Bull. Inst. Zool., Academia Sinica **31**(3): 163-168 (1992)

DECREASE OF PLATINUM ACCUMULATION IN A CISPLATIN-RESISTANT CHINESE HAMSTER OVARY CELL LINE

HAIMEI HUANG, HSUEH-WEI CHANG, HSIAN-GUEY HSIEH and LAI-CHEN TSAI[1]

Institute of Radiation Biology, National Tsing Hua University, Hsinchu, Taiwan and Department of Medical Research, Veterans General Hospital, Taipei, Taiwan, Republic of China,

(Accepted September 26, 1991)

Haimei Huang, Hsueh-Wei Chang, Hsian-Guey Hsian and Lai-Chen Tsai (1992) Decrease of platinum accumulation in a cisplatin-resistant Chinese hamster ovary cell line. *Bull. Inst. Zool., Academia Sinica* **31**(3): 163-168. Cisplatin-resistant cells, Pt-r6, were developed through the chronic exposure of Chinese hamster ovary (CHO-K1) cells to increasing concentrations of cisplatin. The LD_{50} of clonogenic survival after a 2-h cisplatin treatment was 16.3 and 7 μM for resistant Pt-r6 and parental CHO-K1 cells, respectively. An analysis of cellular DNA adducts by cisplatin-DNA monoclonal antibodies using the ELISA or flow cytometric methods of adduct-antibody conjugation with fluorescent dye revealed that more adducts were induced in CHO-K1 cells than in Pt-r6 cells. Moreover, CHO-K1 cells also accumulated more cisplatin than Pt-r6 cells, as determined by inductively coupled plasma-atomic emission spectrometry. Thus, resistance to cisplatin was clearly correlated with decreased amounts of platinum and cisplatin-DNA adducts in the resistant cells as compared to the sensitive cells. These results suggest that the reduced aecumulation of cisplatin may be involved in the cisplatin resistance of Pt-r6 cells.

Key words: Cisplatin resistance, Cisplatin accumulation, Cisplatin-DNA monoclonal antibody.

Cisplatin [*Cis*-diamminedichloroplatinum (II)] is a clinically potent antitumor drug which is effective against testicular, ovarian, head, and neck cancers (Zwelling *et al.*, 1979). However, in a relatively high percentage of patients who initially respond to cisplatin, their tumors become unresponsive to cisplatin upon continuing treatment (Ozol *et al.*, 1985; Krakker and Moore, 1988). Although studies of other drug-resistant cells have demonstrated that the potential mechanisms of drug resistance include alterations in drug transport (Andrews *et al.*, 1988), metabolism effects leading to less active compounds (Shebani *et al.*, 1989), gene amplification (such as a multi-drug resistant gene), and indication of a cellular protective agent such as glutathione, little is known regarding the operating mechanism leading to the development of a resistance to cisplatin (Bruchovsky and Goldie, 1983).

We have developed cisplatin-resistant cell lines with low levels of drug resistance *in vitro* from Chinese hamster ovary cells (CHO-K1). One subline—Pt-r6

maintained normally in 6 μM of cisplatin-containing medium—was used to investigate the characteristics of resistance to cisplatin. Using atomic emission spectrophotometry (AES), we found that Pt-r6 cells transport lower drug amounts than do CHO-K1 cells. Using monoclonal antibodies, we also found fewer cisplatin-DNA adducts in Pt-r6 cells than in CHO-K1 cells (according to enzyme-linked immunosorbent assay (ELISA) and flow cytometric assay). Experimental details are described in this report.

