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Reduced drug accumulation in a newly established human lung squamous-carcinoma cell line resistant to *cis*-diamminedichloroplatinum(II)

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Abstract—A human lung squamous-carcinoma cell line resistant to *cis*-diamminedichloroplatinum(II) (CDDP), designated PC10-B3, has been established from the original cell line PC10 by a stepwise increment of the CDDP concentration. This is the first report, to our knowledge, to establish a CDDP-resistant lung squamous-carcinoma cell line. PC10-B3 has continued to proliferate in the presence of 0.5 μg/mL CDDP, whereas PC10 could not survive. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay revealed that PC10-B3 was 11.4-fold more resistant to CDDP than PC10 and cross-resistant to diammine(1,1-cyclobutanecarboxylate)platinum(II) (CBDCA) and 254-S, but not to doxorubicin or etoposide. PC10-B3 was characterized by a smaller DNA index and a larger cell size compared to PC10. The level of intracellular platinum accumulation was reduced by about 5- to 8-fold in PC10-B3 when compared with PC10, suggesting that reduced drug accumulation may be one of the important factors in contributing to CDDP resistance in PC10-B3.

cis-Diamminedichloroplatinum(II) (CDDP*)-based combination chemotherapy has been widely used for its clinical potential on a broad spectrum of human malignancies [1, 2]. However, repetitive administration of CDDP is likely to reduce its efficacy even in patients dramatically responsive to CDDP initially. This decline in efficacy is mainly due to the genesis or predominance of CDDP-resistant subpopulations. Therefore, uncovering the mechanisms of CDDP resistance seems indispensible to improving the outcome of chemotherapy based on CDDP. Thus, a variety of experimental approaches have been conducted, and many of them have taken advantage of *in vitro* established CDDP-resistant cell lines to seek their resistant machinery by comparison with their respective original cell lines. These methods may also provide information in uncovering more complicated resistant phenomena in clinics. Actual mechanisms of CDDP resistance detected so far are more complex and versatile than was anticipated. These include increased expression in the non-protein sulfhydryl glutathione [3–5] and its related enzymes [6, 7], protein sulfhydryl metallothionein [8, 9], and enhanced repair of CDDP-induced DNA damage [10–12]. Other unknown factors involved in CDDP resistance cannot be denied [13].

In the present study we have developed a CDDP-resistant cell line designated PC10-B3 from a human lung squamous-carcinoma cell line PC10. To our knowledge, this is the first report to establish a pulmonary squamous-carcinoma cell line resistant to CDDP. Moreover, the characterization of this cell line PC10-B3 was examined, and reduced drug accumulation was implicated as one of the important contributors to the CDDP resistance in PC10-B3.

Materials and Methods

Drugs. CDDP and etoposide were provided by Nippon Kayaku, Tokyo, Japan. Diammine(1,1-cyclobutanecarboxylato)platinum(II) (CBDCA) and 254-S were obtained from Bristol Myers, New York, while doxorubicin was from Kyowa Hakko, Tokyo, Japan.

Cell lines and development of resistance to CDDP. The original human squamous-carcinoma cell line PC10 (provided by Prof. Y. Hayata, Tokyo Medical College) was maintained in an RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum at 37°, 5% CO_2. A CDDP-resistant subline, PC10-B3, was developed by a stepwise increment of the CDDP concentration as follows. The initial concentration of CDDP was 0.1 μg/mL. After the cells reached confluence, they were passaged into a CDDP-free medium. When the cells entered the

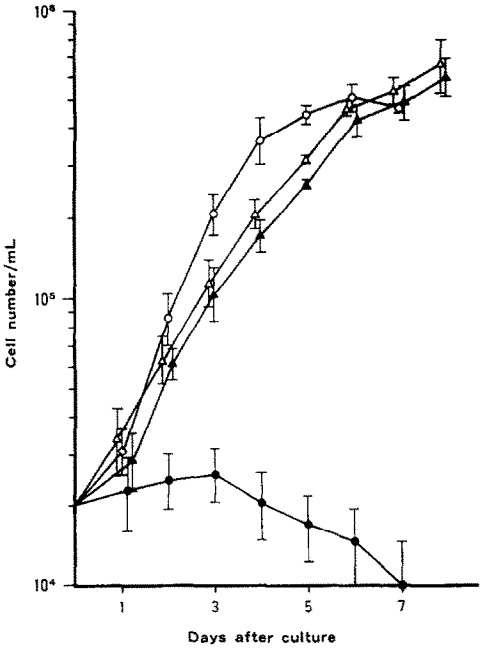

Fig. 1. Growth curves of PC10 (○) and PC10-B3 (△) in CDDP-free medium, and PC10 (●) PC10-B3 (▲) in the presence of 0.5 μg/mL CDDP. Points and bars represent means ± SD of 3 independent experiments.

