent with the cellular levels of GST activities. In R-1 cells, GST-α mRNA level was not completely reverted to that of CHO cells. Southern blot analysis shows no amplification of the GST-α gene in both CDDP-resistant cell lines (Fig. 7).

**DISCUSSION**

In this study, we isolated two CDDP-resistant cell lines, C/CDP-1 and C/CDP-2, after a stepwise exposure of CHO cells to increasing doses of CDDP. The CDDP-resistant phenotype, C/CDP-2, has been stably maintained during long culture in the absence of CDDP. A CDDP-sensitive revertant, R-1, was isolated when CDDP-2 cells were continuously cultured for 5 months in the absence of CDDP. CDDP forms interstrand DNA-DNA and DNA-protein cross-links (2, 3, 19). The cytotoxicity of CDDP was correlated with the formation of DNA-DNA cross-link formation (8, 9). Our present study demonstrates that DNA cross-links are formed in CHO cells, but not in C/CDP-2 cells, when the cells are cultured with CDDP. In contrast, CDDP-induced DNA cross-links are formed in CHO and C/CDP-2 cells when their isolated nuclei are treated with CDDP, suggesting involvement of altered CDDP uptake or any cytotoxic factor in CDDP resistance in CHO cells. A CDDP-resistant variant derived from a human squamous carcinoma cell line shows plasma membrane changes (26), and CDDP-resistant human ovarian cancer cells have decreased accumulation of CDDP (27).

Analysis by the flameless atomic absorption spectrophotometry shows a slightly decreased accumulation of CDDP with 70-80% of the control activity in C/CDP-2 cells compared with CHO cells, suggesting no appreciable change in CDDP uptake in C/CDP-2 cells. With respect to the membrane permeability of anticancer agents, expression of a membrane P-glycoprotein with a molecular weight of 170,000

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* M. Nakagawa, unpublished data.

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![Fig. 6. Western blot analysis of GSTs from CHO, C/CDP-1, C/CDP-2, and R-1 cells. Protein fractions of each cell line were run on 10% SDS-PAGE and transferred to the nitrocellulose filters. Nitrocellulose membranes were incubated with antibodies against GST-α, GST-μ, and GST-γ (1:2000) and biotinylated goat anti-rabbit IgG. GST-α (A), GST-μ (B), GST-γ (C), and preimmune (D). CHO cells (Lane 1), C/CDP-1 (Lane 2), C/CDP-2 (Lane 3), and R-1 (Lane 4). MW, molecular weight.
is often correlated with the multidrug resistance phenotype, resulting in net decrease in the accumulation of the anticancer agents (28). CDDP is not a substrate for the P-glycoprotein, and its expression is supposed to be unrelated to CDDP resistance (28). Consistent with this notion, a gene (mdr-1) coding the P-glycoprotein is not amplified in C/CDP-1, C/CDP-2, and P/CDP-5. Further study should be required to understand the specific pathway for the cellular uptake of CDDP in relation to acquisition of CDDP resistance. Cytosolic changes are also expected as plausible mechanism(s) to acquire CDDP-resistant phenotypes in tumors. Cellular thiols are often referred to influence drug resistance to anticancer agents (22, 24).

In particular, cellular GSH levels influence CDDP or melphalan resistance in human ovarian cancer cells, and GSH depletion can overcome CDDP or melphalan resistance (21, 29). Our CDDP-resistant variant of CHO cells, however, shows GSH levels similar to those of parental CHO cells. On the other hand, cellular levels of various GST isoenzymes may influence the differential sensitivity of tumor cells to the toxic effects of anticancer agents including alkylating agents and CDDP (24). GST activity is often elevated in cancer cell lines resistant to nitrogen mustard, cyclophosphamide, and other anticancer agents (24). By contrast, CDDP-resistant human ovarian cancer cell lines which are cross-resistant to melphalan and irradiation have similar activities of GSH-dependent enzymes including GST, GSH peroxidase, and GSH reductase as their parental ovarian cancer A2780 cell line, suggesting other mechanism(s) responsible for the acquisition of CDDP resistance (29, 30).

Overexpression of GST-\(\tau\) is demonstrated in a multidrug-resistant cell line of the MCF-7 human breast cancer cell line (31). However, MCF-7 cells transfected with the GST-\(\tau\) gene show no increased resistance to CDDP or melphalan (32). C/CDP-1 and C/CDP-2 cells which are cross-resistant to melphalan (Table 1) increased GST activity as compared with CHO cells while a CDDP-sensitive revertant, R-1, shows activity similar to that for CHO. Increased expression of GST-\(\tau\) mRNA is also observed in both CDDP-resistant cell lines of CHO. Consistent with this result, amounts of GST-\(\tau\) are specifically increased in C/CDP-1 and C/CDP-2 cells but not in CHO and R-1 cells (Fig. 6). Among the three classes of GSTs, GST-\(\tau\), GST-\(\mu\), and GST-\(\alpha\), there was no enhanced expression of GST-\(\mu\) and GST-\(\alpha\) in CDDP-resistant cell lines derived from CHO. Our present study favors a mechanism that enhanced expression of GST-\(\tau\) is closely related to acquired CDDP resistance in the CDDP-resistant cell lines of CHO cells. Transfection of human GST-\(\tau\) cDNA into CHO cells with GST-\(\tau\) expression vector should be critical to certify the above supposition.

There appears to be no difference in the cellular levels of GST activity between PC-3 and its CDDP-resistant counterpart, PC/CDP-5. The mechanism for the acquired CDDP-resistance in C/CDP-2 cells and in P/CDP-5 cells appears to be different. A CDDP-resistant cell line derived from human squamous cell line has approximately 2-fold higher content of sulphydryl protein and 2- to 3-fold higher GST activity than its parental cell line (26). The mechanism of CDDP resistance in the squamous cancer cell lines appears to be multifactorial, involving altered activities of plasma membranes, cytosolic binding to CDDP and DNA cross-linking (26). Masuda et al. (33) showed increased DNA repair as a plausible mechanism for acquired CDDP resistance in the human ovarian cancer cell lines. Other different mechanism besides these are also involved in acquired CDDP resistance in human cancer cell lines (34) and human lung cancer cell lines (35). These data suggest involvement of multiple mechanisms for acquisition of CDDP resistance in human tumors as presented by Eastman (36).

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