Characterization of *cis*-Diamminedichloroplatinum (II)-Resistant Murine Cell Lines Derived from Metallothionein Null Cells

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Metallothionein (MT) is known to play an important role in the resistance of tumor cells to *cis*-diamminedichloroplatinum (II) (CDDP, cisplatin). To identify non-MT factors that play important roles in CDDP resistance, we established CDDP-resistant cell lines from simian virus 40-transformed MT null cells. Subclones of CDDP-resistant MT null cells, designated as MKCr-3, -12, and -18, exhibited 24- to 62-fold resistance to CDDP compared with parental cells. The contents of glutathione (GSH) and the activities of GSH-related enzymes and antioxidant enzymes in MKCr-12 and -18 were higher than those in parental cells. However, MKCr-3 cells did not show any significant increase in the levels of GSH nor in enzyme activities except for superoxide dismutase. Accumulation of platinum in CDDP-resistant subclones was 15–26% of that in parental cells 24 h after administration of CDDP. Eleven other subclones of CDDP-resistant MT null cells also exhibited low accumulation of platinum. These results suggest that the decreased accumulation of CDDP may be a predominant factor in the resistance to CDDP in the absence of MT, an intracellular metal- and free radical-scavenger.

Key words —— metallothionein, *cis*-diamminedichloroplatinum, resistance, platinum

INTRODUCTION

cis-Diaminedichloroplatinum (II) (CDDP, cisplatin) is a widely used anticancer drug for the treatment of testicular, ovarian, bladder, and small cell lung carcinoma.[1)] The anticancer activity of CDDP is attributed to its ability to form DNA-platinum adducts, which leads to the disruption of DNA synthesis.[2)] Generation of reactive oxygen species (ROS) by CDDP is also involved in its cytotoxic actions.[3,4)] Although CDDP is a potent anticancer drug, various malignant tumors frequently acquire resistance to CDDP, limiting its clinical application.[5–7)] Therefore, elucidation of the mechanism underlying CDDP resistance is crucial for efficient cancer chemotherapy. The resistance factors so far examined include enhanced repair of DNA lesions,[8,9)] scavenging of ROS by antioxidant enzymes,[10,11)] altered accumulation of CDDP,[12–15)] and detoxification of CDDP by cellular sulfhydryl compounds such as glutathione (GSH),[16)] thioredoxin (Trx),[17,18)] and metallothionein (MT).[19–22)]

MT is a metal-binding protein that is inducible by heavy metals, cytokines, and anticancer drugs including CDDP.[21)] MT suppresses the toxicity of anticancer drugs as well as that of metals. Previous studies reported from our laboratory demonstrated that preinduction of MT by treatment with bismuth compounds in the kidney, but not in tumor tissues, effectively suppresses the renal toxicity of CDDP without compromising its antitumor activity.[23)] However, when MT is induced in tumor tissues by treatment with zinc or transfection of the MT gene, the antitumor activity of CDDP was markedly reduced.[21,22)] Cross-resistance to CDDP is frequently observed in cadmium-resistant cells[19)] and other drug-resistant cancer cell lines that contain high concentrations of MT.[24)] On the other hand, MT null mice[25)] or primary cultured cells derived from MT null mice[26)] exhibit greater sensitivity to CDDP compared with control animals or cells. In addition to

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the evidence obtained from experimental animals, examinations of clinical samples have also shown the association of increased expression of MT with CDDP resistance in human cancer tissues.[27–29] Thus MT is now recognized as an important CDDP resistance factor.

Recently, we have established simian virus 40 (SV40)-transformed MT null cells[30] from primary cultured embryonic cells of MT null mice,[31] in which the genes for MT-I and MT-II were disrupted.[32] This cell line is easier to manipulate than primary cultured cells, and can be used to develop drug-resistant cell lines due to its apparently immortalized property. Previously, we established a cadmium-resistant cell line from SV40-transformed MT null cells, and demonstrated that this cell line exhibited a distinct change in cadmium accumulation,[33] which was caused by an alteration in the transport system common to manganese and cadmium.[34] These results indicate that the utilization of SV40-transformed MT null cells for establishing drug-resistant cell lines is useful for elucidating novel resistance factor(s) that is masked by the presence of MT. The purpose of this study was therefore to establish CDDP-resistant cell lines from MT null cells and identify CDDP resistance factor(s) in the absence of MT.

