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Active Efflux System for Cisplatin in Cisplatin-resistant Human KB Cells

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Mutants, KCP-4 and PC-5, resistant to an anticancer agent, cisplatin, were selected in multiple steps from human epidermoid KB carcinoma cells and human prostate PC-3 carcinoma cells, respectively. KCP-4 and PC-5 were 63 and 10 fold more resistant to cisplatin than the parental cells, respectively. KCP-4 cells exhibited increased resistance to cisplatin analogues and were also slightly cross-resistant to melphalan, cyclophosphamide, mitomycin C and methotrexate. KCP-4 cells were not cross-resistant to doxorubicin, daunorubicin, vincristine or $CdSO_4$. The accumulations of cisplatin in KCP-4 cells and PC-5 in medium containing 50 μM cisplatin were approximately 20% of those in the parental cells. Revertant analysis suggested that a defect in cisplatin accumulation may be related to cisplatin resistance in PC-5 cells. The uncoupling agent of oxidative phosphorylation, 2,4-dinitrophenol, increased the accumulation of cisplatin in KCP-4 and cisplatin-resistant human prostate carcinoma PC-5 cells to nearly the same level as in their parental KB-3-1 and human prostate carcinoma PC-3 cells without 2,4-dinitrophenol, but did not increase accumulation in KB-3-1 and PC-3 cells. Addition of glucose in the medium inhibited the enhancement of cisplatin accumulation in KCP-4 cells by 2,4-dinitrophenol. Enhanced active efflux of cisplatin from KCP-4 cells was observed. A cell-cell hybridization test showed that the cisplatin resistance and the accumulation defect behaved as codominant traits. These data suggest that an active efflux system for cisplatin exists in cisplatin-resistant KCP-4 cells.

Key words: Cisplatin resistance — Accumulation defect — Enhanced efflux

Cisplatin is one of the most effective agents in cancer chemotherapy, and is useful in the treatment of testicular, ovarian, bladder, and head and neck cancers. However, resistance to cisplatin frequently appears in these tumors.[1)] Many cisplatin-resistant cells have been isolated from different cell lines *in vitro* and some mechanisms of cisplatin resistance have been elucidated.[2)] These mechanisms of resistance include inactivation of the drug by thiol compounds,[3,4)] decreased cisplatin accumulation,[5–10)] accelerated DNA repair,[11–16)] and an increase in metallothionein.[17,18)] Cisplatin accumulation was decreased in many cisplatin-resistant cells, but there was no obvious relationship between the decrease of cisplatin accumulation and the degree of resistance.[2)] Andrews *et al.* showed that decreased cisplatin accumulation is an early change associated with the acquisition of cisplatin resistance in a human ovarian cancer cell line.[19)] The reduced accumulation seems to be related to the low-level cisplatin resistance that is commonly observed in tumors from patients treated with cisplatin. However, the mechanism of cisplatin transport is not clearly elucidated. Cisplatin is generally believed to enter cells by passive diffusion, although there is some evidence that entry may be energy-dependent.[19)] Gately and Howell[20)] postulated that some component of transport occurs through a gated ion channel. Enhanced efflux of cisplatin from cisplatin-resistant cells was reported,[8)] but there has been no information on the molecular basis of the efflux.

We have isolated cisplatin-resistant KB human cancer cells, in which the accumulation of cisplatin was decreased. We thus investigated whether the decreased accumulation is due to enhanced efflux, and also whether the transport is energy-dependent or not. We find that an active efflux pump for cisplatin exists in the cisplatin-resistant KB cells.

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[7] The abbreviations used are: MEM, minimal essential medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HAT, hypoxanthine, aminopterin and thymidine; PBS, phosphate-buffered saline; HBSS, Hanks' balanced salt solution; ICP-MS, inductive coupled plasma mass spectrometry; EMS, ethylmethanesulfonate; MMC, mitomycin C; MTX, methotrexate; UV, ultraviolet.

MATERIALS AND METHODS

Cell culture and cell lines The cells were grown in monolayer in MEM[7] (Nissui Seiyaku Co., Tokyo) containing 10% newborn calf serum (Sera-lab Ltd., Sussex, England), 1 mg Bactopeptone/ml (Difco Laboratories, Detroit, MI), 0.292 mg glutamine/ml and 100 U penicillin/ml.

