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ORIGINAL ARTICLE

Cisplatin sensitivity of oral squamous carcinoma cells is regulated by Na^+,K^+-ATPase activity rather than copper-transporting P-type ATPases, ATP7A and ATP7B

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Abstract
Purpose Cisplatin (CDDP) is one of the major chemotherapeutic drugs, but tumor cells' acquired resistance to CDDP limits its therapeutic potentials. One of the main reasons of resistance is reduced drug accumulation. The mechanism by which tumor cells accumulate reduced CDDP is not well elucidated yet. The aim of this study was to investigate what regulates intracellular CDDP accumulation.
Methods Six different types of oral squamous carcinoma cells were used in this study. Assessment of CDDP sensitivity was determined by measuring the ATP level of the cells. Intracellular CDDP and copper (Cu) accumulation were measured and CDDP efflux study was conducted. Assessment of Na^+,K^+-ATPase α and β subunits, ATP7A and ATP7B was done by western blotting. Specific activities of Na^+,K^+-ATPase and copper-transporting P-type ATPase (Cu^{2+}-ATPase) were detected and a role of Na^+,K^+-ATPase inhibitor in intracellular CDDP accumulation was examined.
Results Among the cells HSC-3 and BHY cells were found most CDDP-sensitive and CDDP-resistant, respectively. The most CDDP-sensitive HSC-3 cells exhibited an increased intracellular cisplatin accumulation, high Na^+,K^+-ATPase activity and over-expressed Na^+,K^+-ATPase α and β subunits, ATP7A and ATP7B, compared to the most CDDP-resistant BHY cells, but there were no such differences between the two in the CDDP efflux level or Cu^{2+}-ATPase activity. Moreover, pretreatment with Na^+,K^+-ATPase inhibitor markedly reduced intracellular cisplatin accumulation.
Conclusions Na^+,K^+-ATPase activity is responsible for regulating intracellular CDDP accumulation in oral squamous carcinoma cells rather than Cu^{2+}-ATPase.

Keywords Cisplatin · Oral squamous cell carcinoma · Na^+,K^+-ATPase · Copper-transporting P-type ATPase · ATP7A · ATP7B

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Introduction

The chemotherapeutic drug cisplatin [*cis*-diamminedichloroplatinum(II); CDDP] has been in widespread use for many years to treat several forms of cancer including testicular, ovarian, cervical, head and neck and non-small-cell lung cancers [1]. However, rapid development of both inherent and acquired resistance to the drug limits its application. Emergence of resistance in oral squamous cell carcinoma is commonly accompanied by reduced drug accumulation with changes in the profile of uptake/efflux. However, the mechanism by which reduced CDDP enters tumor cells is not yet clarified [2].

Cellular uptake and efflux of CDDP should be mediated by transporters and carriers. The uptake may involve the carriers or channels as well as various endocytic routes; particularly efflux by macropinocytosis [3] is energy-dependent and biphasic with the rapid early phase and the very long secondary phase [4, 5]. At present only a small number of transporters are known to influence the uptake and efflux of this drug [6].

The Na^+,K^+-ATPase, sometimes called Na^+ pump or Na^+,K^+ pump, is a transmembrane enzyme acting as an electrogenic ion transporter in the plasma membrane of all

mammalian cells. It performs several vital physiological functions that are implicated in numerous pathological states. Each cycle of Na^+,K^+-ATPase activity extrudes three Na^+ from a cell, moves two K^+ into the cell and utilizes one ATP [7]. The primary role of the Na^+,K^+-ATPase is therefore to maintain high intracellular K^+ and low intracellular Na^+ [8]. The Na^+,K^+-ATPase is also involved in CDDP transport, and several reports already suggested that Na^+,K^+-ATPase mediated transport of CDDP into cells [9, 10].

ATP7A and ATP7B are the Cu^{2+}-ATPases, homologous both in structure and in function, and mediate the efflux of Cu^{2+}. They are important constituents of the Cu^{2+} homeostasis system that evolves to deliver Cu^{2+} to Cu^{2+}-requiring proteins while it protects cells from toxic effects of Cu^{2+}. Like other P-type ATPases, these proteins utilize the energy of ATP hydrolysis to transport Cu^{2+} across the vesicular membrane via a process that involves the formation of a transient acylphosphate intermediate [11]. The major Cu^{2+} uptake transporter is the copper transporter 1 (CTR1) [12]. Recent data indicate that the Cu^{2+} homeostasis system also regulates the uptake, intracellular compartmentalization and efflux of CDDP [13–15].