MATERIALS AND METHODS

Establishment of CDDP-Resistant Cell Lines —— SV40-transformed MT null cells established by Kondo *et al.*[30] were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum under 5% CO_2 at 37°C. CDDP-resistant MT null cells were developed by continuous exposure of MT null cells to CDDP. CDDP concentration in the medium was increased stepwise to 10 μM. Subclones of MKCr-3, MKCr-12, and MKCr-18 were used in the following experiments. These cells were cultured for 10 days in CDDP-free medium before the assay.

Assay for Sensitivity to CDDP —— Cells were plated on 96-well microplates at a density of 1×10^4 cells per well, incubated for 24 h, and then treated with various concentrations of CDDP for 48 h. The sensitivity to CDDP was measured by colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described previously.[33]

Measurement of GSH Concentration and Antioxidant Enzyme Activities —— Cells (2×10^5 cells in a 6-cm dish) were cultured for 24 h, washed three times with phosphate-buffered saline (PBS) (–) 2 ml, and harvested using a cell scraper with PBS (–) 1 ml. Cell lysates were prepared by repetitive freezing and thawing of cells in 10 mM Tris–HCl (pH 7.4). After sonication, the supernatant fraction was analyzed for GSH and antioxidant enzymes. Total GSH content was determined by the method of Tietze.[35] Activities of catalase, superoxide dismutase (SOD) and GSH-peroxidase (GSH-Px) were measured by the methods of Johansson and Borg,[36] Oyanagui,[37] and Lawrence and Burk,[38] respectively. Glutathione reductase (GSSG-red) activity was assayed by the method of Wheeler *et al.*[39] GSH S-transferase (GST) activity was measured by the method of Habig and Jakoby using 1-chloro-2,4-dinitrobenzene as a substrate.[40] Thioredoxin reductase (Trx-red) activity was determined by the method of Kitaoka *et al.*[41] The protein concentration in each sample was determined by the method of Lowry *et al.*[42]

Immunoblotting for Trx —— Cells (2×10^6 cells in a 10-cm dish) were cultured for 24 h, washed three times with PBS (–) 5 ml, harvested using a cell scraper with PBS (–) 1 ml, and transferred into microcentrifuge tubes. Cell pellets were lysed by sonication in lysis buffer containing 10 mM Tris–HCl (pH 7.4), 1% NP-40, 0.1% SDS, 0.15 M NaCl, 1 mM EDTA, and aprotinin 10 μg/ml. Cell lysates were separated by 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting using rabbit polyclonal antibody raised against mouse Trx, which was a generous gift from Professor Junji Yodoi, Kyoto University.

Determination of Intracellular Platinum Contents —— Cells (1×10^6 cells in a 6-cm dish) were cultured for 24 h, and then treated with CDDP 5 μM for 1, 3, 7, and 24 h. After washing three times with ice-cold PBS (–), cells were immediately harvested using a cell scraper with PBS (–) 500 μl. Cell pellets were digested in nitric acid 5 ml by heating to dryness, and resolved in 0.1 N hydrochloric acid. Platinum concentrations in digested samples were determined by inductively coupled plasma mass spectrometry (ICP/MS) (HP-4500, Yokogawa Analytical Systems, Inc., Japan).

