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Report

Effect of Cadmium on the Expression of ABCB1 Transporter in Human Proximal Tubular Cells

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Cadmium (Cd) is an ecotoxic heavy metal that predominantly causes renal failure. Proximal tubular cell damage is typical of chronic Cd toxicity. Proximal tubular cells play important roles in maintaining a stable balance of body chemicals through the functions of various transporters. ABC transporter subfamily B member 1 [ABCB1; also called MDR1 (multiple drug resistance 1)], one of the ATP binding cassette (ABC) multidrug efflux transporters, is expressed in the proximal tubular epithelium and is involved in the extracellular clearance of various chemicals. In this study, we demonstrate that Cd significantly increases the levels of *ABCB1* mRNA and P-glycoprotein protein (the *ABCB1* gene product) in the HK-2 human proximal tubular cells. Our results suggest that Cd affects the transportation function in the proximal tubules. However, *ABCB1* knockdown did not affect Cd toxicity in the HK-2 cells. Therefore, the Cd-induced increase in ABCB1 may affect the transportation function in the kidney but not the Cd toxicity.

Key words cadmium, proximal tubular cell, *ABCB1* gene

INTRODUCTION

Cadmium (Cd) is a harmful heavy metal widely distributed in the environment, including in soil, water, and air.[1)] Humans ingest Cd daily through various foods, such as rice, vegetables, and seafood, and through smoking.[1,2)] Several decades ago, many areas in Japan were affected by Cd contamination caused by mining, and long-term Cd ingestion is known to cause *itai-itai* disease.[3)] Cd accumulates in the kidney and liver during chronic dietary exposure over a lifetime, because it has a long biological half-life (15-30 years).[4)] The major effects of Cd poisoning are acute hepatic and testicular toxicity and chronic renal and bone toxicity.[1)] Cd is filtered by the glomerulus and reabsorbed by the proximal tubular cells, so they are the target of Cd toxicity.[4)] It has been shown that the changes in gene expression induced by Cd cause nephrotoxicity by inducing apoptosis and autophagy, the generation of reactive oxygen species (ROS), and the disruption of cell-cell adhesion.[5,6)]

The kidney contains various transporter molecules that excrete and reabsorb numerous compounds. The ABC (ATP-binding cassette) transporters are expressed as membrane proteins in the kidney, and use the energy of ATP hydrolysis to transport exogenous and endogenous chemical substances and reduce the burden of harmful materials on the body. They thus performs a protective function.[7)] ABC transporters are classified into seven subfamilies (ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, and ABCG), among which ABCB1, also known as MDR1 (multidrug resistance 1), is expressed at the brush border membrane of proximal tubular cells. The protein encoded by the *ABCB1* gene is called P-glycoprotein (P-gp), and excretes hydrophobic, amphipathic, and cationic xenobiotics to the outside of the cell.[7)] Part of the resistance to anticancer agents has been demonstrated to be attributable to the overexpression of P-gp in the tumor cells.[8,9)] Renal cell carcinomas express high levels of P-gp.[8,9)] A previous study reported that the increased expression of the *ABCB1* gene induced by Cd may protect cells against Cd toxicity by effluxing Cd-induced apoptosis-related factors from the cells.[10)]

However, the association between P-gp and renal Cd toxicity is still unclear. Therefore, in this study, we examined not only the effect of Cd on the expression levels of *ABCB1* and P-gp, but also the effect of *ABCB1* knockdown on Cd toxicity in the HK-2 human proximal tubular cells.

MATERIALS AND METHODS

Cell Culture and Treatment HK-2 cells, purchased from the American Type Culture Collection (Manassas, MA, USA), were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DMEM/F-12) (Sigma-Aldrich, St. Louis, MO, USA), containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 25 μg/mL streptomycin (DS Pharm, Osaka, Japan), 25 U/mL penicillin (DS Pharm), 10 ng/mL epidermal growth factor (Sigma-Aldrich), 1% insulin-transferrin-selenium X (Gibco), and 5 ng/mL hydrocortisone, at 37°C in a humidified incubator under CO_2 (5%). The HK-2 cells were grown in test plates at a density of 250 cells/mm^2 and cultured for 48 h. After the culture medium was discarded, the cells were treated with Cd ($CdCl_2$; Fujifilm Wako Pure Chemical Co., Tokyo, Japan) in serum-free culture medium.

