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The Isolation and Characterization of Cisplatin-resistant Rat Ovarian Cancer Cells*

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Abstract—*A rat ovarian cancer cell subline (Cis-Pt^r), which became approximately 20-fold resistant to cisplatin, was developed after continuous exposure of the cisplatin-sensitive parent cell line (ROT68/C1) to increasing doses of the drug* in vitro. *Both the ROT68/C1 and Cis-Pt^r cells were tumorigenic in isologous (Sprague–Dawley strain) newborn rats and only the tumors developed by inoculation of Cis-Pt^r cells showed resistance to cisplatin* in vivo. *Resistance towards cisplatin was accompanied by phenotypic changes of the undifferentiated adenocarcinoma cells including enlargement of the cell and nucleus and a slower growth rate both* in vitro *and* in vivo. *Compared to the ROT68/C1 cells, the Cis-Pt^r showed an early recovery of DNA synthetic activity after exposure to cisplatin. Cross-resistance of the Cis-Pt^r cells was found only to a cisplatin analog, carboplatin. These results suggest that our cisplatin resistant rat ovarian cancer cells are useful in the investigation of the characteristics and mechanisms of cisplatin resistance.*

INTRODUCTION

CISPLATIN, a new type of anticancer drug, was developed in 1960s [1]. Cisplatin has been shown to be one of the most effective anticancer drugs in a variety of human solid tumors including ovarian cancer [2]. Although the initial response rate of ovarian cancer to cisplatin is extremely high, the 5 year survival rate has not been significantly improved [3]. Frequent recurrence of cisplatin-resistant ovarian cancer in the patients treated previously with cisplatin seems to be the major cause. Thus, the study of the mechanism of cisplatin-resistance and devising ways to overcome it are urgent problems in current chemotherapy of ovarian cancer. In this paper, we report the isolation and characterization of rat ovarian cancer cells resistant to cisplatin.

MATERIALS AND METHODS

Cell line

A cloned ovarian adenocarcinoma cell line (ROT68/C1) arising in a Sprague–Dawley rat was used [4]. The ROT68/C1 cells produced undifferentiated adenocarcinomas when injected subcutaneously [5] and intraperitoneally [6] in isologous newborn rats within 48 h after birth. Monolayer cultures were maintained in RPMI 1640 medium supplemented with 10% newborn bovine serum (Flow Laboratories, New South Wales, Australia) and antibiotics (penicillin 100 units/ml and streptomycin 100 μg/ml) in a humidified mixture of 5% carbon dioxide and 95% air at 37°C.

Anticancer drugs

A solution of cisplatin in saline (0.5 mg/ml) was provided by Nippon Kayaku Company (Tokyo, Japan). A freeze-dried vial (150 mg) of carboplatin was provided by Bristol-Myers K.K. (Tokyo). The stock solutions of these drugs were made by diluting or dissolving with serum-free medium and stored in the dark at room temperature. Other anticancer drugs (melphalan, busulfan, actinomycin D, adriamycin, 5-fluorouracil, methotrexate and vincristine) were purchased from Sigma Chemical Company (St. Louis, MO) and the stock solutions were kept at −20°C.

Survival studies in vitro

Two methods, growth curve and colony forming efficiency, were employed to examine the cell survival after exposure to cisplatin *in vitro*. Monolayer cells (1.5×10^5) growing in 25 cm^2 Falcon culture flasks (Becton Dickinson & Company, Oxnard, CA)

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were exposed for 1 h to 1, 5 and 10 μg/ml cisplatin, washed with serum-free medium, and then refed with medium containing serum.

Thereafter, the cells were harvested by treatment with 0.25% trypsin solution at 37°C every other day and counted with a hemocytometer after staining with 0.5% crystal violet. A survival study of colony forming efficiency was performed as follows. Approximately 5×10^2 cells were plated into 60 × 15 mm Falcon culture dishes and incubated for 24 h. The cells were exposed for 1 h to cisplatin, carboplatin and other anticancer drugs at various concentrations. Immediately after treatment, the cells were washed with serum-free medium and incubated for 7 days. Macroscopic colonies (usually consisting of more than 32 cells) were counted after methanol fixation and Giemsa staining. The percentage survival was calculated with reference to controls (cells not exposed to drug) and all experiments were performed in duplicate or triplicate per dose of drug.