RESULTS

Establishment of CDDP-Resistant MT Null Cells

We isolated several CDDP-resistant subclones by chronic exposure of SV40-transformed MT null

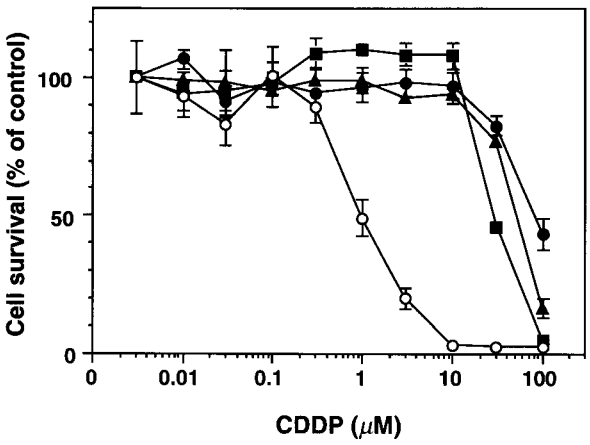

Fig. 1. Sensitivity of CDDP-Resistant and Parental Cells to CDDP

MKCr-3 (closed circles), MKCr-12 (closed triangles), MKCr-18 (closed squares), and parental cells (open circles) were treated with various concentrations of CDDP for 48 h, and then cell viability was determined by MTT assay.

cells to CDDP at progressively increasing concentrations. The sensitivity to CDDP of three resistant subclones, MKCr-3, MKCr-12, and MKCr-18, was examined by MTT assay. Figure 1 shows that these MKCr subclones were 24- to 62-times more resistant than their parental MT null cells in terms of IC_{50} value. MKCr-3 exhibited the highest resistance to CDDP.

Levels of Sulfhydryl Compounds and Activities of Enzymes

As shown in Table 1, MKCr-12 had an approximately three times higher level of GSH compared with parental cells. In addition, MKCr-12 showed higher activities of GST, GSSG-red, GSH-Px, SOD, and catalase than parental cells, and these activities were the highest among the three CDDP-resistant subclones. The GSH content in MKCr-18 was 2.3-

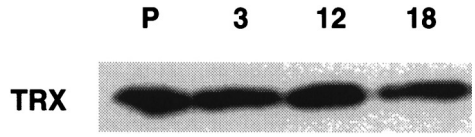

Fig. 2. Expression Level of Trx in CDDP-Resistant and Parental Cells

Whole cell extracts prepared from MKCr-3 (3), MKCr-12 (12), MKCr-18 (18), and parental cells (P) were subjected to Western blot analysis using rabbit anti-Trx antibody.

fold higher than that in parental cells. The activities of GSH-Px and SOD in MKCr-18 were significantly higher than those in parental cells, but no increase was observed in GST, GSSG-red, or catalase activities. On the other hand, MKCr-3, which showed the highest resistance to CDDP, exhibited no change in GSH content, GSH-related enzyme activities, or antioxidant enzyme activities except for SOD. No change in the levels of Trx (Fig. 2) or activities of Trx-red (Table 1) was observed in MKCr-3, MKCr-12, or MKCr-18 subclones compared with parental cells. These data suggest that increases in the level of GSH and activities of GSH-related and antioxidant enzymes might have contributed to the resistance to CDDP in MKCr-12 and MKCr-18, but not in MKCr-3. On the other hand, Trx and Trx-red system may not be involved in CDDP resistance in these subclones.

Intracellular Content of Platinum in CDDP-Resistant and Parental Cells

To examine the change in CDDP accumulation in resistant cells, we measured the intracellular concentration of platinum in CDDP-resistant and parental cells at different time points after exposure to CDDP 5 μM (Fig. 3). The platinum concentration in parental cells increased linearly up to 24 h. On the other hand, MKCr-3, MKCr-12, and MKCr-18

Table 1. Contents of Sulfhydryl Compounds and Activities of GSH-Related and Antioxidant Enzymes in CDDP-Resistant and Parental Cells