Human epidermoid KB carcinoma cells were obtained from Dr. M. M. Gottesman (National Cancer Institute, Bethesda, MD). KB cells were subcloned two times; a single recloned line, KB-3-1, was used as the parental cell line for the present study.[21] Cisplatin-resistant mutants from the KB-3-1 cell line were isolated by culturing with cisplatin after EMS mutagenesis. Human KB-3-1 cells were treated with 200 μg/ml EMS for 24 h and then incubated in the absence of any drug for 5 days. The KB-3-1 cells were plated (1×10^6/dish) in 100 mm plastic dishes in MEM containing 10% fetal calf serum and incubated overnight at 37°C. After the incubation, cisplatin was added to a final concentration of 200 mg/ml. Six clones were isolated and purified (KCP-1, -2, -3, -4, -5 and -6). In view of its higher plating efficiency and growth rate, KCP-4 was further incubated in the selection medium with increasing concentrations of cisplatin. Finally, KCP-4 cells were cultured in medium containing 10 μg cisplatin/ml. Another cisplatin-resistant cell line, PC-5 (P/CDP-5) was spontaneously isolated from a human prostate carcinoma PC-3 cell line and cultured as described previously.[3,22] A revertant, KCP-4R, was isolated from KCP-4 cells by culturing in non-selective medium for about 6 months. Another revertant, PC-5R, was isolated from PC-5 cells by culturing in non-selective medium for 5 months.[21] HeLaD98^{OR}-1 cells resistant to ouabain and sensitive to HAT medium were from E. Stanbridge.[23]

Drugs and chemicals Cisplatin and carboplatin were gifts from the Bristol-Myers Research Laboratory. Other cisplatin analogues, DWA2114R and 254-S were donated to us by Chugai Pharmaceutical Co., Tokyo, and Shionogi Pharmaceutical Co., Osaka, respectively. Other drugs and chemicals were obtained from Sigma Chemical Co., St. Louis, MO.

Determination of drug sensitivity MTT colorimetric assay was used to assess chemosensitivity *in vitro* as described.[24] Since the growth rates of the cisplatin-resistant cells are slower than those of the parental cells, 2×10^3 cells for KB-3-1 and PC-3, and 5×10^3 cells for KCP-4 and PC-5 in 0.18 ml of culture medium were inoculated into each well.

Cell-cell hybridization The hybridization of KB cells and HeLa D98^{OR}-1 cells in monolayer was carried out with polyethylene glycol by the method described by Davidson and Gerald.[25] KB and HeLa cells (5×10^5 each) were plated together in a 60-mm plastic dish. The cells were exposed for 1 min to 50% polyethylene glycol (Baker, PEG-1000) in alpha-modified Eagle's medium (α-MEM) (Flow), washed three times with α-MEM containing 10% fetal bovine serum, and incubated for 24 h in the same medium. The cells were then selected in α-MEM with HAT and 0.1 μM ouabain as described previously. Colonies derived from various fusions were surrounded by a steel ring and trypsinized from the dishes. The cell volume was determined using a Coulter Counter Model ZM and Channelyzer 256 (Coulter Electronics, Hialeah, FL).

Cisplatin accumulation Cells were incubated overnight in MEM medium then incubated with cisplatin at the indicated concentrations for 2 h at 37°C. Cells were washed three times with cold PBS and immediately harvested with a rubber scraper. The harvested cells were washed 2 times with cold PBS. Cells were counted with a hemocytometer before the last wash, and viability determinations were made by standard trypan blue exclusion techniques. For accumulation studies in the presence of 2,4-dinitrophenol, cells were incubated for 10 min in glucose-free HBSS with 1 mM 2,4-dinitrophenol,[26] then 50 μM cisplatin was added and the cells were incubated for the time indicated.

Drug efflux studies Cells were incubated overnight in MEM medium, and incubated with 500 μM cisplatin for 10 min at 37°C in glucose-free HBSS with 1 mM 2,4-dinitrophenol, then the cells were incubated for the time indicated at 37°C in HBSS and harvested.

Platinum determination Cell pellets were hydrolyzed in nitric acid and the platinum content was determined by ICP-MS using a model SPQ 6500 apparatus (Seiko Instruments, Tokyo).

RESULTS

Sensitivity of KB-3-1 and KCP-4 cells to cisplatin and its analogues The sensitivity of the cells to cisplatin is shown in Fig. 1. The IC_{50} for KB-3-1 cells was 0.4 μM and that for KCP-4 cells was 25 μM. KCP-4 cells were 63 times more resistant to cisplatin than the parental KB-3-1 cells. An incomplete revertant, KCP-4R, isolated from KCP-4 was about 4 times more resistant to cisplatin compared to the parental KB-3-1 cells (Fig. 1A). The IC_{50} values for PC-3, PC-5 and PC-5R were 5.1, 50 and 3.2 μM, respectively. PC-5 cells were about 10 times more resistant to cisplatin compared to the parental PC-3 cells, and cisplatin resistant had completely reverted in PC-5R cells (Fig. 1B). The effect of cisplatin analogues on survival of KB-3-1 and KCP-4 cells was also tested (Table I). KCP-4 cells showed significant resistance to other platinum complexes. KCP-4 cells were 29, 35, and 65 times more resistance to carboplatin, DWA2114R, and 254-S respectively than KB-3-1 cells.