RNA Extraction The cells were washed twice with ice-

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cold phosphate-buffered saline (−) [PBS(−)] and the total RNA was extracted with the PureLink™ RNA Mini Kit (Ambion, Grand Island, NY, USA), according to the instructions of the manufacturer. The concentration and purity of the RNA were determined with a BioSpec-nano spectrophotometer (Shimadzu, Kyoto, Japan).

Real-Time Reverse Transcription (RT)-PCR The total RNA was treated with the PrimeScript RT reagent Kit (Perfect Real Time) (Takara Bio, Shiga, Japan) to generate cDNA. Real-time PCR was performed with SYBR® Premix Ex Taq™ II (Perfect Real Time) (Takara Bio) on the Thermal Cycler Dice Real Time System (Takara Bio). The thermal cycling conditions were: 10 s hot-start at 95°C followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. Gene expression was normalized to *GAPDH* mRNA levels. The oligonucleotide sequences of the primers used were: sense, 5′-CAGAGGGGATGGTCAGTGTT-3′, and antisense, 5′-CCTGACTCACCACACCAATG-3′, for the human *ABCB1* gene; sense, 5′-GCACCGTCAAGGCTGAGAAC-3′, and antisense, 5′-TGGTGAAGACGCCAGTGGA-3′, for the human *GAPDH* gene.

Western Blotting Analysis After treatment, the cells were washed twice with ice-cold PBS(−) and harvested in RIPA buffer [25 mM Tris (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS); Thermo Fisher Scientific, Waltham, MA, USA]. The protein concentrations were measured with the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). The protein samples were separated on SDS-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane. The membrane was probed with primary antibodies and then with horseradish-peroxidase-conjugated secondary antibody (diluted 1:10000; GE Healthcare, Little Chalfont, UK). The proteins were detected with enhanced chemiluminescence using Chemi-Lumi One Super (Nacalai Tesque, Kyoto, Japan). The chemiluminescent images were taken with a LAS-500 device (GE Healthcare). The primary antibodies were anti-GAPDH (diluted 1:1000) from American Research Products (Waltham, MA, USA) and anti-ABCB1 (diluted 1:1000) from Cell Signaling Technology (Danvers, MA, USA).

Small Interfering RNA (siRNA) Transfection The Silencer Select Predesigned siRNAs s10419 and s10420 directed against human *ABCB1* mRNA were purchased from Ambion. The control siRNA (Silencer Select Negative Control #1 siRNA) was also purchased from Ambion. siRNA transfection was performed with Lipofectamine RNAiMAX (Invitrogen, Grand Island, NY, USA). After the siRNA mixture was incubated for 15 min with Lipofectamine RNAiMAX and Opti-MEM I Reduced Serum Medium (Opti-MEM; Gibco), HK-2 cells were transfected with the siRNA mixture (1 nM siRNA per sequence, 0.2% Lipofectamine RNAiMAX, 10% Opti-MEM) for 48 h.

Cell Viability HK-2 cells treated with the siRNA mixture were grown in 96-well plates and cultured for 48 h. After treatment, Alamar blue (10%; Invitrogen) was added and the cells incubated for 4 h at 37°C. Fluorescence was measured at an excitation wavelength of 540 nm and an emission wavelength of 595 nm with a SpectraMax® iD3 microplate reader (Molecular Devices, San Jose, CA, USA).

Statistical Analysis Statistical analyses were performed with one- or two-way ANOVA. When the F value was significant ($P < 0.05$), Bonferroni's multiple t test was performed for *post hoc* comparison ($P < 0.05$). Statistical analyses were performed with Statcel3 (OMS, Saitama, Japan).