	Parent	MKCr-3	MKCr-12	MKCr-18
GSH (nmol/mg protein)	0.441 ± 0.023	0.589 ± 0.190	1.354 ± 0.105**	1.011 ± 0.142**
GST (unit/mg protein)	0.347 ± 0.030	0.370 ± 0.044	0.633 ± 0.091**	0.457 ± 0.039
GSSG-red (unit/mg protein)	19.7 ± 2.6	17.1 ± 2.3	34.9 ± 4.8**	21.6 ± 3.5
GSH-Px (unit/mg protein)	0.101 ± 0.001	0.092 ± 0.016	0.162 ± 0.026*	0.124 ± 0.009*
SOD (unit/mg protein)	39.2 ± 6.3	62.1 ± 11.8*	98.9 ± 16.1**	76.3 ± 18.9*
Catalase (unit/mg protein)	0.135 ± 0.016	0.118 ± 0.010	0.187 ± 0.010**	0.122 ± 0.005
Trx-red (unit/mg protein)	8.0 ± 0.8	8.8 ± 2.6	8.5 ± 2.1	6.2 ± 1.7

Values are expressed as mean ± S. D. ($n = 3$). Asterisks show significant differences from the parental cells ($*p < 0.05$ and $**p < 0.01$, respectively, by t-test).

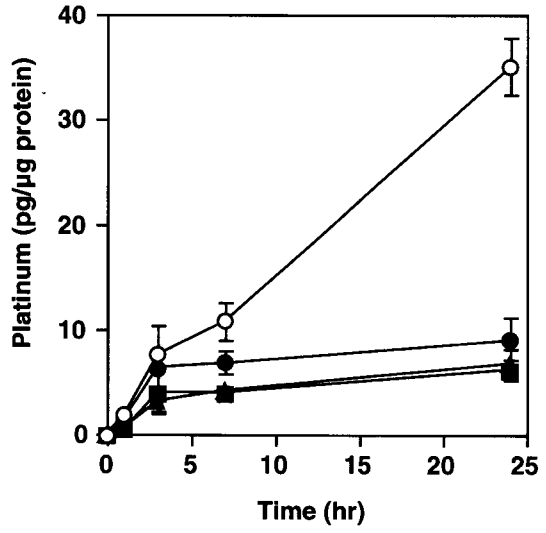

Fig. 3. Accumulation of Platinum in CDDP-Resistant and Parental Cells

MKCr-3 (closed circles), MKCr-12 (closed triangles), MKCr-18 (closed squares), and parental cells (open circles) were treated with CDDP 5 μM for 0, 1, 3, 7, and 24 h, and then platinum concentrations in the cells were determined by ICP/MS after acid digestion.

Table 2. Intracellular Contents of Platinum in CDDP-Resistant and Parental Cells

Cell	Platinum concentration (% of control)
Parent	100.0± 8.1
MKCr-1	35.1± 2.4
2	44.4± 4.3
3	56.5± 8.4
4	58.8±10.5
5	26.6± 1.3
6	36.4± 1.8
9	37.7± 1.1
10	19.0± 3.6
11	25.4± 3.6
12	28.8± 4.6
13	32.2± 4.5
14	65.0± 5.9
18	28.7± 5.2
19	28.7± 5.5

Cells were exposed to CDDP (5 μM) for 7 h. Platinum concentrations were determined by ICP/MS after acid digestion. Values are expressed as mean ± S. D. ($n = 3$).

showed a different pattern of time-dependent increase in cellular platinum concentration. In the initial phase (0–3 h), the rates of platinum accumulation in CDDP-resistant cells were similar to or slightly lower than that of parental cells, but the rates were markedly lowered in a later phase (3–24 h). Thus the difference in platinum concentration between CDDP-resistant subclones and parental cells increased at 24 h. The relative concentrations of platinum at 24 h in MKCr-3, MKCr-12, and MKCr-18 were 26%, 19%, and 15% of those in parental cells, respectively. These data suggest that the efflux of CDDP in CDDP-resistant subclones may be enhanced. Since all three subclones exhibited decreased accumulation of platinum, 11 other subclones that exhibited CDDP resistance to an extent similar to MKCr-3, MKCr-12, and MKCr-18 (data not shown) were also examined for platinum accumulation after 7-h exposure to CDDP 5 μM. As shown in Table 2, the other subclones also showed decreased platinum accumulation, suggesting that the change in CDDP transport may commonly occur in CDDP-resistant cells derived from MT null cells.

