Up-Regulation of MDR1 Function and Expression by Cisplatin in LLC-PK₁ Cells

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To examine whether cisplatin affects the multidrug transporter MDR1/P-glycoprotein in the kidneys, the effects of cisplatin on cell sensitivity to an anticancer drug, MDR1 function and expression were examined by assessing the growth inhibition by the MDR1 substrate paclitaxel, the uptake and efflux of the MDR1 substrate Rhodamine123 and the level of MDR1 mRNA, respectively. Porcine kidney epithelial LLC-PK₁ cells were used, as they have a structure and function similar to those of renal proximal tubular cells and physiologically express low levels of MDR1. The growth inhibitory curve of LLC-PK₁ cells by paclitaxel shifted to a higher concentration range by pretreatment with 1 μM cisplatin for 48 h. The uptake and efflux of Rhodamine123 were significantly reduced and enhanced, respectively, by pretreatment with 1 μM cisplatin for 48 h. This enhanced efflux was suppressed by the representative MDR1 substrate/inhibitor ciclosporin. The expression of MDR1 mRNA was increased by the existence of cisplatin for 48 h. These observations taken together suggested that the transient exposure to cisplatin could cause the up-regulation of MDR1 in LLC-PK₁ cells.

Key words MDR1; P-glycoprotein; cisplatin; LLC-PK₁ cell; MDR1 up-regulation

Cisplatin (cis-dichlorodiammineplatinum (II); CDDP) is an effective anticancer drug used in the treatment of various solid tumors. However, the chief limit to its efficacy is its severe nephrotoxicity, which has made it necessary both to lower its dosage and to actively hydrate patients. The vulnerability of the kidneys to CDDP is almost certainly related to their primary role in the excretion of CDDP. The major site of renal injury is the S3 segment of the proximal tubule, located in the outer stripe of the outer medulla of kidney. CDDP concentrations in proximal tubular epithelial cells exceed plasma concentrations by a factor of five, resulting in nephrotoxicity. This is considered to be at least in part due to active uptake of CDDP into cells via energy-dependent processes, such that mediated by the probenecid-inhibitable organic base transport protein.

On the other hand, multidrug resistance (MDR) is one of the most serious problems responsible for the failure of chemotherapy. Among the cellular factors involved in MDR, acceleration of the efflux system (e.g. multidrug resistance transporter 1, MDR1/P-glycoprotein) is known to be the major mechanism. MDR1 was isolated from resistant tumor cells in 1976, and is a glycosylated membrane protein of 1280 amino acids (170 kDa) consisting of two similar regions containing six putative transmembrane segments and two intracellular binding sites for ATP. MDR1 has been shown to act as an efflux pump to remove anticancer drugs from cells, resulting in resistance to anticancer drugs. MDR1 was also shown to be expressed in the apical membranes of normal tissues including the liver, kidneys, intestine and brain. As MDR1 exports unnecessary or toxic exogenous substances or metabolites out of the body, it is thought to be involved in protection against various types of toxins and stress. However, it is unclear whether MDR1-dependent protection is activated under cytotoxic conditions induced by CDDP, which is not a substrate for MDR1, and there is also little information regarding whether CDDP affects the function and/or expression of MDR1 in renal tubular cells.

Therefore, we examined the effects of CDDP on the function and expression of MDR1 in the porcine kidney epithelial cell line LLC-PK₁, as a model of proximal tubular cells by assessing growth inhibition by the MDR1 substrate paclitaxel, the uptake and efflux of the MDR1 substrate Rhodamine123 and the level of MDR1 mRNA. LLC-PK₁ cells have a structure and function similar to those of renal proximal tubular cells and physiologically express low levels of MDR1.

MATERIALS AND METHODS

Chemicals CDDP was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). WST-1 and 1-methoxy PMS were purchased from Dojindo Laboratories (Kumamoto, Japan). Rhodamine123 was purchased from Molecular Probes, Inc. (Eugene, OR, U.S.A.). All other agents were obtained commercially and were of analytical grade requiring no further purification.

Cells and Cell Culture The porcine kidney epithelial LLC-PK₁ cells (219–228 passages) were maintained in the culture medium consisting of Medium199 (Invitrogen Corp., Carlsbad, CA, U.S.A.) supplemented with 10% fetal bovine serum (Lot. No. 09017, BioWhittaker, Walkersville, MD, U.S.A.) without antibiotics. LLC-PK₁ cells (1.0×10⁶ cells) were seeded on culture dishes (100 mm in diameter) in 10 ml of culture medium. Cells were grown in a humidified atmosphere of 5% CO₂–95% air at 37°C, and passaged every 3 or 4 d with 0.02% EDTA–0.05% trypsin solution (Invitrogen).