* Abbreviations: CDDP, *cis*-diamminedichloroplatinum(II); CBDCA, diammine(1,1-cyclobutanecarboxylato)platinum(II); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; and ELISA, enzyme-linked immunosorbent assay.

logarithmic phase, new CDDP was added and the concentration of CDDP was increased by 0.1 μg/mL. Gradually escalating doses of CDDP was carried out every few passages, and the concentration of CDDP reached 1.5 μg/mL over 6 months. Subsequently, the cells were exposed to continuous challenge of 0.5, 1.0 and 1.5 μg/mL CDDP, respectively. After long-term passages for 3 months, cells under 1.0 μg/mL and 1.5 μg/mL CDDP became gradually unstable and then could no longer survive, while those in the constant presence of 0.5 μg/mL CDDP were persistently stable enough to proliferate to confluence from a very sparse cell density. This stable mass population was subjected to the limiting dilution technique and the resultant subclone PC10-B3 resistant to CDDP was obtained.

Characteristics of cell growth, DNA index and cell size. Subconfluent cells were transferred to 24-well plates so as to adjust an initial cell number to 2×10^4/mL. The number of cells was counted every 24 hr for 7–10 days without changing the medium so as to plot growth curves. To determine cellular DNA content and cell size, exponentially growing cells were fixed with 70% ethanol for at least 30 min at 4°, and resuspended in phosphate-buffered saline containing 50 μg/mL propidium iodide and 100 μg/mL RNase, followed by analysis using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). DNA index was calculated as the ratio of DNA content of each cell line to that of the normal human lymphocytes. Cell size was expressed as the relative value to normal lymphocytes.

MTT assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay modified by Mosmann was utilized [14]. Cells were harvested at their exponential phase and transferred to 96-well plates so that cell density was optimal at the time of the assay. On the following day, chemotherapeutic drugs at various concentrations were added to each well. After a 4-day incubation, MTT solution was added to each well, followed by a further 4-hr culture allowing for the formazan crystals to generate from MTT by living cells. Immediately after formazan products were dissolved by dimethyl sulfoxide (DMSO), spectrophotometric absorbance was measured at 540 nm with an enzyme-linked immunosorbent assay (ELISA) reader (ImmunoReader NJ-2000, Nippon InterMed K.K., Tokyo, Japan).

Platinum content. Intracellular platinum content was measured using a Hitachi model Z-8000 polarized Zeeman atomic absorption spectrometer (Hitachi, Ltd., Tokyo, Japan). Determined platinum content was corrected for cellular protein as measured by the Bradford method.