MATERIALS AND METHODS

Materials

Chemicals for the cell culture were obtained from Gibco. *Cis*-diamminedichloroplatinum(II) [cisplatin] was provided by Strem Chemicals Inc., Newburyport, MA 01950 USA. Freshly prepared cisplatin was filtrated using a 0.2 μm filter and diluted with culture medium before use. Murine monoclonal antibodies (MoAb 62-5) elicited against calf thymus DNA modified with cisplatin were developed (Hsieh, 1990). The immunoglobulin subclass of MoAb 62-5 is IgG. Goat anti-mouse IgG-peroxidase and rabbit anti-mouse IgG-FITC, obtained from Cappel, were chosen as secondary antibodies against MoAb 62-5 for enzyme-linked immunosorbent assay (ELISA) and flow cytometry (FCM), respectively. The substrate for peroxidase, 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) [diammonium salt] (ABTS), was freshly prepared.

Cell culture and cell lines

Chinese hamster ovary (CHO-K1) cells were maintained in exponential monolayer cultures at 37°C in McCoy's 5A medium supplemented with 10% fetal calf serum, sodium bicarbonate (2.2 g/liter), penicillin G sodium (100 units/ml), and streptomycin sulfate (100 μg/ml). Cisplatin-resistant Pt-r6 cells were established from CHO-K1 cells by progressively increasing the concentration of cisplatin in culture medium as follows: 0.5, 1, 2, 4, and 6 μM, At each concentration, the cells were subcultured at least ten times. The resistant cell lines were maintained in complete medium containing 6 μM cisplatin. Pt-r6 cells were incubated in cisplatin-free medium for one week before each experiment to assure the use of drug-free cells. Both CHO-K1 and Pt-r6 cells were treated with drugs during the exponential growth phase.

Clonogenic survival assay

The clonogenic assay was performed as described previously (Wilson *et al.*, 1989). Cells were trypsinized and seeded at densities of 200–200,000 cells/60 mm dish. The colonies were harvested after cells were grown for seven days. Dishes were then fixed with ethanol and stained with crystal violet, after which the colonies were counted (Lee *et al.*, 1989). Untreated CHO-K1 and Pt-r6 cells exhibited clone plating efficiencies of approximately 85 and 65%, respectively.

Cellular and nuclear Pt determinations

For cellular Pt measurements, 5×10^6–10^8 cells were incubated for 2–4 h with cisplatin concentrations ranging from 100–400 μM for CHO-K1 cells, and 200–700 μM for Pt-r6 cells. After three washes with PBS at 4°C, pellets were dissolved in 150 μl 65% HNO_3 at 70°C for 2 h. The digested cells were then mixed with distilled water to 3 ml per sample. The amount of Pt was determined with a Plasmakon S 35 "inductively coupled plasma-atomic emission spectrometer" (ICP-AES). Standard solution ($H_2PtCl_6 \cdot 6H_2O$ in H_2O, from Merck) was used in each experiment to calibrate the unknown

samples. Experiments were also performed to measure the Pt in the nuclei and cytoplasmic fractions of the cells. After treating the cells with NP-40, nuclei and cytoplasmic fractions were collected separately (Favaloro *et al.*, 1980). The Pt in each fraction was also measured by the ICP-AES.

Enzyme-linked immunosorbent assay (ELISA) and flow cytometry (FCM)

For the ELISA assay, 8×10^4 cells were seeded in each well of a 96-well microtiter plate. For flow cytometry, $1\text{-}2\times10^6$ cells were plated in 100 mm petri dishes. After treatment, cells were fixed overnight in 70% ethanol at 4°C. Nonspecific binding was minimized by incubating wells with PBS containing 5% normal goat serum for 1 h at 37°C. Hybridoma supernatants (1:10) were transferred into individual wells or tubes for 30 min. For ELISA, the binding of antibodies to DNA was detected with peroxidase-conjugated goat anti-mouse IgG and ABTS as a substrate. Results were obtained after measurement with a Biotek microtiter plate reader at O. D. 405 nm (Hsieh, 1990). For flow cytometry, fluorescein-conjugated goat anti-mouse IgG (1:50) was used as a secondry antibody, and 10,000 cells were counted with FACStar (Becton-Dickinson). For double-parameter assays, DNA was stained with PBS containing 10 μg/ml propidium iodide and 100 μg/ml RNase. The intensity of green fluorescence of fluorescein-conjugated antibodies was measured with the excitation wavelength at 488 nm and emission wavelength at 530 nm. Red fluorescence of propidium iodide was estimated with the excitation wavelength at 488 nm and emission wavelength at 620 nm. The mean intensity of green fluorescence of bound antibodies was calculated from linear-histograms using Consort 30 programs. Fluorescence signals are reported as fluorescence channel numbers. Mean fluorescence was calculated as a mean channel number divided by the total number of cells (Frankfurt, 1987).