Electron microscopy

Cells were scraped off the wall of culture flasks with a rubber policeman and centrifuged at 1000 r.p.m. for 5 min to obtain cell pellets. The pellets were fixed and embedded, and ultrathin sections were made as described previously [7]. The ultrathin sections were doubly stained with uranyl acetate followed by Reynold's lead acetate. The sections were examined with a Hitachi (Tokyo) Model HS 12 electron microscope.

Transplantation

Approximately 5×10^5 cells suspended in 0.2 ml of medium were inoculated subcutaneously into the interscapular region of newborn Sprague–Dawley strain rats, within 48 h after birth.

Cisplatin administration

At 28 days after inoculation, 2 mg/kg body weight cisplatin was injected into the abdominal cavity and the dose repeated twice every 3 weeks during the 90-day observation period. The dose and administration interval of cisplatin showed the most effective inhibition against the growth of tumors developed after inoculation of ROT68/C1 cells [5]. The animals were killed and the wet weight of the tumors was measured. Parts of the tumor tissues were fixed in 10% neutral formaldehyde solution and stained with hematoxylin and eosin for histologic observation.

DNA synthesis

Cells (1.5×10^5) in a logarithmic growth phase were seeded into 60 × 15 mm culture dishes and incubated for 24 h. Then the cells were exposed for 1 h to 1 or 5 μg/ml cisplatin, washed and re-incubated in the medium containing 2 μCi/ml [^{3}H]thymidine (specific activity 2 Ci/mmole, New England Nuclear Corporation, Boston, MA) at 1, 3 and 6 h after exposure to cisplatin. After incubation for 30 min, the radioactive medium was removed and the cells were washed with ice-cold 5% trichloroacetic acid. The incorporated tritium radioactivity was counted with a Beckman LS-235 liquid scintillation spectrometer, according to the method described elsewhere [8].

RESULTS

Approximately 90% of the cisplatin-sensitive parent ROT68/C1 cells were killed by continuous exposure to 0.5 μg/ml cisplatin for 16 h (data not shown). Therefore, the ROT68/C1 cells were initially cultured with medium containing 0.5 μg/ml cisplatin. A routine medium change was performed every other day, since approximately 50% of free cisplatin remained in the medium at 48 h after administration of 1 μg/ml cisplatin (data not shown). The surviving cells began to proliferate and entered a logarithmic growth phase after 2 weeks of continuous exposure to 0.5 μg/ml cisplatin. Thereafter the cells were subcultured with medium containing rising cisplatin concentrations, in steps of 0.5 μg/ml successively. The cells that could proliferate in the presence of 4.5 μg/ml cisplatin were obtained after 35 weeks (10 subcultures) of continuous exposure to cisplatin. This cisplatin-resistant subline of the ROT68/C1 cell line was designated Cis-Pt^r. Before use, the Cis-Pt^r cells were subcultured a further 10 times in cisplatin-free medium. The morphology, growth and cisplatin resistance were stable during that period (data not shown).

The ROT68/C1 cells were small polygonal cells with large nuclei (Fig. 1a). Several ultrastructural characteristics such as surface microvilli, abundant free ribosomes in the cytoplasm, an interdigitated nucleus and desmosome-like cell–cell junctions were visible on electron photomicrography (Fig. 2a). Morphologic alterations such as the enlargement of the cells and nuclei appeared in cisplatin-resistant Cis–Pt^r cells (Fig. 1b). Larger, round nuclei with prominent nucleoli were the ultrastructural features characteristic of the Cis-Pt^r cells (Fig. 2b).

The growth curves and the effect of cisplatin *in vitro* are presented in Fig. 3. The population doubling times of ROT68/C1 and Cis-Pt^r cells in the logarithmic growth phase were 27 h and 31 h, respectively. The time lag of growth was prominent in Cis-Pt^r cells. The growth of ROT68/C1 cells was completely arrested after 48 h of exposure to $\geq$ 5 μg/ml cisplatin for 1 h. In contrast, only a slight growth inhibition was observed in Cis-Pt^r cells even when they were treated with as much as 10 μg/ml cisplatin for 1 h.