RESULTS

Effects of Cd on *ABCB1* mRNA and ABCB1 Protein Levels in the HK-2 Cells To clarify the effects of Cd on the expression of ABCB1 in the HK-2 cells, the levels of *ABCB1* mRNA and ABCB1 protein (known as P-gp protein) were examined after HK-2 cells were treated with Cd. Cd treatment for 3 and 6 h significantly and dose- and time-dependently increased the *ABCB1* mRNA levels in the HK-2 cells (Figs. 1A and B). In addition, the treatment with Cd for 6 h markedly increased the ABCB1 protein levels in the HK-2 cells (Fig. 1C). These results indicate that Cd increases the level of ABCB1 protein by inducing of the expression of its gene in the HK-2 cells.

Effect of *ABCB1* Knockdown on the Toxicity of Cd in the HK-2 Cells The effect of *ABCB1* knockdown on Cd toxicity was examined to investigate the involvement of ABCB1 in Cd toxicity in the HK-2 cells. *ABCB1* siRNA treatment significantly reduced the level of *ABCB1* mRNA in the HK-2 cells (Fig. 2A). The level of ABCB1 protein in the HK-2 cells was completely reduced by the siRNA treatment (Fig. 2B). However, the cytotoxicity of Cd in the *ABCB1* knockdown cells was similar to that in the control cells (Fig. 2C). Therefore, although Cd increased ABCB1 levels by inducing the expression of *ABCB1*, the increase in ABCB1 protein may be independent of the Cd toxicity in the HK-2 cells.

DISCUSSION

This study demonstrates that Cd treatment increases both the *ABCB1* mRNA and ABCB1 protein levels in the HK-2 cells. This implies that when proximal tubules are exposed to Cd, the substrates of ABCB1 may be discharged from the cells. However, *ABCB1* knockdown did not alter the toxicity of Cd in the HK-2 cells. Therefore, the Cd-induced increase

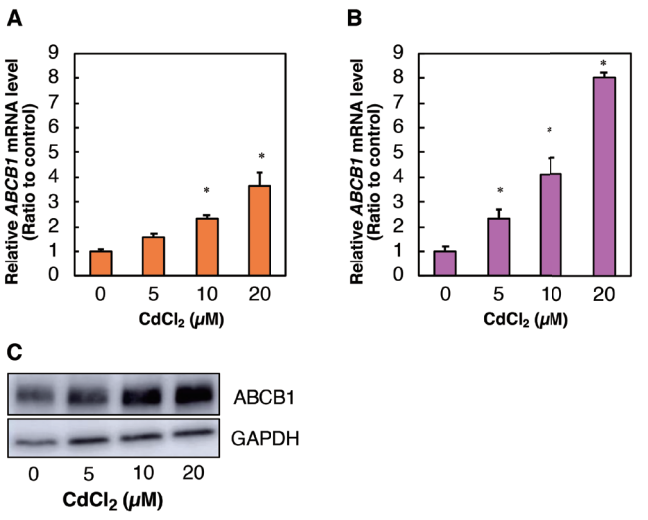

Fig. 1. Effect of Cd on *ABCB1* mRNA and Protein Levels in the HK-2 Cells

HK-2 cells were treated with Cd for 3 or 6 h. *ABCB1* mRNA levels were examined with real-time RT–PCR after Cd treatment for 3 h (**A**) and 6 h (**B**). *ABCB1* mRNA levels were normalized to *GAPDH* mRNA levels. Values are means ± SD (n = 3). *Significantly different from the control group, $P < 0.05$. (**C**) Protein levels were examined with western blotting after Cd treatment for 6 h. GAPDH was used as the loading control.