RESUNTS AND DISCUSSION

The LD_{50} of CHO-K1 and Pt-r6 cells after 2 h cisplatin treatment were 7 and 16.3 μM, respectively (Fig. 1). Therefore, Pt-r6 cells were about twice as resistant to the cell killing effects of cisplatin as CHO-K1 cells. Increased amounts of cisplatin-DNA adducts with increasing cisplatin-treatment time or cisplatin-treatment dosage were detected by both ELISA (Fig. 2) and FCM assays (Fig. 3). Increasing amounts of cellular accumulation of cisplatin with increasing cisplatin-treatment dosage was also detected by ICP-AES (Fig. 4A). In all experiments, Pt-r6 cells were found to be lower in cisplatin-DNA adducts and in cellular cisplatin accumulation than were CHO-K1 cells (Figs. 2A, 2B, 3 and 4). The present results suggest that the lower

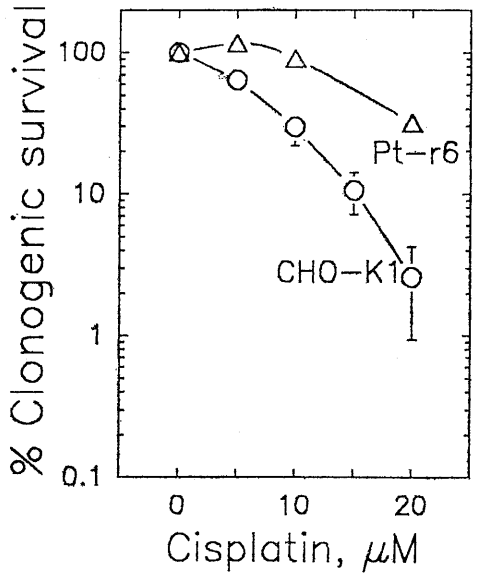

Fig. 1. Clonogenic survival of CHO-K1 and Pt-r6 cells treated with various doses of cisplatin for 2 h.

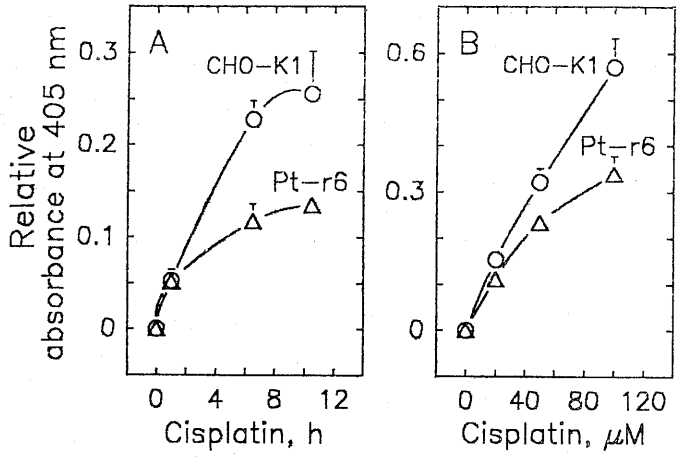

Fig. 2. Effect of cisplatin treatment time and dosage on cisplatin-DNA adduct formation as assayed by ELISA using MoAb 62-5. (A) Cells treated with cisplatin 100 μM for 1, 6, and 10 h. (B) Cells treated with various doses of cisplatin for 8 h.

cellular accumulation of cisplatin in Pt-r6 cells may be responsible for its resistance to the killing effects of cisplatin. The lower cellular accumulation of cisplatin in Pt-r6 cells may be due to a lower uptake or higher efflux of cisplatin. Thus, a decrease in drug accumulation may be involved in the development of cisplatin resistance in Pt-r6 cells.