Growth Inhibition Assay The cytotoxicity of paclitaxel in LLC-PK₁ cells pretreated with CDDP was evaluated by WST-1 colorimetric assay. LLC-PK₁ cells were pretreated with or without 0.1 or 1 μM CDDP for 48 h, and then the cells (5000 cells/well) were seeded into 96-well plates (Corning Inc., NY, U.S.A.) in 100 μl of culture medium without paclitaxel on Day 0, and 24 h later the culture medium was exchanged for that containing paclitaxel at various concentrations (on Day 1). After incubation for 3 d at 37°C, the WST-1 colorimetric assay was performed. The culture medium was exchanged for 110 μl of that containing WST-1...
reagent solution (10 µl WST-1 + 100 µl culture medium), and 3 h later the absorbance was determined at 450 nm with a reference wavelength of 630 nm using a SpectraFluor microplate reader (Tecan Switzerland, Switzerland). The 50% growth inhibitory concentration (IC₅₀) of paclitaxel in LLC-PK₁ cells was calculated according to the sigmoid inhibitory effect model; \( E = E_{\text{max}} \times \left[ 1 - C^7 (C^7 + IC_{50}^7) \right] \), by means of a nonlinear least-squares fitting method (Solver, Microsoft Excel 2001). \( E \) and \( E_{\text{max}} \) represent the surviving fraction (% of control) and its maximum, respectively, and \( C \) and \( \gamma \) represent the drug concentration in the medium (µM) and the sigmoidicity factor, respectively.

### Uptake and Efflux of Rhodamine123

Uptake and efflux of Rhodamine123 were determined as described previously.²⁰ LLC-PK₁ cells (5×10⁶ cells/well) were seeded into 24-well plates (Corning) in 1 ml of culture medium, and incubated for 48 h in a humidified atmosphere of 5% CO₂–95% air at 37°C. Then, the culture medium was exchanged for fresh culture medium containing 0.1 or 1 µM CDDP, and further incubated for 48 h in a humidified atmosphere of 5% CO₂–95% air at 37°C. After pretreatment with CDDP, cells were washed twice with warmed Hanks’ balanced salt solution (HBSS), and the uptake experiments were started by addition of fresh HBSS containing 10 µM Rhodamine123. The reaction was stopped by aspiration of HBSS from the wells, followed by washing three times with ice-cold phosphate buffered saline (PBS).

In the efflux experiments, LLC-PK₁ cells were cultured in the same manner as described for the uptake experiments. Cells were washed twice with warmed HBSS and incubated in fresh HBSS containing 10 µM Rhodamine123 for 1 h (Loading time). After loading, HBSS was removed from the wells and cells were washed twice with fresh HBSS. Efflux experiments were started immediately by addition of fresh warmed HBSS without Rhodamine123, and further incubated for the indicated times at 37°C. The reaction was stopped by aspiration of HBSS from the wells, followed by washing three times with ice-cold PBS.

After finishing the uptake or efflux experiments, cells were lysed with 500 µl of 0.3 M NaOH, and neutralized with 500 µl of 0.3 M HCl. Aliquots (100 µl) of cell lysate were transferred into 96-well black plates (Corning), and the fluorescence intensity of Rhodamine123 measured using the SpectraFluor (Tecan) with an excitation wavelength of 485 nm and emission wavelength of 535 nm. Protein content was determined by the method of Lowry,²¹ and bovine serum albumin was used as the standard.