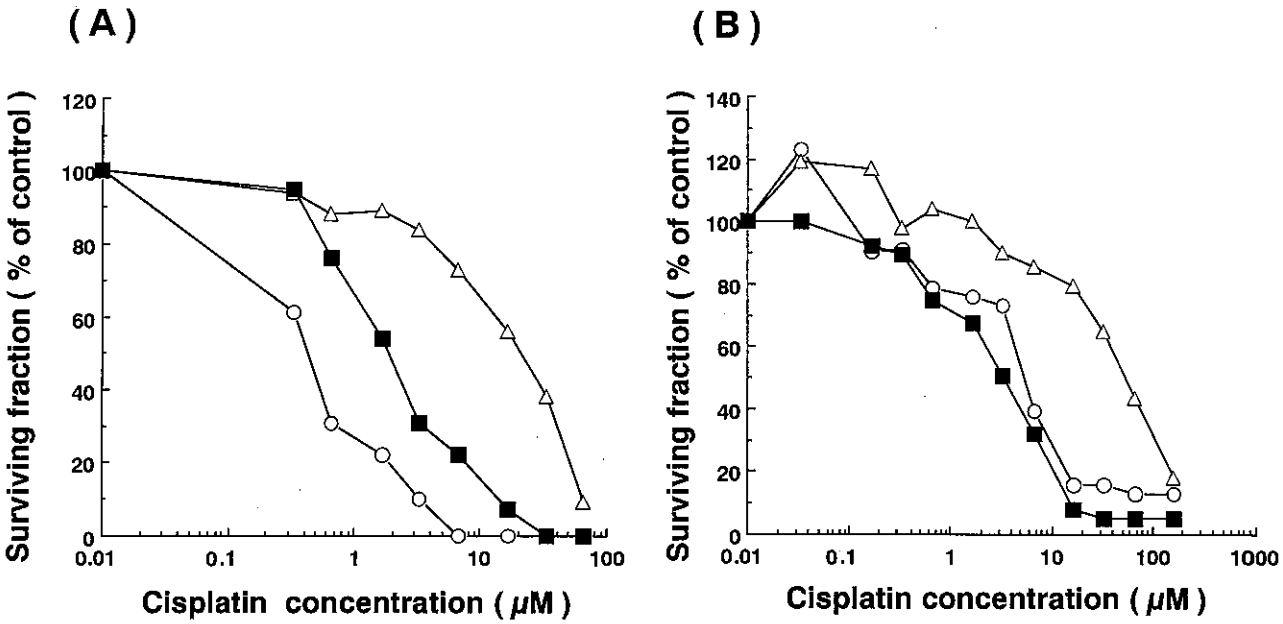

Fig. 1. Sensitivity of human carcinoma cells to cisplatin. Cells were exposed to various concentrations of cisplatin and incubated for 4 days. The cytotoxic effect of cisplatin was examined by MTT assay. (A) Parent KB-3-1 (○), cisplatin-resistant mutant KCP-4 (△), revertant KCP-4R (■). (B) Parent PC-3 (○), cisplatin-resistant PC-5 (△), revertant PC-5R (■). Each point represents the mean of duplicate determinations.

Table I. Drug Resistance in Cisplatin-resistant KCP-4 Cells

Agent	IC_{50} (μM)		RR[a]
	KB-3-1	KCP-4	
Cisplatin	0.4	25	62.5
Carboplatin	5.5	160	29.1
DWA2114R	2.9	100	34.5
254-S	0.52	34	65.4
$CdSO_4$	15.0	21	1.4
Melphalan	39.4	197	5.0
Cyclophosphamide	1.34	3.64	2.7
MMC	1.5	6.0	4.0
MTX	0.013	0.066	5.1
Doxorubicin	0.050	0.053	1.1
Vincristine	0.017	0.023	1.4
Daunorubicin	0.040	0.042	1.1

a) RR: Relative resistance was determined by dividing the IC_{50} value of the drug for KCP-4 cells by that for KB-3-1 cells.

Cross-resistance to other anticancer agents Cell lines resistant to one drug are often cross-resistant to structurally and functionally unrelated cytotoxic agents because of altered membrane permeability. We tested the sensitivity of KCP-4 cells to melphalan, cyclophosphamide, MMC, MTX, $CdSO_4$, doxorubicin, daunorubicin and vincristine (Table I). KCP-4 cells were slightly more resistant to melphalan, cyclophosphamide, MMC and MTX than the parental cells. The sensitivity of KCP-4 cells to $CdSO_4$, doxorubicin, daunorubicin, and vincristine was similar to that of KB-3-1 cells. The dose response to UV light exposure was also the same between KCP-4 and KB-3-1 cells (data not shown).