The aim of this work was to investigate in detail what regulates intracellular CDDP accumulation. In an attempt to examine the intracellular CDDP accumulation, we used six different types of oral squamous carcinoma cells and determined CDDP sensitivity of the cells. Then we took the most sensitive and resistant variants among the cells and measured their intracellular CDDP and copper accumulation, CDDP efflux and the Na^+,K^+-ATPase and Cu^{2+}-ATPase activity. We observed that there was no difference in Cu^{2+}-ATPase activity between them, although there was a marked difference in the level of Na^+,K^+-ATPase activity. Moreover pretreatment with a specific inhibitor of Na^+,K^+-ATPase markedly reduced the intracellular CDDP accumulation. Our results strongly suggest that the reduction in cellular CDDP accumulation is regulated by Na^+,K^+-ATPase activity and that Cu^{2+}-ATPase may play a marginal role, if any, in CDDP transport. This result may help to develop the strategies to overcome CDDP resistance and hence improve the therapeutic outcome of platinum-based chemotherapy.

Materials and methods

Drugs, reagents and antibodies

We used the following drugs, reagents and antibodies. Cisplatin, Tween 20 (Sigma, St Louis, MO, USA); Trypsin-EDTA (GibcoBRL, Bethesda, MD, USA); Dulbecco's modified eagle's medium (D-MEM) (ICN Biomedicals Inc., Aurora, OH, USA); Minimum essential medium eagle (MEM) (ICN Biomedicals Inc., Costa Mesa, CA, USA); Fetal bovine serum (FBS) (HyClone, Perbio, Canada); Kanamycin sulfate (Meiji, Japan); Antibodies against Na^+,K^+-ATPase α (anti- Na^+,K^+-ATPase α-1; Upstate Biotechnology, Lake Placid, NY, USA); Antibodies against Na^+,K^+-ATPase β (anti- Na^+,K^+-ATPase β-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA); Antibodies against ATP7A (ATPase alpha polypeptide Cu^{2+} transporting (ATP7A) polyclonal antibody; Orbigen Inc, San Diego, CA, USA); Antibodies against ATP7B (ATPase alpha polypeptide Cu^{2+} transporting (ATP7B) polyclonal antibody; Orbigen Inc); Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG1, IgG2a, IgG (H + L); Goat anti-rabbit IgG (H + L) (ZYMED, San Francisco, CA, USA). All other drugs and chemicals were obtained from Wako Pure (Osaka, Japan).

Cells and culture conditions

Six different types of human oral squamous carcinoma cells were used in this study, which were provided by the Japanese Cancer Research Resources Bank (JCRB, Tokyo, Japan). The cells were HSC-2 (epithelial-like squamous cell carcinoma of mouth, with neither invasive nor metastatic potential), HSC-3 (squamous cell carcinoma of tongue, low metastatic type), HSC-4 (squamous cell carcinoma of tongue, non-metastatic type), Ca9-22 (epithelial-like gingival carcinoma), BHY (epithelial-like squamous cell carcinoma of mouth, non-metastatic but highly invasive to muscle and bone) and TYS cells (salivary gland tumor).

The cells were grown in a humidified incubator gassed with 5% CO_2–95% air at 37°C. The media used for HSC-2, HSC-3, HSC-4, Ca9-22 and BHY cells were D-MEM supplemented with 1 mM L-glutamine, 4.5 g/l glucose, 3.7 g/l $NaHCO_3$, 66.5 mg/l kanamycin sulfate and 10% FBS. For TYS cells the medium was MEM supplemented with 1 mM L-glutamine, 2.2 g/l $NaHCO_3$, 66.5 mg/l kanamycin sulfate and 10% FBS. The cells were maintained in 100 × 20 mm tissue culture dish (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) and media were renewed every second day. Subcultures were obtained by trypsin-EDTA treatment. Experiments were performed when the dishes became at least 80% confluent.