Since cisplatin uptake or efflux may

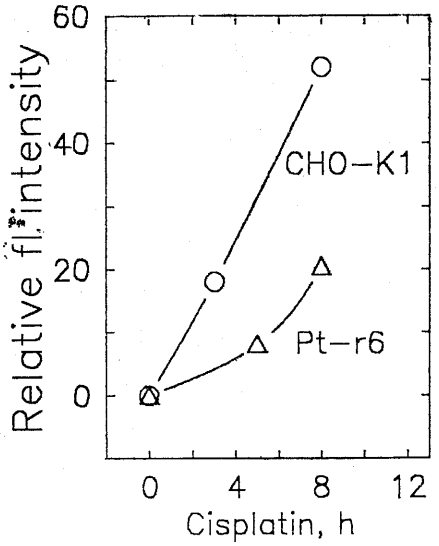

Fig. 3. Effect of cisplatin treatment time on cisplatin-DNA adduct formation as analyzed by flow cytometric determination of relative fluorescence intensity of FITC-conjugated goat anti-mouse IgG against MoAb 62-5. Cells were treated with cisplatin 100 μM for various periods of time.

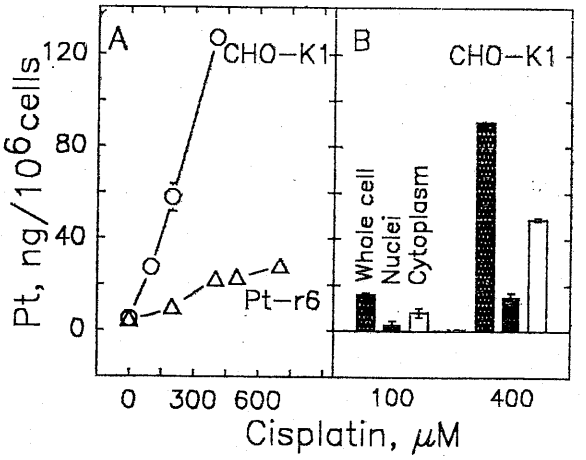

Fig. 4. (A) Pt accumulation in cells treated with various doses of cisplatin for 2 h. Pt was measured by ICP-AES method. (B) CHO-K1 cells were treated with cisplatin (100 or 400 μM) for 2 h; Pt accumulations within the whole cell (narrow crosshatch), nucleus (filled box), and cytoplasmic fraction (blank box) were then determined.

be associated with cell membranes, the role of the drug target (such as P-glycoprotein, as a result of gene amplification) on the membranes of resistant and sensitive cells should be further investigated. Alternatively, platinum may undergo a very rapid and relatively loose associaiton with intracellular components in sensitive cells that somewhat retards its return diffusion out of the cells (Mann *et al.*, 1990). As reported in previous studies, cisplatin resistance which is associated with the amount of intracellular glutathione (Behrens *et al.*, 1987), rate of drug uptake (Kuppen *et al.*, 1988) and efflux (Mann *et al.*, 1990), or the formation and repair of cisplatin-DNA adducts (Masuda *et al.*, 1988; Shebani *et al.*, 1989) has been demonstrated in several cell lines. Multifactorial resistance to cisplatin involving more than one of above-mentioned mechanisms was also found in some cell lines (Richon *et al.*, 1987; Teicher *et al.*, 1987; Hospers *et al.*, 1988; Andrews *et al.*, 1990).