Dominance analysis of cisplatin resistance using somatic cell hybrids To test for the dominance or recessiveness of the cisplatin-resistant trait in the presence of a wild-type genome, cisplatin-resistant KCP-4 cells were hybridized with cisplatin-sensitive HeLaD98^{OR}-1 cells (HAT^S, oua^R). As the control, cisplatin-sensitive KB-3-1 cells were hybridized with HeLaD98^{OR}-1 cells. The hybrids were formed and maintained in HAT medium containing 0.1 mM ouabain. Hybrids cells appeared at a frequency of 10^{-5}, while HAT^S, oua^R mutants appeared at a rate of 5×10^{-8} from each parental cell line. The mean cell volumes of KB-3-1, KCP-4, HeLaD98^{OR}-1, KB-3-1$\times$HeLaD98^{OR}-1, and KCP-4$\times$HeLaD98^{OR}-1, were 1.83, 1.90, 1.33, 2.70 and 2.50 μm^3, respectively.

We examined the cisplatin sensitivity of the hybrid clones, HeLaD98^{OR}-1, KB-3-1, and KCP-4 cells. When the sensitivity was determined one month after cell fusion, the hybrid cell line between KCP-4 and HeLaD98^{OR}-1 was more resistant to cisplatin than the control hybrid cell line KB-3-1$\times$HeLaD98^{OR}-1, but less resistant than KCP-4 cells (Fig. 2). It therefore appears that the cisplatin-resistance is incompletely dominant.

Accumulation and efflux of cisplatin The amounts of cisplatin accumulated in 2 h in KB-3-1 and KCP-4 cells and also in human prostate carcinoma PC-3 cells and the cisplatin-resistant subline, PC-5 cells, were examined. When the cells were incubated in medium containing 10, 20 and 50 μM cisplatin, the intracellular level of Pt in KCP-4 cells was about one-third, one-fourth and one-fifth that in KB-3-1 cells (Fig. 3A). Similar results were obtained for the accumulation of cisplatin in PC-3 and PC-5 cells (Fig. 3B). To study whether the decreased accumulation is related to cisplatin resistance, we isolated revertants KCP-4R and PC-5R from KCP-4 and PC-5, respectively, and measured the cisplatin accumulation in these revertant cells. KCP-4R cells had not completely reverted and were still 4 times more resistant to cisplatin than KB-3-1 cells, but PC-5R cells had completely reverted. The accumulation of cisplatin in KCP-4R cells were about 2 times that in KCP-4 cells and 40% of that in KB-3-1 cells when the cells were treated with 20 to 50 μM cisplatin for 2 h. Meanwhile, the accumulation of cisplatin in PC-5R cells was about three times that in PC-5 cells, and 70% of that in PC-3 cells (Fig. 3).

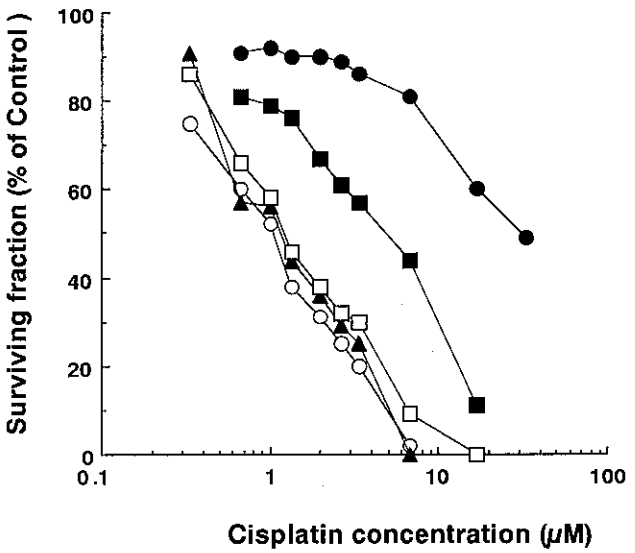

Fig. 2. Dose-response of hybrid cell lines and their parental cells to cisplatin. Cytotoxic effect of cisplatin on KB-3-1 (▲), HeLaD98^{OR}-1 (○), HeLaD98^{OR}-1×KB-3-1 (□), HeLaD98^{OR}-1×KCP-4 (■), and KCP-4 (●) was analyzed by MTT assay. Each point represents the mean of duplicate determinations.