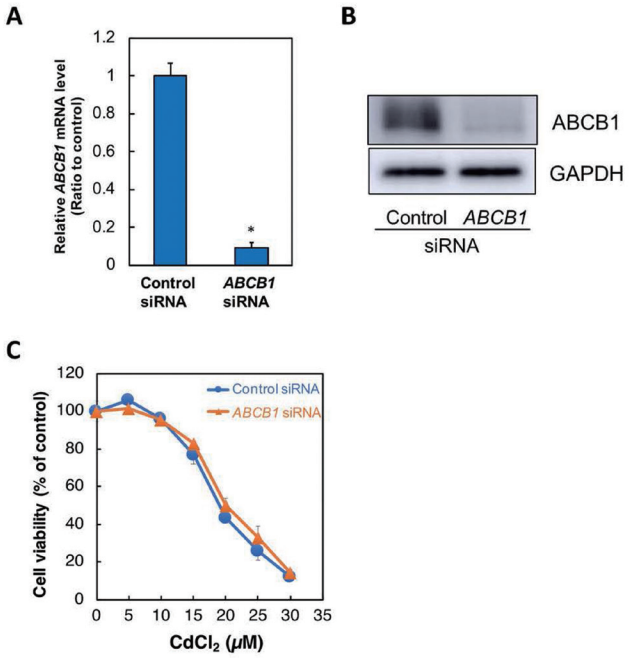

Fig. 2. Effect of *ABCB1* Knockdown on the Viability of HK-2 Cells Treated with Cd

The efficiency of *ABCB1* knockdown was examined after HK-2 cells were treated with siRNA for 48 h. (**A**) *ABCB1* mRNA levels were examined with real-time RT–PCR and normalized to *GAPDH* mRNA levels. Values are means ± SD (n = 3). *Significantly different from the control group, $P < 0.05$. (**B**) Protein levels were examined with western blotting. GAPDH was used as the loading control. (**C**) After treatment with siRNA for 48 h, HK-2 cells were treated with Cd for 24 h. Cell viability was examined with an Alamar blue assay. Values are means ± SD (n = 3).

in ABCB1 may affect the transportation function in the kidney but not the toxicity of Cd.

The expression of ABCB1 is regulated by a stress reaction that involves NF-κB (nuclear factor kappa B), AP-1 (activator protein 1), and WNT signal transduction.[10] Several previous studies have suggested that NF-κB, AP-1, and WNT signaling are involved in Cd renal toxicity.[11-13] Therefore, further study is expected to clarify the transcription pathway regulating the expression of ABCB1 induced by Cd treatment.

Similar to Cd, inorganic mercury (Hg^{2+}), another exogenous metal toxicant that targets the kidney, increases *MRP2* (ABCC2) and *ABCB1* mRNA levels in the kidney.[14] The renal cell toxicity of inorganic mercury is also reduced by the MRP2-mediated extracellular excretion associated with the increased expression of MRP2.[15] Therefore, we speculated that the increased expression of ABCB1 induced by Cd may reduce the cytotoxicity of several toxic compounds.

In a previous study, we demonstrated that long-term exposure to Cd reduced the expression of *Abcb1b* (mouse orthologue of human *ABCB1*) in the mouse kidney.[16] Moreover, the transcription of *Abcb1b* was suppressed in the mouse kidney by Cd exposure.[17] On the contrary, in the present study, the *ABCB1* expression in the human proximal tubule cells was increased by Cd treatment. It has previously been shown that the expression levels of the *UBC* gene (encoding ubiquitin c) in the HK-2 cells and the *Ubc* gene in the mouse liver caused opposite changes after Cd treatment.[18,19] These data imply that gene expression levels may vary between whole tissues and single cell colonies, and between different tissues.

The current findings imply that excessive increases in ABCB1 expression by Cd may accelerate the excretion of drugs acting in the kidney, resulting in a loss of the full pharmacological actions of the drug. One of the substrates of ABCB1 is everolimus, an anticancer agent that is indicated for renal cell carcinoma, neuroendocrine tumor, breast cancer, gastrointestinal neuroendocrine tumor, pulmonary neuroendocrine tumor, and other cancers.[20] Everolimus causes renal toxicity as a side effect.[21] Therefore, it is expected that the increase in ABCB1 levels induced in the kidney by Cd may enhance the excretion of everolimus by the strongly expressed ABCB1 and thus reduce the renal toxicity of everolimus. Further studies should clarify whether the Cd-induced transporter gene expressed in the proximal tubules can regulate transportation of materials, including medicines.

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Conflict of interest The authors declare no conflict of interest.

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