Assessment of cellular sensitivity to CDDP at various concentrations and incubation periods

The cells were sub-cultured in 96-well plates (Falcon, Becton Dickinson Labware). After the cells reached confluence (at least 80%), they were treated with various concentrations of freshly prepared CDDP up to 1 mM dissolved in respective media and incubated for 24 h. Then

cellular sensitivity was assayed by determination of the ATP (adenosine triphosphate) level in the cells, with luminometer using ViaLight Plus (Cambrex Bio Science Inc., Rockland, ME, USA) according to the procedure recommended by the manufacturer. ATP values were expressed as percentage (%) in comparison with controls and the values shown are the means ± SD of three independent experiments. For time course assay, the cells were treated with 1 mM CDDP, incubated for various time frames up to 24 h and then the ATP level in the cells was measured.

Intracellular accumulation of CDDP and copper

For characterization of CDDP and copper uptake, 2×10^5 cells were seeded in 100 mm tissue culture dishes and the cells were exposed to freshly prepared CDDP or $CuSO_4$ dissolved in respective media at various concentrations up to 1 mM. After 3 h of exposure to the drugs, the media were discarded quickly and the cells were washed three times with 5 ml ice-cold PBS buffer. Then cells were trypsinized, scraped and resuspended in fresh drug-free medium and centrifuged at 1,000 rpm for 4 min. The supernatant was discarded and the pellet was washed twice in 1 ml ice-cold PBS buffer. After centrifugation for 2 min at 4°C and 6,000×*g* the supernatant was discarded again and the cell pellet was frozen at −20°C until analysis. Immediately after thawing the cells were lysed with concentrated nitric acid for 20 min in the water bath at 60°C. The lysed samples were then diluted to 3.5 ml of distilled water and intracellular platinum (Pt) and Cu concentrations were measured by the inductively coupled plasma atomic emission spectrometer (ICP-AES) (Hitachi P-4010, Tokyo, Japan). Pt and Cu levels were expressed as μM/2×10^5 cells, with the cell number determined by counting (Burker-Turk cell counter, SLGC, Tokyo) in parallel cultures. Experiments were performed in duplicate and the values expressed were the means ± SD of the three independent experiments. The effects of preincubation with ouabain, a specific inhibitor of Na^+,K^+-ATPase, on the intracellular accumulation of Pt were also investigated. Ouabain of 0.1 mM concentration was added at 60 min prior to 3 h exposure to different concentrations of CDDP and Pt accumulation was determined as described above.

CDDP efflux study

For characterization of the efflux of cisplatin, 2×10^5 cells were incubated with 1 mM cisplatin for 3 h as described above. Subsequently the cells were washed three times with 5 ml ice-cold PBS buffer and incubated in a fresh drug-free medium for various times up to 3 h. The further procedure was same as described above.

Assessment of Na^+,K^+-ATPase and Cu^{2+}-ATPase activities

Microsomes of HSC-3 and BHY cells were prepared according to Jorgensen's method [16] with some modifications [17].

Na^+,K^+-ATPase activities of the microsomes from HSC-3 and BHY cells were measured in triplicate at 37°C in the same procedure as we recently described [18]. The concentrations of various components in the reaction mixture were 1–10 μl microsome, 25 mM sucrose, 0.1 mM EDTA, 50 mM tris–HCl at pH 7.4, 160 mM NaCl, 16 mM KCl and 5 mM $MgCl_2$. The reaction was started by adding 5 mM ATP and was stopped by adding 0.3 ml of 12% sodium dodecyl sulfate (SDS). The amount of released inorganic phosphate was measured by Chifflet method [19].

Cu^{2+}-ATPase activities of the microsomes from HSC-3 and BHY cells were detected by following the method of Takeda et al. [20]. The concentrations of various components in the reaction mixture were 30 μl microsome, 25 mM sucrose, 50 mM acetate buffer at pH 5.0, 10 mM reduced glutathione (GSH), 300 mM KCl, 3 mM $MgSO_4$ and 0.5 μM $CuSO_4$. Released inorganic phosphate was measured in the same method described above.

The protein concentration was estimated by using Bio-Rad Protein Assay (Bio-Rad Laboratories, CA, USA) according to the manufacturer's protocol with bovine serum albumin as a standard.