2,4-Dinitrophenol is an uncoupling agent of oxidative phosphorylation. It allows electron transport to continue but prevents the phosphorylation of ADP to ATP. The effect of 2,4-dinitrophenol on the accumulation of cisplatin was compared in KB-3-1 and KCP-4 cells in glucose-free medium to determine if the decreased accumulation of cisplatin in KCP-4 cells is due to enhanced active efflux or reduced uptake of the drug by the cells. Viability of the cells was not decreased after 60 min treatment with 2,4-dinitrophenol in the glucose-free

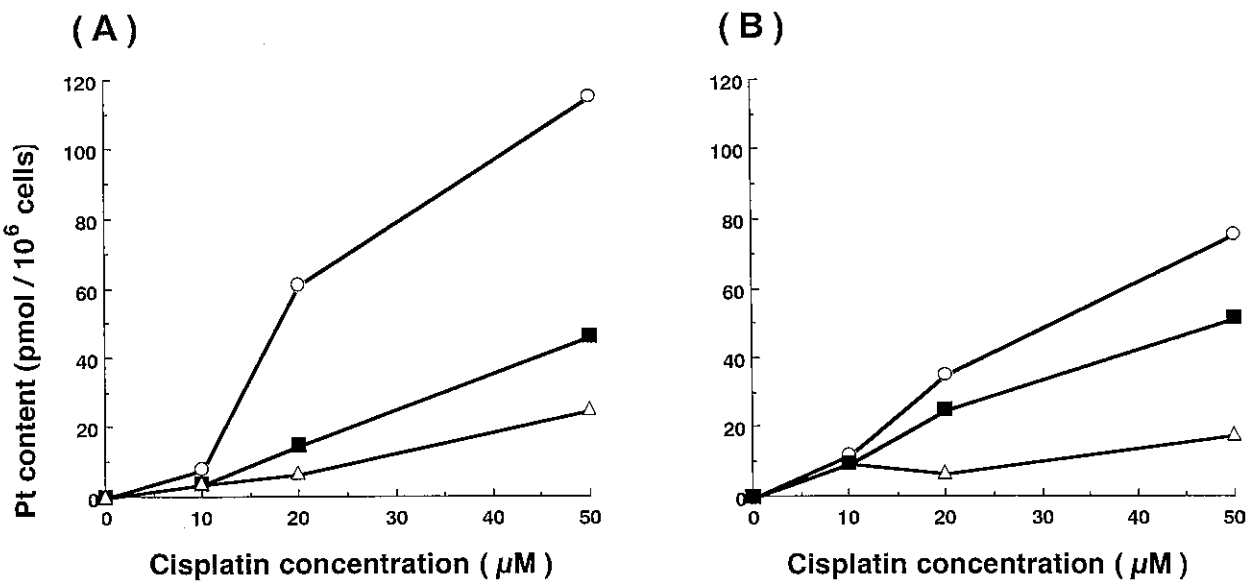

Fig. 3. Accumulation of platinum in human cancer cells. (A) Accumulation of cisplatin in KB-3-1 (○), KCP-4 (△) and KCP-4R (■) at the indicated concentrations. (B) Accumulation of cisplatin in PC-3 (○), PC-5 (△) and PC-5R (■) at the indicated concentrations. Each point represents the mean of duplicate determinations.