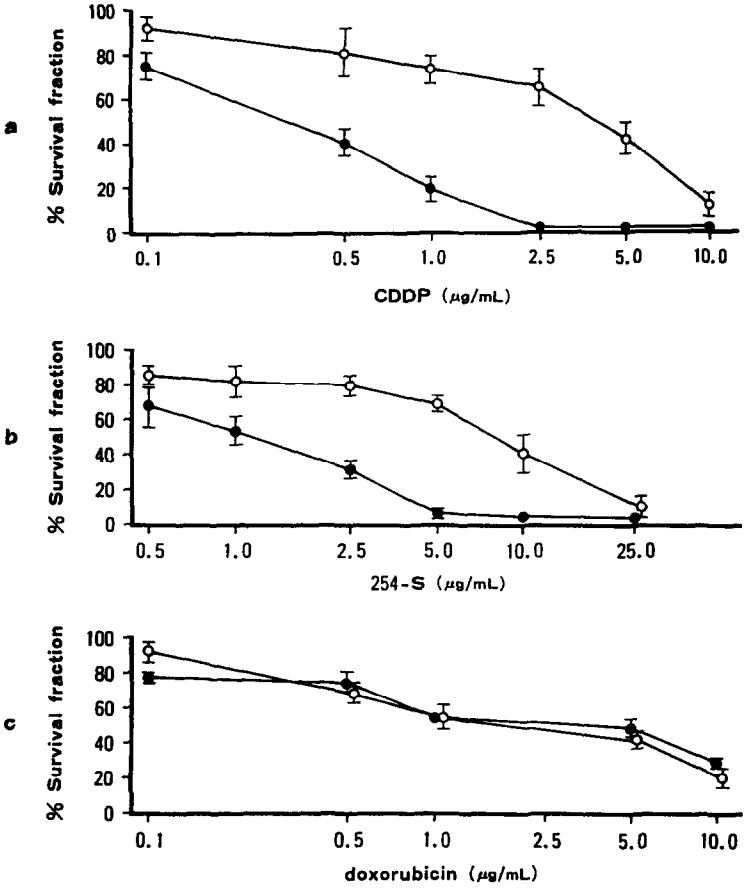

Fig. 2. Concentration–response curves of PC10 (●) and PC10-B3 (○) to CDDP (a), 254-S (b) and doxorubicin (c). Points and bars represent means ± SD of 2–3 experiments performed in duplicate.

Table 1. Relative resistance of PC10-B3 compared to PC10 for CDDP and other chemotherapeutic drugs

Drugs	IC_{50} (μg/mL) PC10	PC10-B3	Relative resistance
CDDP	0.35	4.0	11.4
CBDCA	11.8	55.3	4.7
254-S	1.2	8.5	7.1
Doxorubicin	2.4	2.4	1.0
Etoposide	2.2	1.8	0.8

Values are means of 2–4 determinations performed in duplicate.

Results and Discussion

The characteristics of CDDP-resistant subline PC10-B3 were studied and compared to those of the original cell line PC10. Figure 1 shows the growth curves of PC10 and PC10-B3 both in CDDP-free medium and in the presence of 0.5 μg/mL CDDP. The growth of PC10-B3 was inhibited slightly in the presence of 0.5 μg/mL CDDP, whereas PC10 could not grow at all and died within 2 weeks. Doubling times of PC10 and PC10-B3 were estimated to be 23.5 and 33.0 hr, respectively, with a significantly prolonged doubling time of the latter ($P < 0.01$). In addition, cellular DNA content and cell size were determined. Compared to PC10, PC10-B3 had a smaller DNA index (2.15 vs 1.93), and a larger cell size (1.98 vs 2.57).

Concentration–response curves were made by MTT assay to estimate the degree of CDDP resistance in PC10-B3. PC10-B3 proved to be 11.4-fold more resistant than PC10 to CDDP (Table 1, Fig. 2a). The cross-resistance pattern of PC10-B3 to other chemotherapeutic drugs, including CDDP derivatives, was confirmed. As shown in Table 1 and panels b and c of Fig. 2, the cross-resistance of PC10-B3 to CDDP derivatives, CBDCA and 254-S, was less than to CDDP (resistance was 4.7- and 7.1-fold, respectively), whereas no cross-resistance was observed against doxorubicin or etoposide.

Finally, intracellular platinum accumulation was measured in PC10 and PC10-B3. CDDP was added at a final concentration of 5.0 μg/mL to exponentially growing PC10 and PC10-B3 in 10-mL tissue culture plates. Intracellular platinum content after being exposed to CDDP for 1, 3 and 6 hr was calculated. Platinum accumulation in PC10-B3 was reduced by about 5–8 times as compared to that in PC10 at every time point examined, i.e. reduced 5.2-, 7.7- and 6.0-fold in PC10-B3 on the average, after 1-, 3- and 6-hr exposures, respectively (Fig. 3). Therefore, reduced drug accumulation is considered to be one of the important factors inducing CDDP resistance in PC10-B3.