Western blot analysis

Twenty micrograms of microsomes of HSC-3 cells and BHY cells were boiled for 5 min, fractionated by 10% polyacrylamide gel electrophoresis and then transferred into polyvinylidene difluoride membranes (Millipore corporation, MA, USA) by electroblotting. The transferred membranes were blocked with blocking reagents (Immuno Block®, DS Pharma Biomedical, Osaka, Japan) for 1 h at room temperature. After washing three times with TBST [50 mmol/l tris (pH 7.5), 150 mmol/l NaCl, and 1% tween 20] buffer at pH 7.5 at room temperature, the membranes were incubated overnight at 4°C with anti-Na^+,K^+-ATPase α, anti-Na^+,K^+-ATPase β, anti-ATP7A and anti-ATP7B antibodies. All the antibodies were diluted to the ratio of 1:1000. After washing with TBST the membranes were incubated with HRP-conjugated rabbit anti-mouse IgG1, rabbit anti-mouse IgG2a, rabbit anti-mouse IgG (H + L) or goat anti-rabbit IgG (H + L) for 1 h at room temperature. After washing with TBST bands were detected by Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer, Boston, MA, USA) according to manufacturer's instruction. Medical X-ray films were used to visualize the chemiluminescence. Kaleidoscope Prestained Standards (Bio-Rad) was used for molecular mass determination. The

antibodies were diluted in immune-reaction enhancer solution (Can Get Signal, Toyobo, Japan); solution 1 was used for primary antibodies and solution 2 was used for secondary antibodies. Purified rat Na^+,K^+-ATPase (1:100; diluted in 0.25 M sucrose) was used as a positive control for Na^+,K^+-ATPase both α and β subunits. Hela and HepG2 cell lysates (BD Biosciences) were used as positive control for ATP7A and ATP7B, respectively.

Results

Assessment of cellular sensitivity to CDDP concentration

Figure 1 shows the effects of varying concentrations of CDDP on cellular ATP contents (% of control) in the six different types of oral squamous carcinoma cells (HSC-2, HSC-3, HSC-4, Ca9-22, BHY and TYS). The cells were treated with 0–1 mM final concentrations of CDDP and cellular ATP level was measured after 24 h. Reduction was hardly observed in the cellular ATP content with 0–10 μM CDDP. When the dose of CDDP was increased from 100 μM to 1 mM, ATP levels started to decline in all the cells, and the amount and pattern of the decline varied among the cells. At the dose of 1 mM, HSC-3 cells appeared most sensitive to CDDP with about 99% declined ATP level, while BHY cells appeared most resistant to CDDP with 59% declined ATP level. HSC-2, HSC-4, Ca9-22 and TYS cells showed about 92, 79, 88 and 95% declined ATP levels, respectively. These data demonstrated that ATP levels started to decline by addition of 0.1 mM CDDP, suggesting that a lower dose has no inhibitory effect while a higher dose causes the inhibition.

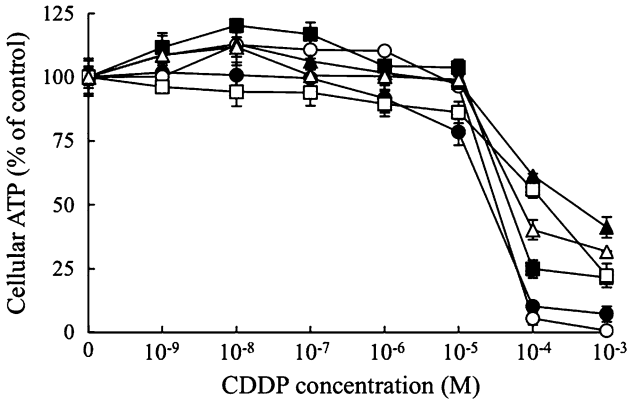

Fig. 1 Assay of cellular sensitivity to various concentrations of CDDP. Cells were treated with different concentrations of CDDP for 24 h and sensitivity assay was performed by determination of the ATP level of the cells with luminometer. Symbols used are as follows: HSC-2 (*filled circle*), HSC-3 (*open circle*), HSC-4 (*filled square*), Ca9-22 (*open square*), BHY (*filled triangle*) and TYS (*open triangle*). HSC-3 (*open circle*) and BHY (*filled triangle*) were most sensitive and most resistant to CDDP, respectively. ATP values were expressed as percentage (%) in comparison with controls and the values shown are the means ± SD of three independent experiments

Time course experiments with a higher dose (1 mM) of CDDP

In separate experiments, all cells were harvested for determination of time-related changes after administration of 1 mM CDDP. No inhibitory effect was observed in ATP levels within the first 6 h of exposure, although some proliferative effect was observed. However, at 12 h ATP levels started to decline and at 24 h ATP levels were significantly low (Fig. 2). These data confirmed that among the six kinds of cells HSC-3 cells and BHY cells were most sensitive and most resistant to CDDP, respectively.