The killing curves of cisplatin demonstrated by

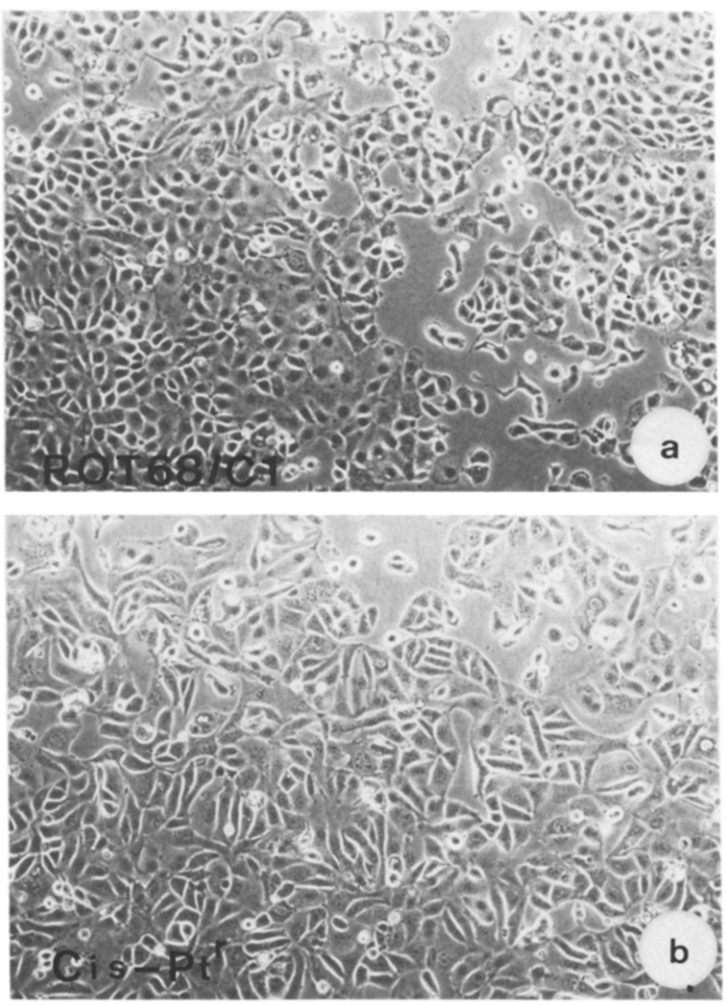

Fig. 1. *Phase-contrast photomicrographs of ROT68/C1 cells (a) and Cis-Pt^r cells (b). Phase-contrast,* × 80.

