The Mechanism of Acquired Resistance to Cisplatin by a Human Ovarian Cancer Cell Line

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The present study was designed to elucidate the mechanism of resistance to cisplatin. A cisplatin-resistant cell line (KFr) was established from KF cells derived from human serous cystadenocarcinoma of the ovary. The DNA histogram revealed an increase of S-phase cells and a decrease of G_1-phase cells in cultured KFr cells, compared to that in cultured KF cells. Although the cisplatin content in the KF cells incubated with cisplatin at 10 μg/ml increased in a time-dependent manner, that in the KFr cells remained unchanged during the experimental period. When 0.5 mg of cisplatin was administered ip to nude mice with KF or KFr tumor, the cisplatin content in the KFr tumor was significantly lower than that in the KF tumor. The KFr cells showed a cross-resistance to L-phenylalanine mustard, while no cross-resistance to vincristine or 5-fluorouracil was observed. These findings suggest that the mechanism of cisplatin resistance in the KFr cells involves a decrease of cisplatin accumulation in the tumor cells.

Key words: Cisplatin — Acquired resistance — Cross resistance — Human ovarian cancer cell line

Cisplatin [cis-diamminedichloroplatinum (II)] has demonstrated a broad range of activity against several malignancies in humans,[1, 2] particularly ovarian and testicular cancer. In patients with advanced epithelial ovarian cancer cisplatin-based regimens have produced increased complete response rates, and in some studies prolonged overall survival compared to single agent therapy.[3, 4] However, their effectiveness is frequently limited by the development of acquired drug resistance.[5] The mechanisms responsible for the development of resistance to cisplatin in ovarian cancer cells have been only partially characterized. We have developed a human ovarian cancer cell line (KFr) which exhibits relatively stable resistance to cisplatin after repeated exposure to escalating doses of the drug.[6] The studies reported here were designed to elucidate the mechanism(s) by which the KFr cell line is resistant to cisplatin. We now report that the mechanism(s) of acquired resistance to cisplatin involves a decrease of cisplatin accumulation into tumor cells.

MATERIALS AND METHODS

Agents Cisplatin was obtained from Bristol-Banyu Pharmaceutical Co., Ltd., Tokyo. LPAM was obtained from the Wellcome Foundation Ltd., London. 5FU was purchased from Kyowa Hakko Industrial Co., Ltd., Tokyo. Vincristine was obtained from Shionogi Pharmaceutical Co., Ltd., Osaka.

Cells and Cell Culture KF cells were established from tissue of a patient with serous cystadenocarcinoma of the ovary.[7] The cell line had a plating efficiency of 20–40% and a doubling time of 18–24 hr *in vitro*. After repeated exposure to escalating doses of cisplatin, cells that could proliferate in the presence of cisplatin at 1.0 μg/ml were obtained and designated "KFr."[6] The KFr cell line has been maintained for 3 months in the absence of cisplatin and the resistance of this cell line to cisplatin has remained stable for that period. The cells were incubated in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2mM glutamine, 100 U penicillin/ml, and 100 μg streptomycin/ml (Grand Island Biological Co., Grand Island, NY)

The abbreviations used are: LPAM, L-phenylalanine mustard; 5FU, 5-fluorouracil.

in 5% CO_2 at 37°. The medium was changed every 3 days, and the cells were passaged when they reached confluence.

Flow Cytometry Cell cycle phase distribution analysis was performed with a Jasco (Japan Spectroscopic Co., Ltd. Tokyo) FCS-1 multiparametric cell sorter using propidium iodide-stained nuclei of the cells. Briefly, control and treated cells were washed twice with ice-cold 0.85% sodium chloride solution and the cell pellet was resuspended in hypotonic propidium iodide staining solution.[8] The nuclei were maintained at 4° for 24 hr in the staining solution prior to analysis for cell cycle traverse perturbation. At least 10^4 nuclei were analyzed in each sample, and the fractions of cells in G_1+G_0, S, and G_2+M of the cell cycle were determined as reported previously.[9]

Determination of Cisplatin Content in Cells and Tumor KF and KFr cells in a preconfluent state were harvested with 0.25% trypsin and counted with a hematocytometer. The cell number was adjusted to 10^7 cells/ml with medium RPMI 1640 and cisplatin (10 μg/ml) was added to the medium. After the incubation time indicated, cells were harvested, washed three times with fresh medium and resuspended in 1 ml of medium. Although the cell viability was examined by using the trypan blue dye exclusion test, the incubation time at this cell density did not affect the cell viability. Treatment with 0.25% trypsin did not seem to affect the drug transport. The cell suspension was sonicated (50% duty cycle for 3 min) using a W-225 sonicator (Heat Systems-Ultrasonics, Inc., Farmingdale, NY). After centrifugation at $30,000g$ for 1 hr, the supernatants were taken for platinum assay. To obtain tumor tissue samples, 5×10^5 KF or KFr cells were inoculated sc into the right flank of nude mice. About 21 days after inoculation, tumor nodules with about 1×1 cm diameter were formed. About 4 hr after ip administration of 0.5 mg of cisplatin, the tumor tissues were resected. The tumor tissue samples were digested using nitric and perchloric acids, and after evaporation to dryness the residue was then solubilized in dilute hydrochloric acid. The platinum assay was performed as described previously,[10] on a Perkin-Elmer model 603 atomic absorption spectrometer equipped with a heated graphite atomizer on which the temperature control was modified to produce gradual increases in temperature.