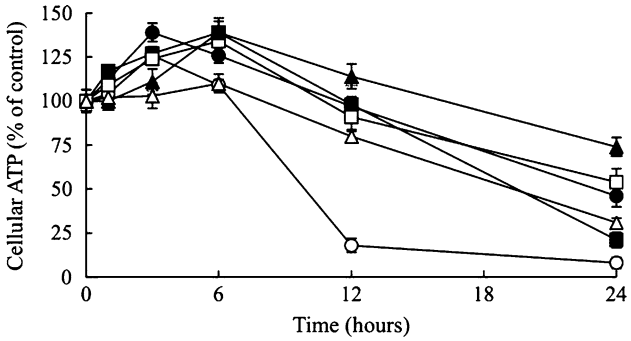

Fig. 2 Assay of time-dependent cellular sensitivity to a higher dose of CDDP. Cells were treated with 1 mM of CDDP for different time frames and sensitivity assay was performed by determination of the ATP level of the cells with luminometer. Symbols used are as follows: HSC-2 (*filled circle*), HSC-3 (*open circle*), HSC-4 (*filled square*), Ca9-22 (*open square*), BHY (*filled triangle*) and TYS (*open triangle*). HSC-3 (*open circle*) and BHY (*filled triangle*) were most sensitive and most resistant to CDDP, respectively. Data were shown as described in the figure legend to Fig. 1

Intracellular CDDP accumulation and efflux from cells

Total intracellular Pt levels after 3 h drug exposure (range 0.1–1 mM) were measured in CDDP-sensitive HSC-3 cells and CDDP-resistant BHY cells (Fig. 3). In both cells, exposure to CDDP resulted in a concentration-dependent linear increase in intracellular Pt levels; and the slope was about threefold steeper in HSC-3 cells, supporting that the uptake was higher in HSC-3 cells than in BHY cells.

For time course studies of Pt efflux, intracellular Pt concentrations were measured after incubation with 1 mM CDDP for 3 h and subsequent incubation with drug-free medium (Fig. 4). Both the cells exhibited a biphasic decline and BHY cells showed slightly a higher efflux level than HSC-3 cells. However, the two types of cells did not show marked differences in the CDDP efflux rate.

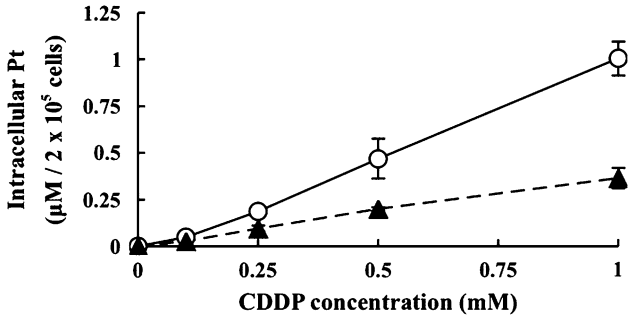

Fig. 3 Intracellular Pt accumulation following CDDP exposure. HSC-3 (*open circle*) and BHY (*filled triangle*) cells were incubated with different concentrations of CDDP for 3 h. The amounts of intracellular accumulated Pt were determined by ICP-AES. Data represent the mean ± SD of triplicated experiments

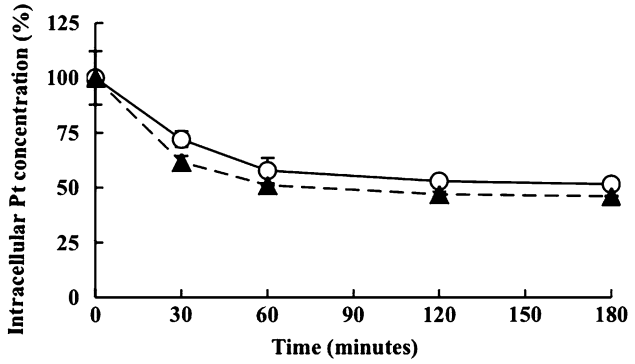

Fig. 4 Efflux of intracellular CDDP. Intracellular Pt concentration in HSC-3 (*open circle*) and BHY (*filled triangle*) cells was measured after incubation with 1 mM CDDP for 3 h and subsequent incubation with drug-free medium. The amount of accumulated Pt was determined by ICP-AES. Results were expressed as percentage in comparison with intracellular platinum accumulation after 3 h CDDP exposure as 100%. Data represent the mean ± SD of triplicated experiments