The reduction in intracellular platinum accumulation has been found in various CDDP-resistant cell lines, e.g. head and neck carcinoma, ovarian carcinoma and leukemia [15–17]. This mechanism was also seen in small-cell lung carcinoma and lung adenocarcinoma cell lines resistant to CDDP [18, 19]. Since the same mechanism was revealed in PC10-B3 originating from a lung squamous-cell carcinoma, it could be involved in CDDP resistance irrespective of the difference in histologies, at least in the lung. Although CDDP is generally thought to influx into cells through passive diffusion, it is not obvious whether reduced platinum accumulation in PC10-B3 is due to a blockage of CDDP passive diffusion or, if any, an increased efficiency of the CDDP efflux system.

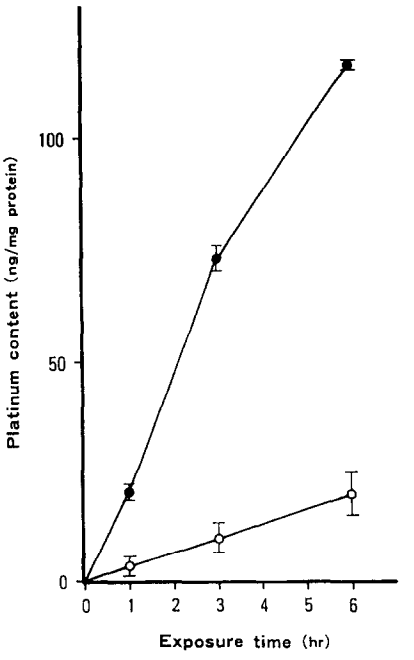

Fig. 3. Accumulation of platinum in PC10 and PC10-B3. PC10 (●) and PC10-B3 (○) were exposed to 5.0 μg/mL CDDP for 1, 3 and 6 hr. Points and bars represent means ± SD of 3–4 independent experiments.

P-glycoprotein is unlikely to contribute to the CDDP resistance in PC10-B3, since PC10-B3 did not show cross-resistance to doxorubicin. Furthermore, additional factors other than reduced drug accumulation contributing to CDDP resistance in PC10-B3 need to be explored, because more than one factor relevant to CDDP resistance could function in one cell line [19]. There may be overexpressions of metallothionein, glutathione and its related enzymes, enhanced DNA repair, or other unknown factors. Further investigation on mechanisms of CDDP resistance in PC10-B3 may be useful to circumvent the problem associated with CDDP resistance and to improve the clinical effectiveness of CDDP.

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‡ Corresponding author: Dr. Motoo Katabami, Laboratory of Molecular Genetics, Cancer Institute, Hokkaido University School of Medicine, Kita-15 Nishi-7, kita-ku, Sapporo 060, Japan. FAX 011-717-1127.

*Laboratory of Molecular Genetics Cancer Institute; and
†First Department of Internal Medicine
Hokkaido University School of Medicine
Sapporo 060, Japan

MOTOO KATABAMI*†‡
HISAKAZU FIJITA*
HITOSHI HANEDA†
HIROTOSHI AKITA†
NOBORU KUZUMAKI*
HIROSHI MIYAMOTO†
YOSHIKAZU KAWAKAMI†