In our experiments, 10^7–10^8 cells per sample were used for the assay of ICP-AES, 10^5 cells per sample for ELISA, and 10^4 cells for the FCM assay. Thus, the immunoassays were at least 1000-fold more sensitive in detecting cellular cisplatin than was the ICP-AES. Because the cisplatin in unbound or bound forms cannot be distinguished by ICP-AES, immunocytochemical methods for the exact determination of cisplatin-DNA adducts were used. Approximately 1% of cisplatin can react with genomic DNA, as reported by Eastman (1990). Therefore, examining cisplatin-DNA adducts with immunossays may be about 100,000 times more sensitive than with ICP-AES. Similar results have also been reported by other investigators (Terheggen *et al.*, 1990; Tilby *et al.*, 1991).

Acknowledgements: This work was supported by grants from the National Science Council, Republic of China (NSC 80-80-0211-B007-01) to H. Huang and the Biomedical Research Foundation of Veterans General Hospital, and from Tsing-Hua University (VGHTH 78-032-2) to H. Huang and L. C. Tsai.

REFERENCES

Andrews, P. A., V. Sriharasha, S. C. Mann and S. B. Howell (1988) *cis*-Diamminechloroplatinum(II) accumulation in sensitive and resistant human ovarian carcinoma cells. *Cancer Res.* **48**: 68-73.

Andrews, P. A. and S. B. Howell (1990) Cellular pharmacology of cisplatin: Perspectives on mechanisms of acquired resistance. *Cancer Cells* **2**: 35-43.

Behrens, B. C., T. C. Hamilton, H. Masuda, K. R. Grotzinger, J. Whang-Peng, K. G. Louid, T. Knutsen, W. M. McKoy, R. C. Young and R. F. Ozols (1987) 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.

Bruchovsky, N. and J. H. Goldie (eds.) (1983) Drug and Hormone Resistance in Neoplasia, Vol. 1. Boca Raton, Fl: CRC Press, Inc.

Eastman, A. (1990) Activation of programmed cell death by anticancer agents: Cisplatin as a model system. *Cancer Cells* **2**(8 & 9): 275-280.

Favaloro, J., R. Treisman and R. Kamen (1980) Transcription maps of polyoma virus-specific RNA: Analysis by two-dimensional nuclease S1 gel mapping. *Methods Enzymol.* **65**: 718.

Frankfurt, O. S. (1987) Flow cytometry analysis of DNA damage and the evaluation of cytotoxicity of alkyating agents. *Cancer Res.* **47**: 5537-5541.

Hospers, G. A. P., N. H. Mulder, B. de Jong, L. de Ley, D. R. A. Uges, A. M. J. Fichtinger-Schepman, R. J. Scheper and E. G. E. de Vries (1988) Characterization of a human small lung carcinoma cell line with acquired resistance to *cis*-diamminedichloroplatinum(II) *in vitro*. *Cancer Res.* **48**: 6803-6807.

Hsieh, H. G. (1990) Probe DNA damage and repair by antibodies against cisplatin-DNA adducts. Master thesis, Institute of Radiation

Biology of the National Tsing-Hua University, Hsinchu, R. O. C.

Krakker, A. J. and C. H. Moore (1988) Accumulation of *cis*-dichlorodiammineplatinum(II) and platinum analogues by platinum-resistant murine leukemia cells *in vitro*. *Cancer Res.* **48**: 9-13.

Kuppen, P. J. K., H. Schuitemarker, L. J. van't Veer, E. A. de Bruijn, A. T. van Oosterom and P. I. Schrie (1988) *cis*-dichlodiammineplatinum(II)-resistant sublines derived from two human ovarian tumor cell lines. *Cancer Res.* **48**: 3355-3359.

Lee, T. C., J. C. Ko and K. Y. Jan (1989) Differential cytotoxicity of sodium arsenite in human fibroblasts and Chinese hamster ovary cells. *Toxicology* **56**: 289-299.