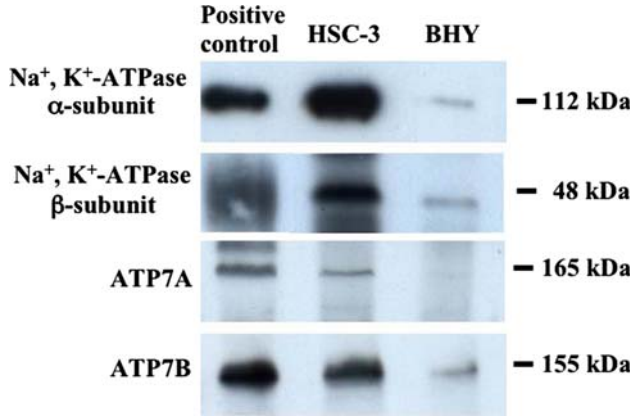

Fig. 5 Expressions of different transport proteins. Twenty micrograms of protein, respectively, from HSC-3 and BHY microsomes were subjected to SDS-PAGE and analyzed by western blotting with antibodies specific for each Na^+,K^+-ATPase α-subunit, Na^+,K^+-ATPase β-subunit, ATPase alpha polypeptide Cu^{2+} transporting ATP7A and ATPase alpha polypeptide Cu^{2+} transporting ATP7B

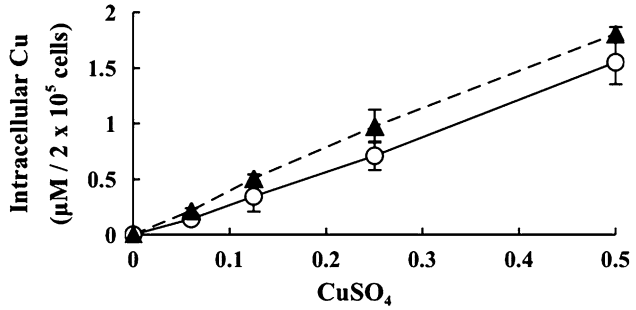

Fig. 6 Intracellular copper accumulation following $CuSO_4$ exposure. Cells were incubated with different concentrations of $CuSO_4$ for 3 h. The amount of accumulated copper was determined by ICP-AES. The symbols used are as follows: HSC-3 cells (*open circle*) and BHY cells (*filled triangle*). Data represent the mean ± SD of triplicated experiments

Western blot

Expressions of Na^+,K^+-ATPase α and β subunits, ATP7A and ATP7B were assessed in the microsomes of HSC-3 and BHY cells (Fig. 5). Immunoblot analysis revealed markedly elevated levels of Na^+,K^+-ATPase α-1 and β-1 subunits, ATP7A and ATP7B in CDDP-sensitive HSC-3 cells compared with CDDP-resistant BHY cells.

Intracellular Cu accumulation

In order to compare the copper transporters in HSC-3 and BHY cells, we measured their copper accumulating ability by exposing the cells to $CuSO_4$, and measured intracellular Cu levels after 3 h $CuSO_4$ exposure (range 0.06–0.5 mM) (Fig. 6). The exposure to $CuSO_4$ resulted in a concentration-dependent linear increase in both the cells. Although BHY cells showed slightly a higher intracellular Cu accumulation than HSC-3 cells, the difference was not significant. Therefore, we concluded that CDDP-sensitive HSC-3 cells possessed an equal or slightly a less amount of Cu transporters than CDDP-resistant BHY cells.

Na^+,K^+-ATPase and Cu^{2+}-ATPase activities

Specific activities of Na^+,K^+-ATPase and Cu^{2+}-ATPase of the microsomes of HSC-3 and BHY cells were measured (Table 1). CDDP-sensitive HSC-3 cells exhibited about three times higher Na^+,K^+-ATPase activity than CDDP-resistant BHY cells. However, both the cells exhibited low Cu^{2+}-ATPase activity and there was no significant difference between the two cells.