### RT-PCR Analysis of MDR1 mRNA

The expression of MDR1 mRNA was determined as described previously.²⁰ LLC-PK₁ cells (2×10⁶ cells) were seeded on plastic culture dishes (100 mm in diameter) in 5 ml of culture medium. After incubation for 48 h, the culture medium was exchanged for that containing CDDP at the indicated concentrations, and further incubated at 37°C for 48 h. Total RNA was isolated using an RNasy® mini kit (Qiagen Inc., CA, U.S.A.), and aliquots (0.1 µg) of RNA were used for reverse transcription and cDNA-PCR, using an RNA PCR kit (AMV) version 2.1 (Takara Shuzo Co., Ltd., Shiga, Japan). PCR oligonucleotide primers for amplification of MDR1 and β-actin cDNA were synthesized by Genset K. K. (Kyoto, Japan) and their sequences are shown in Table 1.¹⁸,²⁰ PCR amplification of cDNA was performed in a total reaction volume of 25 µl. PCR amplification was initiated by denaturation at 94°C for 2 min followed by 35 or 25 sequential cycles for MDR1 or β-actin, respectively, of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 45 s in a thermal cycler (iCycler Thermal Cycler, Bio-Rad Laboratories, Inc., CA, U.S.A.). PCR products were separated on Triac–aceta–EDTA 3% agarose gels containing 100 ng/ml ethidium bromide, and visualized with ultraviolet illumination at 312 nm and photographed with a Polaroid camera. Densitometric analysis was performed on a Macintosh computer using the public domain NIH Image program version 1.62 (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/), and the ratio of band intensity (MDR1/β-actin) was calculated.

### Statistical Analysis

Comparisons among more than three groups were performed by non-repeated one-way analysis of variance (ANOVA) followed by Scheffé F-test, and \( p \) values of less than 0.05 (two-tailed) were considered significant.

### RESULTS

#### Growth Inhibitory Activity of Paclitaxel

The growth inhibition curve of non-treated LLC-PK₁ cells by paclitaxel was shifted to a higher concentration range by pretreatment with CDDP for 48 h (data not shown). Table 2 summarizes the effects of pretreatment with 0.1 or 1 µM CDDP on the 50% growth inhibitory concentration (IC₁₀) for paclitaxel in LLC-PK₁ cells. The IC₁₀ values for paclitaxel in CDDP-treated LLC-PK₁ cells were increased in a concentration-dependent manner, and the values in cells pretreated with 0.1 and 1 µM CDDP were 2.2- and 6.9-fold higher, respectively, than control.

#### Uptake of Rhodamine123

The uptake of Rhodamine123 in LLC-PK₁ cells was increased in a time-dependent manner (Fig. 1). The uptake of Rhodamine123 at 90 min in LLC-PK₁ cells was significantly decreased by pretreatment with 1 µM CDDP for 48 h, although pretreatment with 0.1 µM CDDP significantly increased the uptake at 90 min in LLC-PK₁ cells. The uptake of Rhodamine123 was shifted to a higher concentration range by pretreatment with 0.1 and 1 µM CDDP, and further incubated for 48 h in a humidified atmosphere of 5% CO₂–95% air at 37°C. After pretreatment with CDDP, cells were washed twice with warmed Hanks’ balanced salt solution (HBSS), and the uptake experiments were started by addition of fresh HBSS containing 10 µM Rhodamine123. The reaction was stopped by aspiration of HBSS from the wells, followed by washing three times with ice-cold phosphate buffered saline (PBS).
CDDP showed only a slight effect.

**Efflux of Rhodamine123**
The efflux of Rhodamine123 from LLC-PK1 cells increased in a time-dependent manner (data not shown), and the residual amounts of Rhodamine123 after efflux from LLC-PK1 cells were ca. 80 and 70% at 5 and 15 min, respectively (Fig. 2). These were significantly reduced by pretreatment with 1 µM CDDP for 48 h, although 0.1 µM CDDP showed no remarkable effects. Moreover, this decrease in the residual amount at 15 min was significantly restored by addition of 10 µM ciclosporin, and this restoration was comparable to that in non-treated LLC-PK1 cells (Fig. 3).

**MDR1 mRNA Expression**
Increases of ca. 0.9, 1.0 and 1.7-fold on MDR1 mRNA expression were observed in LLC-PK1 cells treated with 0.1, 1 and 10 µM CDDP for 48 h, respectively (Fig. 4), although the differences were not significant.

**DISCUSSION**

The growth inhibitory curve of the non-treated LLC-PK1 cells by paclitaxel was shifted to a higher concentration range by pretreatment with 1 µM CDDP for 48 h (data not shown), and the IC50 values for paclitaxel increased in a CDDP concentration-dependent manner (Table 2). This indicates the acquisition of the resistance to paclitaxel, which might be one part caused by the up-regulation of MDR1.