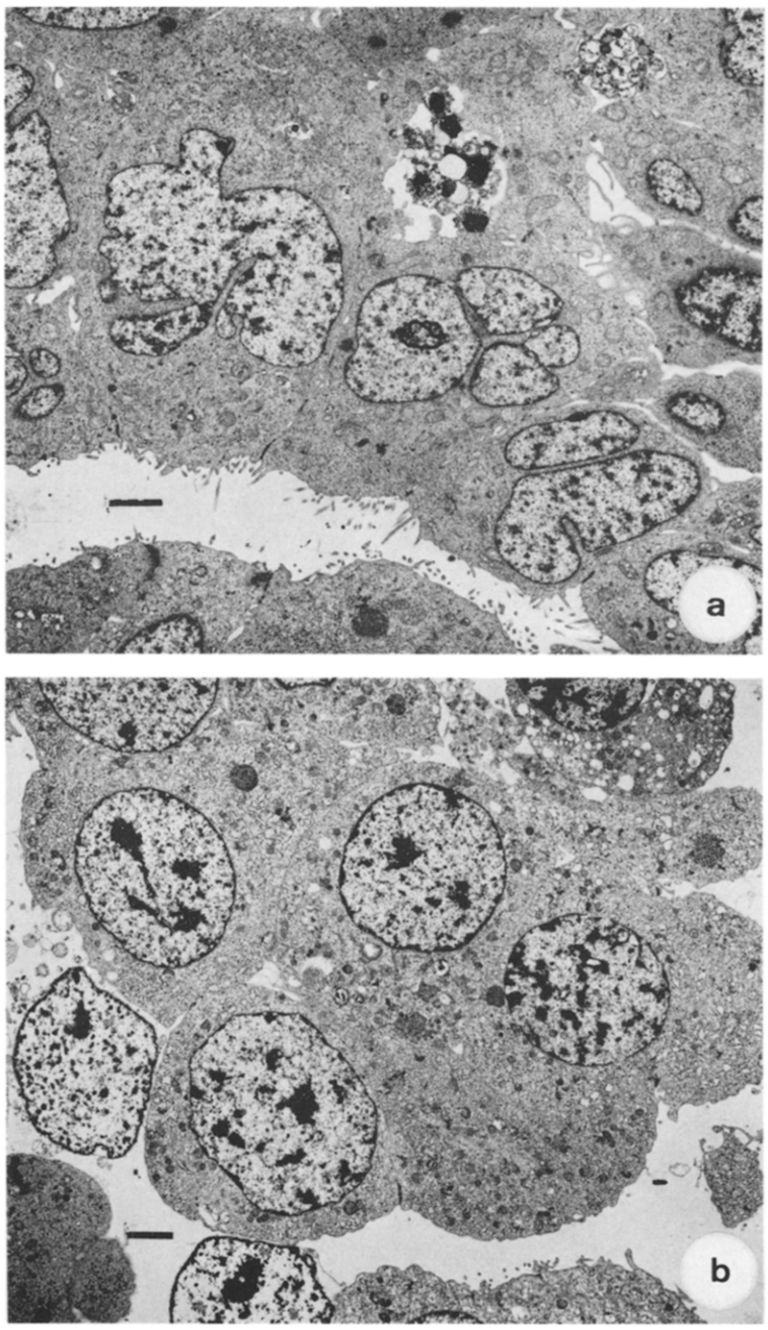

Fig. 2. Electron photomicrographs of ROT68/C1 cells (a, × 2000) and Cis-Pt^r cells (b, × 1600). Bars indicate 2 μm.