Table 1 Specific activities of Na^+,K^+-ATPase and Cu^{2+}-ATPase in HSC-3 and BHY cells

Specific activity (nmol Pi/mg protein/min at 37°C)	HSC-3 cell cisplatin-sensitive	BHY cell cisplatin-resistance
Na^+,K^+-ATPase	8.52 ± 0.67	2.61 ± 0.42
Cu^{2+}-ATPase	0.51 ± 0.10	0.48 ± 0.12

The Na^+,K^+-ATPase and Cu^{2+}-ATPase activities in microsomes of HSC-3 and BHY cells were detected and calculated

Effects of Na^+,K^+-ATPase inhibitor on intracellular CDDP accumulation

Pretreatment of the cells with ouabain, a specific inhibitor of Na^+,K^+-ATPase, resulted in a significant decrease in intracellular Pt accumulation in both the cells (Fig. 7). In the presence of ouabain intracellular Pt accumulation was fourfold lower in HSC-3 cells and twofold lower in BHY cells.

Discussion

There have been many studies on the mechanism of tumor cells' resistance to CDDP and the decreased intracellular accumulation of CDDP has been reported in a large number of them. However, the molecular mechanisms that underlie CDDP resistance are not well understood yet. In the present study, at first we examined the cellular sensitivity to CDDP in six different types of human oral squamous carcinoma cells. After determining the cellular sensitivity, we selected the most sensitive and most resistant variants, with aim to assess the functions of drug uptake and efflux in CDDP sensitivity using an integrated and quantitative approach. In the pair of two selected types of cells, we measured and compared between them intracellular CDDP accumulation and efflux, intracellular Cu accumulation, specific activity of Na^+,K^+-ATPase and Cu^{2+}-ATPase, effect of Na^+,K^+-ATPase inhibitor on intracellular CDDP accumulation and expression of Na^+,K^+-ATPase α- and β-subunits, ATP7A and ATP7B.

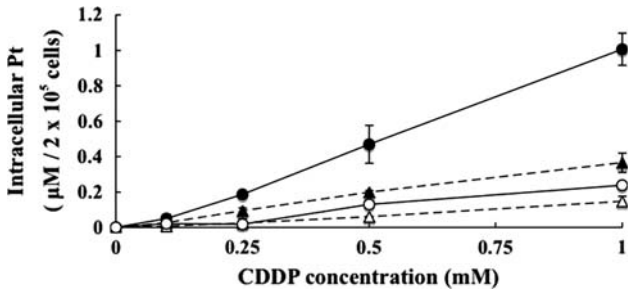

Fig. 7 Influence of ouabain on intracellular CDDP accumulation. Ouabain (0.1 mM) was added 60 min prior to 3 h exposure of the cells to different concentrations of CDDP. The amounts of accumulated Pt were determined by ICP-AES. In HSC-3 cells : intracellular Pt accumulation in absence of ouabain (*filled circle*) and in presence of ouabain (*open circle*). In BHY cells : intracellular Pt accumulation in absence of ouabain (*filled triangle*) and in presence of ouabain (*open triangle*). Data represent the mean ± SD of triplicated experiments

Concentration- and time-dependent profiles of intracellular Pt represent the result of uptake and efflux processes that take place simultaneously. This study showed that, following 3 h exposure to different concentrations of CDDP, the intracellular accumulation of Pt was significantly lower in the CDDP-resistant BHY cells than in the CDDP-sensitive HSC-3 cells. On the other hand, efflux studies did not reveal such obvious differences between them. Therefore, we concluded that alteration in CDDP uptake primarily accounted for the differences in the intracellular Pt concentrations between CDDP-sensitive and -resistant cells. Other investigators also reported decreased uptake and lower intracellular platinum concentrations in CDDP-resistant cells [21–23]. Most of the authors found a decreased uptake without changes in efflux [24–26], but the opposite situation, i.e., no difference in uptake but increased efflux, was also reported [27].

For understanding the mechanisms leading to alter intracellular Pt concentrations, it is crucial to reveal the functions of transport proteins. It has been suggested that CTR1 is implicated in mediating the uptake and acquisition of resistance to CDDP in yeast, and in the cells of mammals [4, 28, 29] but conflicting data recently emerged in contrast with such a hypothesis. In fact, results obtained by Chauhan et al. [30] in the human epidermoid carcinoma cell line KB-3-1 and in the CDDP-resistant variant KB-CP20 revealed no change in CDDP accumulation when CTR1 was overexpressed in KB-CP20 cells. Beretta et al. [31], using cervix squamous cell carcinoma cell line A431 and the CDDP-resistant variant A431/Pt, reported that impairment of intracellular CDDP accumulation was not mediated by CTR1 and that it played a marginal role in cellular pharmacology of CDDP. In this study, copper accumulation was compared between CDDP-sensitive HSC-3 and -resistant BHY cells, following exposure to $CuSO_4$. Although BHY cells showed a markedly lower Pt accumulation than the HSC-3 cells, the copper content in BHY cells following exposure to $CuSO_4$ was slightly higher than in HSC-3 cells. These results suggest that CTR1 was not down-regulated in the cisplatin-resistant variant BHY cells.