DISCUSSION

To identify the non-MT factor responsible for CDDP resistance, several clones of CDDP-resistant cells were established from SV40-transformed MT null cells. Characterization of the obtained CDDP-resistant MT null cells demonstrated that the expression of antioxidant enzymes or sulfhydryl compounds was enhanced in some subclones but not in all. On the other hand, all subclones we have established exhibited decreased accumulation of platinum, suggesting that the change in CDDP accumulation is crucial for the survival of MT null cells that are continuously exposed to CDDP.

Cellular resistance to CDDP has been characterized by the involvement of diverse factors including DNA repair,[8,9] efflux of CDDP,[12–15] activities of antioxidant enzymes and GSH-related enzymes,[10,11] and the levels of GSH,[16] Trx[17,18] and MT.[19–22] Among these factors, MT is especially important because it is readily inducible by a variety of anticancer drugs used frequently in the clinical setting,[21] and relatively small amounts of MT can effectively attenuate CDDP toxicity. Animal experiments reported from our laboratory demonstrated that only 2-fold and 3-fold increases in MT levels by treatment with zinc[21] or by transfer of the MT gene[22] in transplanted tumor tissues were sufficient to confer resistance to CDDP *in vivo*. Furthermore, MT null mice[25] and the primary cultured cells derived from MT null mice[26] exhibited enhanced sensitivity to CDDP, suggesting that even low concentrations of MT present in control tissues or cells contributes to the protection against CDDP cytotoxicity.

To date, however, the mechanism of detoxification of CDDP by MT has not been fully elucidated. It has been postulated that either MT scavenges ROS produced by CDDP, or that MT sequesters CDDP as a metal compound. Although the precise mechanism of CDDP-induced ROS production remains unclear, mounting evidence has indicated that antioxidant enzymes or sulfhydryl compounds are able to reduce the toxicity of CDDP.[11,43,44] We also have reported that combined expression of SOD and GSH-Px by gene transfer caused resistance to CDDPin HeLa cells.[10] Therefore the utilization of MT null cells for developing CDDP-resistant cells may permit the detection of other important antioxidants associated with the acquisition of CDDP resistance. However, the activities of several antioxidant enzymes and GSH contents were increased in MKCr-12 and MKCr-18, but not in MKCr-3 that showed the highest resistance against CDDP among these three subclones (Table 1). These data suggest that there is no specific antioxidant enzyme or sulfhydryl compound that is required for the survival of MT null cells exposed to CDDP.

On the other hand, all subclones examined in the present study exhibited reduced accumulation of platinum (Table 2), and this is the only common feature of CDDP-resistant cell lines obtained from MT null cells. MT is known to have the ability to bind platinum and thereby to confer CDDP resistance.[45,46] Therefore if the primary role of MT in the detoxification of CDDP is sequestering platinum, it seems likely that the reduction of cellular CDDP concentration is crucial for the survival of MT null cells exposed to CDDP. It is noteworthy that we established a cadmium-resistant cell line from SV40-transformed MT null cells in a previous study, and found that the accumulation of cadmium in these cells was markedly suppressed compared with that in parental cells.[33] These results suggest that, in both cases of resistance to cadmium and CDDP, the alteration in transport of the chemical is crucial for the acquisition of drug resistance in MT null cells, which have lost an efficient intracellular sequestering protein for metal compounds and alkylating agents.

As shown in Fig. 3, the enhanced efflux of CDDP may account for the reduced accumulation of platinum in CDDP-resistant subclones. Recent studies have suggested that multidrug resistance-associated proteins (MRPs) are involved in the efflux of CDDP.[47–49] However, which subtype of MRPs actually contributes to CDDP efflux remains to be elucidated. The CDDP-resistant MT null cells we have established in the present study may provide a useful tool to clarify the cellular transport system of CDDP, including the involvement of MRPs.

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