RESULTS

Flow cytometric patterns in the KFr cells showed a decrease of cells in the G_1-phase and an increase of cells in the S phase, compared to those in the parent KF cells (data not shown). When 10^7 KF or KFr cells were incubated
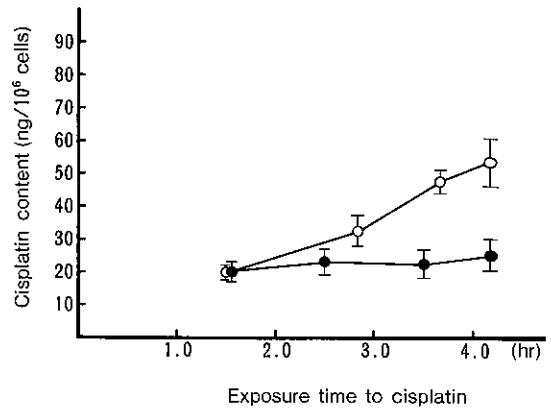

Fig. 1. Changes of cisplatin contents in KF and KFr cells with increasing exposure time to cisplatin. KF and KFr cells (10^7 cells) were incubated with cisplatin at 10 μg/ml for the period indicated. The platinum assay was performed as described in "Materials and Methods." Each point shows the mean±SD from 3 experiments. KF cells, ○; KFr cells, ●.

Table I. Uptake by KF and KFr Tumors of Cisplatin Administered ip to Nude Mice

Tissues	Cisplatin (μg/g dry weight)
Spleen[a]	12.0 ± 1.3[b]
KF tumor	20.4 ± 1.6*
KFr tumor	15.3 ± 1.0

a) Spleen of tumor-bearing mice.
b) Mean±SD from 3 experiments.
* $P<0.01$ (Student's t-test), compared to spleen and KFr tumor.

Table II. The Degree of Resistance of KFr Cells to Various Anticancer Drugs

Drug	Resistance index[a]
Cisplatin	20.6 (3.50/0.17)[b]
LPAM	8.6 (1.60/0.25)
5FU	0.4 (0.36/0.90)
Vincristine	1.0 (0.01/0.01)

a) Ratio of IC_{50} drug dose in resistant KFr cells to that in the parent KF cells.
b) IC_{50} value (μg/ml) in resistant KFr cells/IC_{50} value (μg/ml) in the parent KF cells.

with cisplatin at 10 μg/ml for the period indicated in Fig. 1, the cisplatin content in the KF cells increased with exposure time to cisplatin, while the cisplatin content in the KFr cells remained unchanged during the experimental period. At about 4 hr exposure time, the cisplatin content in the parent KF cells was about 2-fold higher than that in the KFr cells. Concentrations in the KF and KFr tumors of cisplatin administered ip to nude mice are shown in Table I. Cisplatin administered ip was accumulated significantly more effectively in the KF tumor cells than in the KFr tumor cells or the spleen cells of tumor-bearing mice. As shown in Table II, cross-resistance of KFr cells was observed to LPAM but not to 5 FU or vincristine.

DISCUSSION

Human ovarian cancer cells (KFr cells) with induced resistance to cisplatin have been shown to be useful in studying mechanisms of drug resistance and in evaluating pharmacological means by which drug resistance can be decreased. We have already reported that the morphologic characteristics of the KFr cells were an enlarged nucleus and prominent nucleoli, unlike the nucleus and nucleoli of the parent KF cells.[6] Accordingly, KFr cells in the preconfluent state were considered to contain more S-phase cells and fewer G_1-phase cells than KF cells in the preconfluent state. When KF or KFr cells were incubated with cisplatin, cisplatin was time-dependently accumulated in the KF cells while the cisplatin content in the KFr cells remained unchanged. Furthermore, when nude mice bearing KF or KFr tumor were treated with 0.5 mg of cisplatin, greater accumulation of cisplatin in the KF tumor tissues than in the KFr tumor tissues was observed. Similarly, Belehradek et al.[11] reported that an L1210 subline with 15-fold-increased resistance to cisplatin accumulated lower levels of bound cisplatin than the parental line, suggesting restricted cisplatin penetration. Another preliminary report also claimed that uptake of cisplatin in an L1210 leukemia cell variant with 3.5-fold-increased resistance to cisplatin as measured by atomic absorption spectroscopy, was 49% less than in sensitive cells.[12] These results suggest that one of the mechanisms of resistance to cisplatin may be impairment of accu-

mulation systems. In the present study, it is uncertain whether such impairment of the accumulation systems results from an elevation of efflux functions or a decrease of influx functions. However, it has been reported that amino acids could protect cells against cisplatin, suggesting that cisplatin enters cells through an amino acid transport mechanism.[13] In addition, Scanlon et al.[14] showed that cisplatin inhibited membrane transport of methionine and aminoisobutyric acid. Results on cross-resistance to other antitumor drugs revealed that cisplatin-resistant cells had cross-resistance to LPAM, but not to vincristine or 5FU. It has generally been accepted that, as with cisplatin, LPAM transport is inhibited in tumor cells by amino acids, including leucine, methionine, tyrosine and alanine.[15] The findings in the present study are consistent with the fact that tumors resistant to LPAM (presumably because of impaired drug transport) are partially cross-resistant to cisplatin.[16, 17]

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