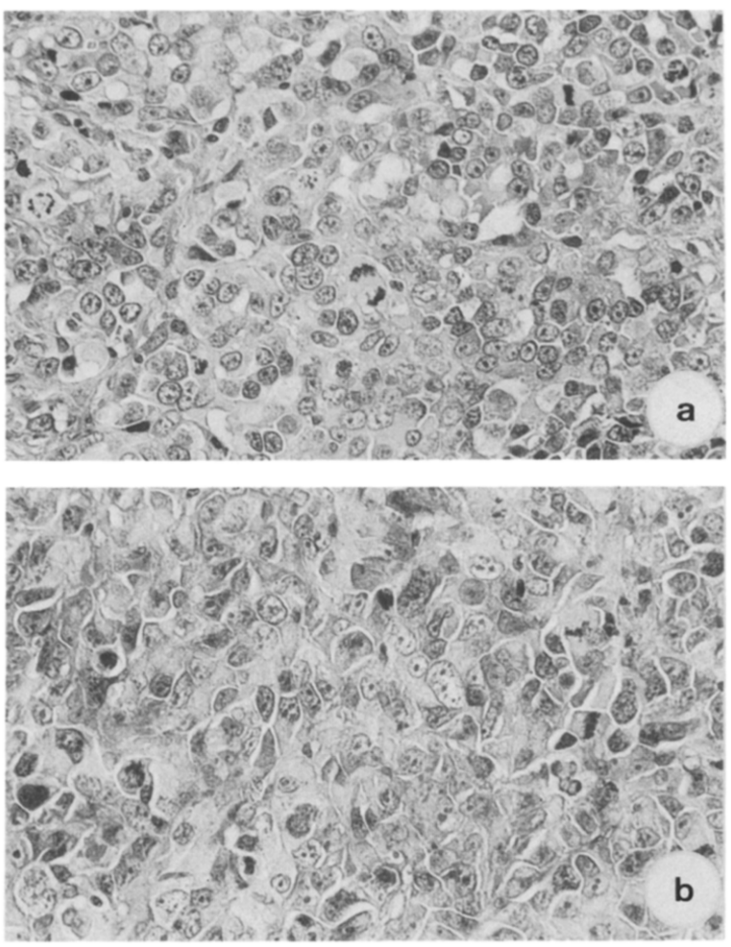

Fig. 6. *Photomicrographs of the tumors developed by inoculation of ROT68/C1 cells* (*a*) *and Cis-Pt*^{r} *cells* (*b*) in vivo. *Hematoxylin and eosin stain*, × 160.

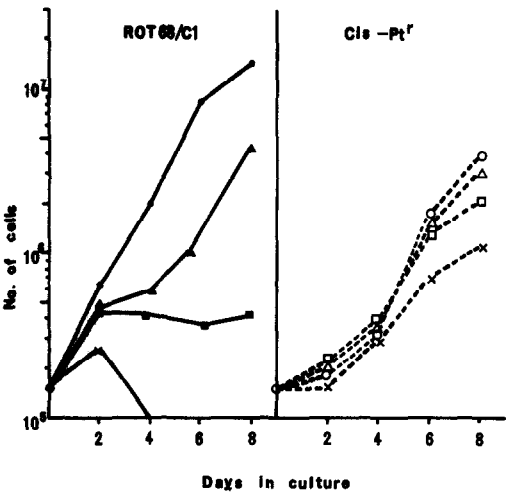

Fig. 3. Effect of cisplatin on the growth of ROT68/C1 and Cis-Pt^r (—●—) and Cis-Pt^r cells in (···○···) in vitro. See Materials and Methods. ▲—, ···△···, 1 μg/ml; —■—, - - - □ - - -, 5 μg/ml; —×—, ···×···, 10 μg/ml.

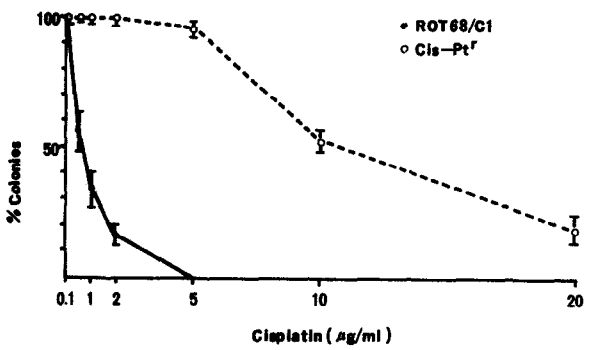

Fig. 4. Effect of cisplatin on the colony forming efficiency of ROT68/C1 (—●—) and Cis-Pt^r cells (- - - ○ - - -) in vitro. See Materials and Methods. Bars indicate means and standard deviation of triplicate samples.

the colony formation of the cells are given in Fig. 4. The Cis-Pt^r cells were about 20-fold more resistant to cisplatin than the ROT68/C1 cells, at a concentration of cisplatin required for 50% colony inhibition.

Both the ROT68/C1 and Cis-Pt^r cells produced tumors when injected subcutaneously in isologous newborn rats. The growth of tumors and the effects of cisplatin on their growth rate are shown in Fig. 5. The tumors which developed after inoculation of ROT68/C1 cells began to grow exponentially 4 weeks after inoculation. Tumor growth was inhibited 3 weeks after the first injection of cisplatin at dose of 2 mg/kg body weight and the inhibition of growth persisted after the two additional injections of cisplatin. Compared to ROT68/C1 cells, the tumors produced by inoculation of Cis-Pt^r cells grew extremely slowly and the growth was not inhibited by injections of cisplatin.