Mann, S. C., P. A. Andrews and S. B. Howell (1990) Short-term *cis*-diamminedichloroplatinum(II) accumulation in sensitive and resistant human ovarian carcinoma cells. *Cancer Chemother. Pharmacol.* **25**: 236-240.

Masuda, H., R. F. Ozols, G. M. Lai, A. Fojo, M. Rothenberg and T. C. Hamilton (1988) Increased DNA repair as mechanism of acquired resistance to *cis*-diamminedichloroplatinum(II). *Biochemistry* **27**: 4730-4734.

Ozol, R. F. (1985) Pharmacologic reversal of drug resistance in ovarian cancer. *Semin. Oncol.* **12** (Suppl. 4): 7-11.

Richon, V. M., N. Schulte and A. Eastman (1987) Multiple mechanisms of resistance to *cis*-diamminedichloroplatinum(II) in murine leukemia L1210 cells. *Cancer Res.* **47**: 2056-2061.

Shebani, N., M. M. Jennerwein and A. Eastman (1989) DNA repair in cells sensitive to *cis*-diamminedichloroplatinum(II) host cell reactivation of damaged plasmid DNA. *Biochemistry* **28**: 3120-3124.

Teicher, B. A., S. A. Holden, M. J. Kelley, T. C. Shea, C. A. Cucchi, A. Rosowsky, W. D. Henner and E. Frei III (1987) Characterization of a human squamous carcinoma cell line resistant to *cis*-diamminedichloroplatinum(II). *Cancer Res.* **47**: 388-393.

Terheggen, P. M. A. B., J. Y. Emondt, B. G. J. Floot, R. Dijkman, P. I. Schrier, L. Den Engelse and A. C. Begg (1990) Correlation between cell killing by *cis*-diammnedichloroplatinum (II) in six mammalian cell lines and binding of a *cis*-diamminedichloroplatinum(II)-DNA antiserum. *Cancer Res.* **50**: 3556-3561.

Tilby, M. J., C. Johnson, R. J. Knox, J. Cordell, J. J. Roberts and C. J. Dean (1991) Sensitive detection of DNA modifications induced by cisplatin and carboplatin *in vitro* and *in vivo* using a monoclonal antibody. *Cancer Res.* **51**: 123-129.

Wilson, R. E., P. C. Keng and R. M. Sutherland (1989) Drug resistance in Chinese hamster ovary cells during recovery from severe hypoxia. *J. Natl. Cancer Institute* **81**(16): 1235-1240.

Zwelling, L. A., M. O. Bradley, N. A. Sharkey, T. Anderson and K. W. Kohn (1979) Mutagenicity, cytotoxicity and DNA cross-linking in V-79 Chinese hamster cells treated with *cis*- and *trans*-Pt(II) diamminedichloride. *Mutation Res.* **67**: 271-280.

藥物累積情形在正常與抗雙氨雙氯鉑化物之中國倉鼠卵巢細胞中的研究

黃海美　張學偉　謝現貴　蔡來誠

Pt-r 6 爲中國倉鼠卵巢細胞生長在含漸次增加雙氨雙氯鉑化物濃度的培養液中，長時期所建立的抗藥細胞。Pt-r 6 與正常細胞以雙氨雙氯鉑化物處理兩小時，其50%致死劑量爲 16.7 與 7 μM。用酵素免疫方法或流動細胞分析法配合酵素或螢光結合抗 Pt-DNA 鍵結物的抗體來檢查細胞，顯示：正常細胞比抗性細胞中之 Pt-DNA 鍵結物含量高。而以原子放射光譜分析鉑含量，亦發現：正常細胞比抗性細胞累積鉑元素較多。因此，Pt-r 6 細胞之抗性與其較少之鉑化物累積以及較少之 Pt-DNA 鍵結物相關。而細胞抗性，可能來自其對雙氨雙氯鉑化物累積量之減少有關。