No apparent difference was observed in the efflux rate between the sensitive and the resistant cells (Fig. 4). However, the concentration- and time-dependent profiles were different between the two cells. This observation is consistent with the results of Loh et al. [25], Parker et al. [27] and Chao [32]. Moreover, the specific activity of Cu^{2+}-ATPase

did not markedly differ between the two types of cells (Table 1), but the expressions of both ATP7A and ATP7B were higher in CDDP-sensitive HSC-3 cells than in -resistant BHY cells (Fig. 5). Although, both transporters have been shown to transport platinum drugs [15], their expression and function in CDDP sensitivity might be different. Samimi et al. [33] reported an increased expression of ATP7A in CDDP-resistant ovarian carcinoma cells which had no reduction in CDDP accumulation. In a recent review Safaei [34] speculates that the function of ATP7A is vesicular sequestration of platinum drugs rather than efflux. In some previous studies, higher expression of ATP7B was found to be related to CDDP resistance [13, 35, 36]. Owatari et al. [37] strongly suggested that ATP7A expression was correlated to CPT-11 resistance, but not to CDDP resistance, in clinical cancer cells. Moreover, recent evidence indicates that ATP7A is up-regulated in pancreatic cancer compared with chronic pancreatitis [38] and that ATP7A and the copper-containing enzyme lysyl oxidase are more highly expressed in invasive breast cancer cell lines than in noninvasive lines [39]. Thus, ATP7A expression might be related to the malignant phenotype in addition to its role in drug resistance. Further study will be necessary to find an answer to this question. Zisowsky et al. [26] reported that the expression profiles of both ATP7A and ATP7B differ depending on the cell type, and our results strongly support these findings.

Na^+,K^+-ATPase plays an important role in active transport through the cell membrane and in supporting physical ion balances [40, 41]. In addition, its enzyme activity is reported to be essential for the intracellular accumulation of CDDP except for the passive transport. Kishimoto et al. [10] reported the role of Na^+,K^+-ATPase α-1 subunit in intracellular accumulation of CDDP. Andrews et al. [9] pointed out the significance of a CDDP transport system being dependent on the function of Na^+,K^+-ATPase. Furthermore, many studies have confirmed the importance of a membrane potential maintained by Na^+,K^+-ATPase in CDDP accumulation [25, 42, 43]. Increased sensitivity to ouabain, a selective inhibitor of Na^+,K^+-ATPase, is observed in CDDP-resistant cancer cells [44], suggesting that lower Na^+,K^+-ATPase activity is essential for CDDP resistance. On the contrary, CDDP-resistant cancer cells were reported to be cross-resistant to ouabain [9]. In this study, we found that CDDP-sensitive HSC-3 cells possessed higher specific activity of Na^+,K^+-ATPase than CDDP-resistant BHY cells (Table 1) and that the expression levels of both α- and β-subunits were also higher in CDDP-sensitive HSC-3 cells than in the -resistant BHY cells (Fig. 5). Moreover, pretreatment with ouabain markedly inhibited intracellular CDDP accumulation to about 80 and 68%, respectively (Fig. 7). This inhibition of CDDP accumulation by ouabain is an intriguing observation. The inhibition of CDDP accumulation by ouabain indicates either that CDDP accumulation is dependent upon the electro-chemical gradient across plasma membranes, or that CDDP is transported by Na^+,K^+-ATPase which ouabain inhibits.

In conclusion, the results obtained in our cell system are consistent with the interpretation that impairment of intracellular drug accumulation is a common event in the development of cellular CDDP resistance and is regulated by its Na^+,K^+-ATPase activity not by Cu^{2+}-ATPase. Cu^{2+}-ATPase plays a marginal role in the cellular pharmacology of CDDP. Moreover, the expression profiles of ATP7A and ATP7B differ depending on the cell type.

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