The histology of tumors grown from ROT68/C1 and Cis-Pt^r cells were both undifferentiated adenocarcinoma. However, Cis-Pt^r tumors had

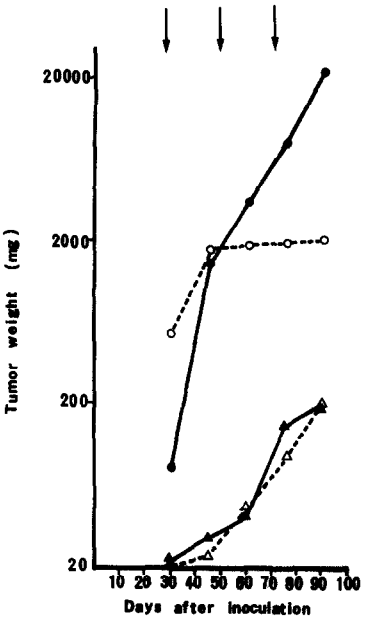

Fig. 5. Effect of cisplatin on the growth of tumors developed by inoculation of ROT68/C1 cells (circle) and Cis-Pt^r cells (triangle) in vivo. —●—, —▲—, untreated; - - - ○ - - -, - - - △ - - -, cisplatin-treated. Arrows indicate cisplatin injections (2 mg/kg body weight).

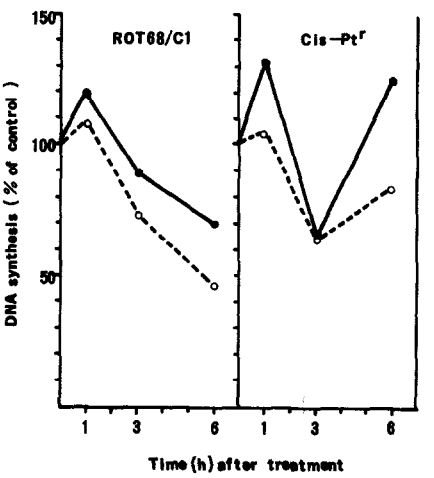

Fig. 7. DNA synthesis of ROT68/C1 cells and Cis-P^r cells after exposure to 1 μg/ml (—●—) or 5 μg/ml (- - - ○ - - -) cisplatin for 1 h in vitro. See Materials and Methods.

larger cells and nuclei than ROT68/C1 tumors (Fig. 6). Thus, the morphologic characteristics of Cis-Pt^r cells when grown *in vitro* were retained *in vivo*.

DNA synthetic activity of cells is one of the indicators for examining cisplatin-resistance. As shown in Fig. 7, DNA synthesis of the ROT68/C1 cells decreased progressively for 6 h after exposure to 1 or 5 μg/ml cisplatin for 1 h *in vitro*. In contrast, DNA synthesis of the Cis-Pt^r cells began to recover as early as 3 h after exposure to cisplatin.

Patterns of cross-resistance of Cis-Pt^r cells to various anticancer drugs *in vitro* are shown in Fig. 8. Both ROT68/C1 and Cis-Pt^r cells showed a similar

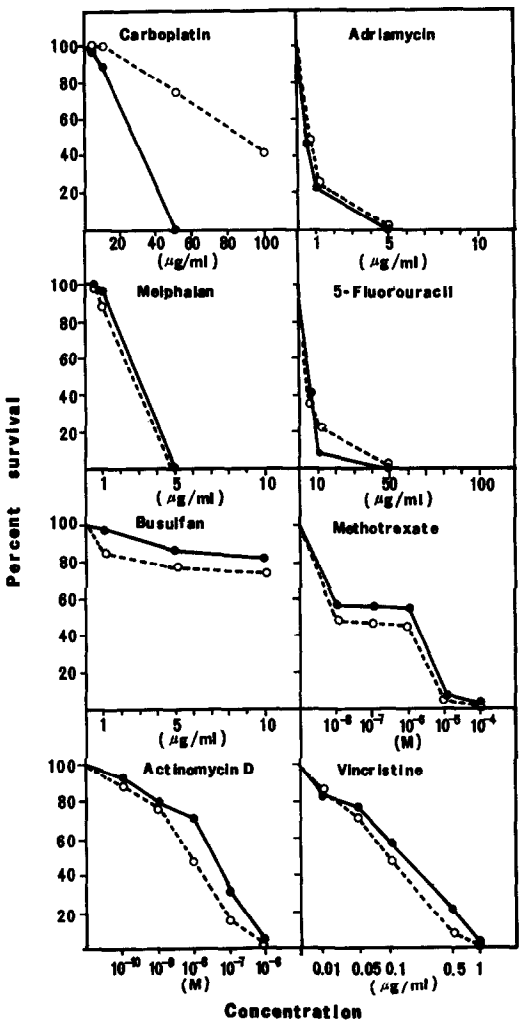

Fig. 8. Sensitivity of ROT68/C1 cells (—●—) and Cis-Pt^r cells (---○---) to various anticancer drugs in vitro. *See Materials and Methods.*