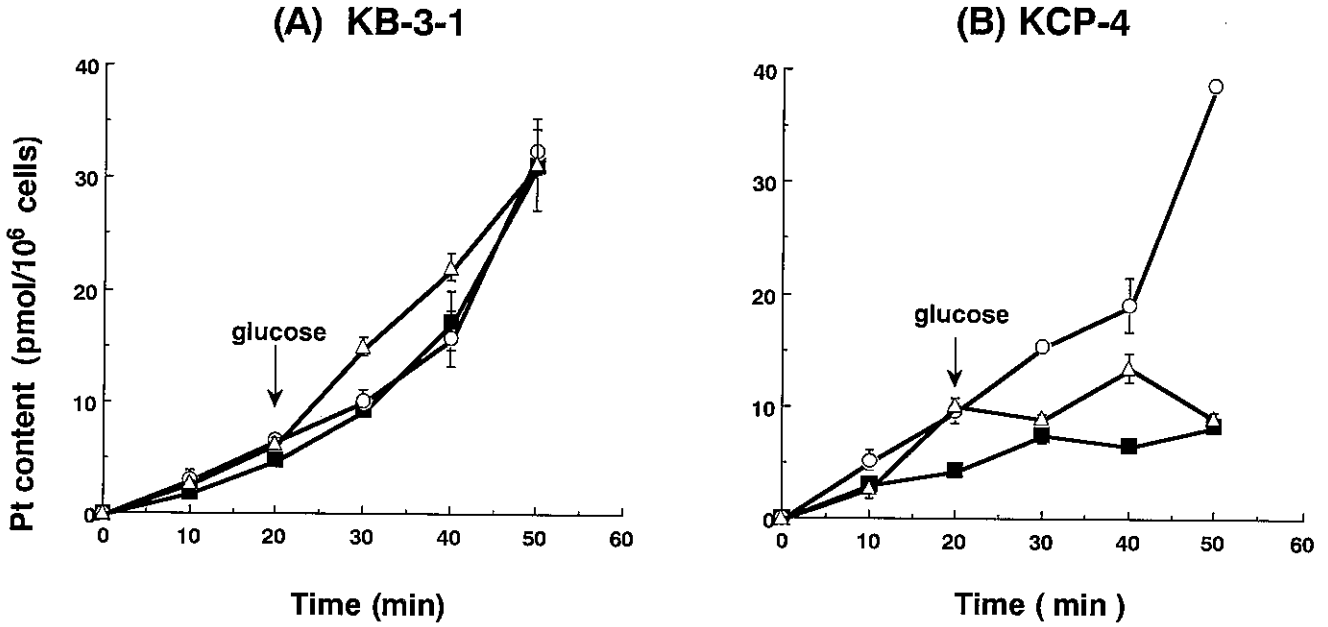

Fig. 4. Effects of 2,4-dinitrophenol on accumulations of cisplatin. KB-3-1 (A) and KCP-4 (B) cells were incubated in HBSS (■) or glucose-free HBSS with 1 m*M* 2,4-dinitrophenol (○,△) for 10 min at 37°C; then 50 μM cisplatin was added and incubated for the indicated times. At 20 min after adding cisplatin, 1 mg/ml glucose was added to the medium (△). Cells were harvested at the indicated times and the Pt content was determined as described in "Materials and Methods." Points, mean of triplicate determinations; bars, SE.

medium. Accumulation of cisplatin was enhanced in KCP-4 cells but not in KB-3-1 cells by 2,4-dinitrophenol. The accumulation of cisplatin in KCP-4 cells in the presence of 2,4-dinitrophenol was nearly the same as that in KB-3-1 cells without 2,4-dinitrophenol. The addition of glucose at 20 min stopped the linear increase of cisplatin accumulation in KCP-4 cells but not in KB-3-1 cells (Fig. 4). Similar results were obtained when the accumulation study was carried out with PC-3 and PC-5 cells (Fig. 5). Accumulation of cisplatin was enhanced by 2,4-dinitrophenol in PC-5 cells but not in PC-3 cells. The accumulation of cisplatin in PC-5 cells in the presence of 2,4-dinitrophenol was similar to that in PC-3 cells with 2,4-dinitrophenol. Next we examined whether the decreased accumulation of cisplatin in KCP-4 cells is due to enhanced efflux. The cells were incubated with cisplatin for 10 min at 37°C, and then further incubated without cisplatin. Very rapid initial efflux of cisplatin from KCP-4 cells was seen when the cells were incubated in the medium with glucose. Within 5 min of incubation in the medium with glucose, 54% of cisplatin accumulated in the KCP-4 cells was released from the cells. After incubation of the cells for 40 min in the presence of glucose, about 60% of cisplatin was lost from KCP-4 cells, whereas all of the cisplatin was retained in KB-3-1

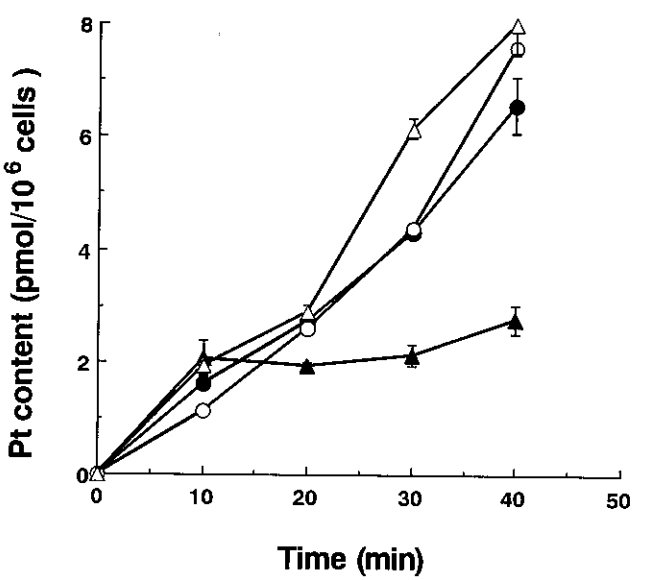

Fig. 5. Effect of 2,4-dinitrophenol on accumulations of cisplatin in PC-3 and PC-5 cells. PC-3 (●,○) and PC-5 (▲,△) were incubated in HBSS (●,▲) or glucose-free HBSS with 1 m*M* 2,4-dinitrophenol (○,△) for 10 min at 37°C, then 50 μM cisplatin was added and incubated for the indicated times. Cells were harvested and the Pt content was determined. Points, mean of triplicate determinations; bars, SE.