REFERENCES

1. Prestayko AW, Crooke ST and Carter SK, *Cisplatin: Current Status and New Developments*. Academic Press, New York, 1980.
2. Loehrer PJ and Einhorn LH, Cisplatin. *Ann Intern Med* **100**: 704–713, 1984.
3. Behrens BC, Hamilton TC, Masuda H, Grotzinger KR, Whang-Peng J, Lonie KG, Knutsen T, McKoy WM, Young RC and Ozols RF, Characterization of a *cis*-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. *Cancer Res* **47**: 414–418, 1987.
4. Hospers GAP, Mulder NH, de Jong B, de Ley L, Uges DRA, Fichtinger-Schepman AMJ, Scheper RJ and de Vries EGE, Characterization of a human small cell lung carcinoma cell line with acquired resistance to *cis*-diamminedichloroplatinum(II) *in vitro*. *Cancer Res* **48**: 6803–6807, 1988.
5. Fujiwara Y, Sugimoto Y, Kasahara K, Bungo M, Yamakido M, Tew KD and Saijo N, Determinants of drug response in a cisplatin-resistant human lung cancer cell line. *Jpn J Cancer Res* **81**: 527–535, 1990.
6. Bedford P, Walker MC, Sharma HL, Perera A, McAuliffe CA, Masters JRW and Hill BT, Factors influencing the sensitivity of two human bladder carcinoma cell lines to *cis*-diamminedichloroplatinum(II). *Chem Biol Interact* **61**: 1–15, 1987.
7. Saburi Y, Nakagawa M, Ono M, Sakai M, Muramatsu M, Kohno K and Kuwano M, Increased expression of glutathione *S*-transferase gene in *cis*-diamminedichloroplatinum(II)-resistant variants of a Chinese hamster ovary cell line. *Cancer Res* **49**: 7020–7025, 1989.
8. Andrews PA, Murphy MP and Howell SB, Metallothionein-mediated cisplatin resistance in human ovarian carcinoma cells. *Cancer Chemother Pharmacol* **19**: 149–154, 1987.
9. Kelley SL, Basu A, Teicher BA, Hacker MP, Hamer DH and Lazo JS, Overexpression of metallothionein confers resistance to anticancer drugs. *Science* **241**: 1813–1815, 1988.
10. Masuda H, Ozols RF, Lai G-M, Fojo A, Rothenberg M and Hamilton TC, Increased DNA repair as a mechanism of acquired resistance to *cis*-diamminedichloroplatinum(II) in human ovarian cancer cell lines. *Cancer Res* **48**: 5713–5716, 1988.
11. Eastman A and Schulte N, Enhanced DNA repair as a mechanism of resistance to *cis*-diamminedichloroplatinum(II). *Biochemistry* **27**: 4730–4734, 1988.
12. Chu G and Chang E, Cisplatin-resistant cells express increased levels of a factor that recognizes damaged DNA. *Proc Natl Acad Sci USA* **87**: 3324–3327, 1990.
13. Teicher BA, Herman TS, Holden SA, Wang Y, Pfeffer MR, Crawford JW and Frei E III, Tumor resistance to alkylating agents conferred by mechanisms operative only *in vivo*. *Science* **247**: 1457–1461, 1990.
14. Mosmann T, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**: 55–63, 1983.
15. Teicher BA, Holden SA, Kelley MJ, Shea TC, Cucchi CA, Rosowsky A, Henner WD and Frei E III, Characterization of a human squamous carcinoma cell line resistant to *cis*-diamminedichloroplatinum(II). *Cancer Res* **47**: 388–393, 1987.
16. Waund WR, Differential uptake of *cis*-diamminedichloroplatinum(II) by sensitive and resistant murine L1210 leukemia cells. *Cancer Res* **47**: 6549–6555, 1987.
17. Andrews PA, Velury S, Mann SC and Howell SB, *cis*-Diamminedichloroplatinum(II) accumulation in sensitive and resistant human ovarian carcinoma cells. *Cancer Res* **48**: 68–73, 1988.
18. Bungo M, Fujiwara Y, Kasahara K, Nakagawa K, Ohe Y, Sasaki Y, Irino S and Saijo N, Decreased accumulation as a mechanism of resistance to *cis*-diamminedichloroplatinum(II) in human non-small cell lung cancer cell lines: Relation to DNA damage and repair. *Cancer Res* **50**: 2549–2553, 1990.
19. Teicher BA, Holden SA, Herman TS, Sotomayer EA, Khandekar V, Rosbe KW, Brann TW, Korbut TT and Frei E III, Characteristics of five human tumor cell lines and sublines resistant to *cis*-diamminedichloroplatinum(II). *Int J Cancer* **47**: 252–260, 1991.

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Protection against tabun toxicity in mice by prophylaxis with an enzyme hydrolyzing organophosphate esters

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Abstract—We demonstrate here the correlation between protection afforded by pretreatment alone with parathion hydrolase purified from *Pseudomonas* sp. against tabun toxicity in mice and the kinetic parameters which are assumed to determine the *in vivo* detoxification of tabun by the same enzyme. Results show that 15 and 22 μg of parathion hydrolase per animal conferred a protective ratio of 3.94 and 5.65 respectively, against tabun toxicity, without post-exposure treatment.

Pretreatment with as little as 7.5 and 26 μg of parathion hydrolase purified from *Pseudomonas* sp. has been demonstrated in mice to confer, without additional treatment, protection against multiple median lethal doses (LD_{50}) of diethyl *p*-nitrophenyl phosphate (paraoxon) and its P-F-containing analogue, diethyl fluorophosphate