To clarify the participation of MDR1 in the resistance to paclitaxel, the transport characteristics of Rhodamine123, which is a substrate for MDR1, were examined. The uptake of Rhodamine123 in LLC-PK1 cells was decreased by pretreatment with 1 µM CDDP for 48 h (Fig. 1). Further, the efflux of Rhodamine123 from LLC-PK1 cells was significantly enhanced by pretreatment with 1 µM CDDP for 48 h (Fig. 2), and this increase in efflux was suppressed by addition of 10 µM ciclosporin (Fig. 3). However, no remarkable difference between normal and CDDP-treated cells was observed in the transport of Rhodamine123. This might be explained by that the level of MDR1 up-regulated by the transient stimuli was lower than those of the MDR1-transfectant cells or the resistant cells established by the drug selection. Otherwise, it would be considered that the resistance induced by
the transient exposure to CDDP was not regulated by only the acceleration of efflux via the up-regulation of MDR1. Besides, the reason why the acceleration of Rhodamine123 efflux by the transient exposure to cisplatin was observed less than for 15 min remains unclear, although it takes for 90 min to reach the significant decrease in Rhodamine123 uptake.

To examine whether the change in the MDR1 function by the transient exposure to CDDP was dependent on the expression level of MDR1, the level of MDR1 mRNA was evaluated by RT-PCR. As a result, a ca. 2-fold increase in MDR1 mRNA level was observed by treatment with 10 μM CDDP for 48 h (Fig. 4), indicating a discrepancy between the resistance to paclitaxel (Table 2) and the expression level of MDR1 mRNA. This would be explained by that the exposure time (48 h) of CDDP was the same in both experiments or other resistance mechanism(s) might be up-regulated. These observations taken together suggested that the transient exposure to CDDP could cause the up-regulation of the function and expression of MDR1 in LLC-PK1 cells.

Demeule et al.22) reported that single administration of CDDP to rats increased MDR1 levels in the kidney by ca. 3-fold as determined by western blotting assay. Huang et al.23) also demonstrated the up-regulation of MDR1 mRNA expression in the rat kidney under conditions of CDDP-induced nephrotoxicity using a microarray. The present observations confirmed and expanded upon these results as neither of these previous studies evaluated the resistance produced by MDR1 nor its transport function.

Recently, Yang et al.24) reported that the activation of phospholipase C by a variety of cellular stimuli increased the expression of MDR1. CDDP has also been demonstrated to activate phosphatidylincholine-specific phospholipase C but not phospholipase A2 or D.25) On the other hand, the transcription factor YB-1 was reported to be directly involved in MDR1 gene activation in response to genotoxic stress including CDDP, etoposide, and ultraviolet irradiation using CAT reporter gene assay.26) The transcription factors including Sp1, HSF1 and MEF1 were also known to regulate MDR1 expression.27) Taking these reports into consideration, the activation of phospholipase C and/or transcription factor(s) may participate in the induction of MDR1 by CDDP in LLC-PK1 cells. Additional studies of the mechanism of MDR1 up-regulation by CDDP in LLC-PK1 cells are currently in progress in our laboratory.

The resistance to CDDP, which is not a substrate for MDR1,14) in cancer chemotherapy has not been shown to be associated with the induction of MDR1. This implies that the induction of MDR1 observed in the present study is not the mechanism of detoxification or protection against the cytotoxicity induced by CDDP. However, MDR1 has recently been suggested to protect cells against caspase-dependent apoptosis induced by anticancer drugs including CDDP. Fas ligation, tumor necrosis factor and ultraviolet irradiation,27,28) although it is not yet clear whether MDR1 is associated directly with the suppression of apoptotic signaling. The present findings suggested that LLC-PK1 cells might be indirectly protected from CDDP-induced cytotoxicity via induction of MDR1.

Clinically, CDDP is usually administered by continuous infusion over a period of five days.29) For dose adjustment of CDDP based on population pharmacokinetics, its target maximum plasma concentration at the end of infusion is in the range from 1 to 2 mg/l as total platinum (ca. 5–10 μM as CDDP).29) Although the plasma concentrations of CDDP decreased immediately after administration and the amount excreted in the urine for 24 h was ca. 30% of the total dose,29) it was reported that CDDP concentrations in proximal tubular epithelial cells exceed plasma concentrations by a factor of five.11) Thus, continuous infusion of CDDP in clinical use may reach the concentration that causes the up-regulation of MDR1 in the proximal tubular epithelial cells. Therefore, these findings suggested that drug–drug interactions via up-regulation of MDR1 might be caused by use of CDDP in clinical situations. In addition, the administration of CDDP might induce not only resistance to CDDP but also MDR1-mediated resistance in tumor cells.

In conclusion, these results suggested that the transient exposure to CDDP could cause the up-regulation of the function and expression of MDR1 in porcine kidney epithelial LLC-PK1 cells. This will provide useful information for optimal dosage of CDDP in cancer chemotherapy.

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