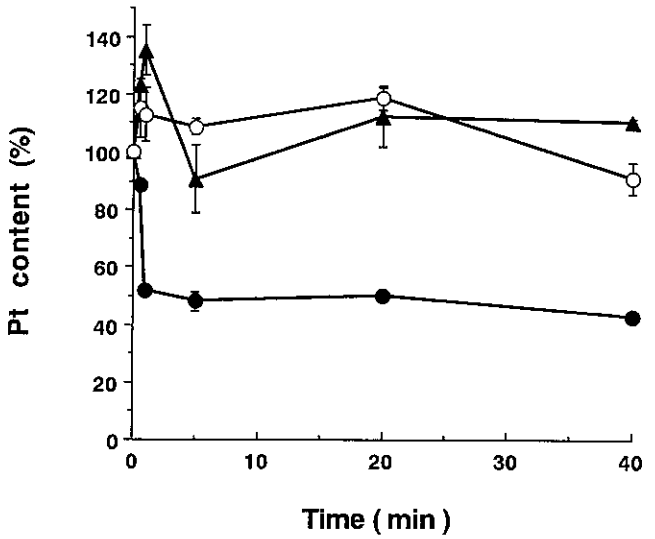

Fig. 6. Effect of 2,4-dinitrophenol on efflux of cisplatin. Release of cisplatin in the absence (●,▲) or presence (○) of 2,4-dinitrophenol from KB (▲) and KCP-4 (●,○). Points, mean of triplicate determinations; bars, SE.

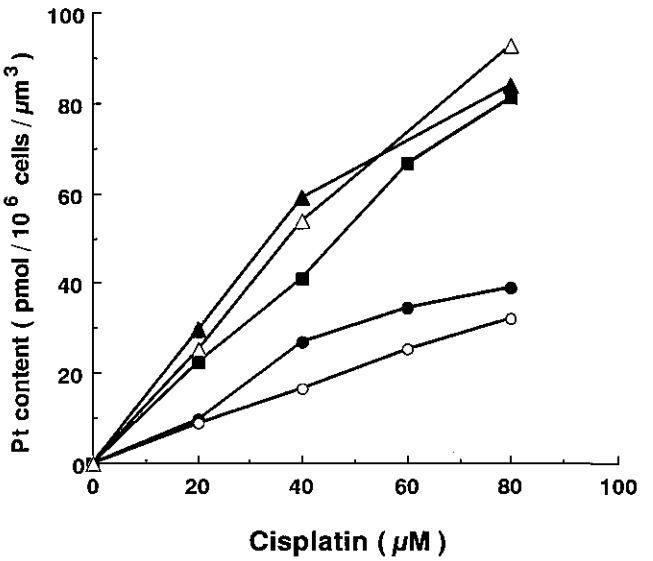

Fig. 7. Accumulation of cisplatin in hybrid cells. Accumulation of cisplatin in KB-3-1 (▲), HeLaD98^{OR}-1 (■), KCP-4 (○), and hybrid clones between HeLaD98^{OR}-1 and KCP-4 (●), and between HeLaD98^{OR}-1 and KB-3-1 (△), was measured at the indicated concentrations of cisplatin in the medium. Each point represents the mean of duplicate determinations.

cells (Fig. 6). The efflux of cisplatin from KCP-4 cells in glucose-free medium was completely inhibited by 2,4-dinitrophenol (Fig. 6). The hybrid cells of KCP-4 and HeLaD98^{OR}-1 showed similar but slightly higher cisplatin accumulation compared to KCP-4 cells (Fig. 7).