sensitivity to a variety of anticancer drugs except to a newly developed cisplatin analog, carboplatin.

DISCUSSION

Cisplatin-resistance has been studied chiefly using cisplatin-resistant sublines derived from a cisplatin-sensitive murine leukemia L1210 cell line *in vitro* [9–11]. Recently, a cisplatin-resistant human squamous cell carcinomal cell line [12, 13] and several cisplatin-resistant human ovarian cancer cell lines [14–16] have been described. Most of these cisplatin-resistant cell lines were isolated from cisplatin-sensitive parent cell lines by continuous or intermittent exposure of the parent cell lines to increasing cisplatin concentrations *in vitro* [11, 12, 14, 16]. We isolated our cisplatin-resistant subline, Cis-Pt^r, by a similar method. Since the parent ROT68/C1 cells were cloned and usually one surviving colony was picked for subculture, the Cis-Pt^r cells were not selected from cisplatin-resistant cells originally mixed in ROT68/C1 cells. Naturally cisplatin-resistance has been reported in human prostate cancer cell lines [17].

In most previous investigations, the increase in resistance was at most 30-fold (20-fold in our studies) [10–12, 14–16, 18, 19]. These low resistance levels seem to be characteristic of platinum coordination complexes, and differ from resistance to other anticancer drugs.

The stability of cisplatin resistance has not been fully discussed in previous studies. Frei *et al.* [12] reported that isolation of stable cisplatin-resistant cells was difficult, as with other alkylating agents, and usually the levels of resistance gradually diminished after removal of cisplatin. In our present studies, cisplatin-resistant Cis-Pt^r cells were stable for a relatively short period in several subcultures with cisplatin-free medium. However, the levels of resistance tended to decrease and seemed to be relatively unstable after removal of the drug's selection pressure (unpublished observations).

Phenotypic characteristics of cells acquiring cisplatin resistance have not been fully described in previous reports. Our cloned ROT68/C1 cells provide a useful model to examine the characteristics of cisplatin-resistant cells both *in vitro* and *in vivo*. As compared to cisplatin-sensitive parent cells, enlargement of the cell and nuclei was a morphological characteristic of the cisplatin-resistant Cis-Pt^r cells. The same observation was reported by Kikuchi *et al.* [16] using a cell line derived from human serous cystadenocarcinoma of the ovary. Another phenotypic characteristic of Cis-Pt^r cells was their slower growth rate *in vitro* and *in vivo*, as also reported by Frei *et al.* [12] and Shinoya *et al.* [19].

The mechanisms of cisplatin-resistance are not fully elucidated. Reduction of DNA cross-linking [20–22], decreased drug uptake [23, 24] and a high content of cisplatin-binding proteins, methallothioneins [25], have been reported to be the causes of resistance. Our observation of early recovery of DNA synthesis in cisplatin-resistant cells strongly suggests that early DNA repair is one of the mechanisms of cisplatin resistance. Recently, Teicher *et al.* [13] suggeted multifactorial mechanisms in combination with plasma membrane changes, increased cytosolic binding and decreased DNA cross-linking.

Cisplatin belongs to the family of alkylating agents and Teicher *et al.* [13] reported a minor cross-resistance between cisplatin and other alkylating agents. Our cisplatin-resistant Cis-Pt^r cells did not show clear cross-resistance to melphalan and busulfan. On the other hand, a partial cross-resistance and Cis-Pt^r cells to carboplatin was found, as reported by Hrubisko *et al.* [11]. These results indicate that other alkylating agents may be effective in recurrent patients previously given cisplatin.

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