DISCUSSION

Many cisplatin-resistant cell lines have been generated and characterized to elucidate the mechanism of cisplatin resistance in human tumors. In most of the resistant cells, cisplatin accumulation was decreased, and the decreased accumulation was considered as one of the mechanisms of the resistance.[20] However, there has been no direct evidence on the relationship between cisplatin resistance and decreased accumulation of cisplatin.[2] Cisplatin accumulation in cisplatin-resistant KCP-4 and PC-5 cells was also decreased compared with that in parental KB-3-1 and PC-3 cells. We have compared the degree of resistance with the decrease in platinum content in cisplatin-resistant sublines. KCP-4 cells were 63 times more resistant to cisplatin and had about 80% less accumulated platinum compared with the parental cells. Meanwhile the revertant KCP-4R, while only 4 times more resistant to cisplatin, showed a 70% decrease in cisplatin accumulation. The lack of correlation between the degree of resistance and the decrease in platinum content does not necessarily mean that the decreased accumulation is not related to cisplatin resistance. Andrews *et al.* showed that decreased cisplatin accumulation is an early change associated with the emergence of the cisplatin-resistance phenotype in a human ovarian cancer cell line.[19] Cisplatin resistance in KCP-4 cells may result not only from decreased cisplatin accumulation but also from other changes in cisplatin-resistant cells. Cisplatin accumulation in a revertant PC-5R was three times that in cisplatin-resistant PC-5 cells and close to that in parental PC-3 cells. This result may suggest that the decreased accumulation of cisplatin is related to the cisplatin resistance in PC-5 cells. KCP-4 cells were not cross-resistant to $CdSO_4$ and UV exposure, suggesting that metallothionein and repair enzymes concerned with excision of thymine dimers are not involved in the resistance.

Most previous studies did not elucidate whether the decrease of cisplatin accumulation was attributed to decreased uptake or increased efflux of cisplatin by cisplatin-resistant cells. Cisplatin accumulation in KB-3-1 cells in glucose-free medium was not affected by 2,4-dinitrophenol, but that in KCP-4 cells was increased to the level of that in KB-3-1 cells by 2,4-dinitrophenol. Addition of glucose abolished this effect of 2,4-dinitrophenol on KCP-4 cells. These results suggest the presence of an active efflux mechanism in KCP-4 cells. We thus examined whether cisplatin is released from KCP-4 cells and whether the efflux is ATP-dependent. Our data showed that cisplatin was actively effluxed from KCP-4

cells but not from KB-3-1 cells. Mann *et al.* reported that retention of platinum in the rapidly effluxing pool of cisplatin-resistant 2008 human ovarian carcinoma cells was also decreased.[8]

Experiments using somatic cell hybrids to test whether cisplatin resistance behaves as a dominant or recessive trait indicate that cisplatin-resistant phenotype is incompletely dominant in the intraspecific hybrid between cisplatin-resistant KCP-4 cells and sensitive HeLaD98^{OR}-1 cells. The sensitivity to cisplatin of KB-3-1 cells and HeLaD98^{OR}-1, and the levels of accumulation of cisplatin in the two cell lines were similar. KCP-4 cells showed about 70% decrease in cisplatin accumulation compared with KB-3-1 cells and HeLaD98^{OR}-1 cells, and the somatic cell hybrid showed similar but slightly higher cisplatin accumulation to that of the KCP-4 cells when corrected for cell volume. These data indicate that the accumulation defect is a codominant trait, and are consistent with the inference that an active efflux pump for cisplatin is expressed in KCP-4 cells. Richon *et al.* previously reported that the cisplatin-accumulation defect in murine leukemia L1210 cells behaves as a recessive trait.[27] However, they did not investigate whether the decrease in platinum accumulation was caused by an alteration in drug uptake or drug efflux. The mechanism of the decreased accumulation of cisplatin in KCP-4 cells and cisplatin-resistant L1210 cells may be different. KCP-4 cells were not resistant to vincristine, doxorubicin and daunorubicin that are transported by P-glycoprotein.[28] Further, we could not detect P-glycoprotein in KCP-4 cells (data not shown). An active drug efflux pump other than P-glycoprotein seems to exist and transport cisplatin in KCP-4 cells.

The increased expression of a 200 kDa membrane glycoprotein in cisplatin-resistant murine cells was reported.[29] However, it is not known whether this glycoprotein is involved in mediating the reduced accumulation of cisplatin in the resistant cells. We analyzed membrane proteins in KCP-4 cells by one- and two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis and found that the expression of some proteins was elevated in the cisplatin-resistant cells, but there was no obvious elevation in the amount of a 200 kDa protein such as that found in the cisplatin-resistant murine lymphoma cells (data not shown; D.W. Shen *et al.*, submitted). Recently, Ishikawa and Ali-Osman suggested that the ATP-dependent glutathione S-conjugate export pump named the GS-X pump[30] may work to efflux the bis-(glutathionato)-platinum(II) complex from L1210 murine leukemia cells.[31] We are now investigating whether the active efflux pump for cisplatin in KCP-4 cells is identical with the GS-X pump. Further study is needed to elucidate the molecular basis for the active efflux system expressed in KCP-4 cells and whether it exists in other cisplatin-resistant cell lines.

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