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Digoxin Up-Regulates MDR1 in Human Colon Carcinoma Caco-2 Cells

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Because MDR1 (P-glycoprotein) plays an important role in pharmacokinetics such as absorption and excretion of xenobiotics and multidrug resistance, an understanding of the factors regulating its function and expression is important. Here, the effects of digoxin on cell sensitivity to an anticancer drug, MDR1 function, and expression were examined by assessing the growth inhibition by paclitaxel, the transport characteristics of the MDR1 substrate Rhodamine123, and the level of *MDR1* mRNA, respectively, using human colon carcinoma Caco-2 cells, which are widely used as a model of intestinal epithelial cells. The sensitivity to paclitaxel, an MDR1 substrate, in Caco-2 cells pretreated with digoxin was lower than that in non-treated cells. The accumulation of Rhodamine123 was reduced by pretreatment with digoxin and its efflux was enhanced. The level of *MDR1* mRNA in Caco-2 cells was increased in a digoxin concentration-dependent manner. These results taken together suggested that digoxin up-regulates MDR1 in Caco-2 cells.

Key Words: **MDR1; P-glycoprotein; digoxin; Caco-2 cells; up-regulation of MDR1.**

Digoxin, a cardiac glycoside, is mainly prescribed for patients with congestive heart failure. The therapeutic range of serum digoxin concentration is very narrow (0.5–2.0 ng/mL), and therefore its pharmacokinetics have been enthusiastically evaluated. Digoxin has been reported to be mainly excreted by tubular secretion and glomerular filtration (1, 2). Its tubular secretion has been demonstrated to be mediated *via* multidrug resistance transporter MDR1 (P-glycoprotein) using kidney epithelial cell lines (3, 4). Recently, it has been demonstrated that the amounts of MDR1 in the small intestine also significantly affect the digoxin pharmacokinetics from the viewpoint of drug–drug interaction and pharmacogenomics (5–8). Rifampicin was reported to induce MDR1 expression in the small intestine in healthy volunteers, following reduction of the serum concentration of digoxin (5). The variability of MDR1 expression in the small intestine was shown to be regulated by the *MDR1* genotypes (7), by following the inverse correlation of the amounts of MDR1 in the intestine with AUC of digoxin. Collectively, the expression levels of MDR1 in not only the kidney but also the intestine were considered to be important in digoxin pharmacokinetics.

MDR1 was first discovered in 1976 in tumor cells resistant to multiple anticancer drugs (9). It has been demonstrated that the continuous exposure of tumor cells to some anticancer drugs induces MDR1 *in vitro* and *in vivo* (10, 11). It was reported in 2000 that aspirin enhanced MDR1 expression in human T lymphoma cells (12). This suggested that the drugs other than anticancer drugs may also induce MDR1. Therefore, we hypothesized that the expression of MDR1 might also be affected by exposure to digoxin.

Here, we investigated whether digoxin affects the function and expression of MDR1 in intestine using the human colon carcinoma cell line Caco-2, which is widely used as a model for intestinal epithelial cells. First, the effects of digoxin on the sensitivity to an anticancer drug and MDR1 function were examined by assessing the growth inhibition by paclitaxel and the transport characteristics of Rhodamine123, both of which are substrates for MDR1. Next, we investigated the effects of digoxin on the expression of *MDR1* mRNA using reverse transcription-polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

Chemicals. Digoxin was purchased from Aldrich Chemical Co. (Milwaukee, WI). Paclitaxel and cyclosporin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). WST-1 and 1-methoxy PMS were purchased from Dojindo Laboratories (Kumamoto, Japan). Rhodamine123 was purchased from Molecular Probes, Inc. (Eugene, OR). All other agents were obtained commercially and were of analytical grade requiring no further purification.

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Cells and cell culture. The human colon carcinoma cell line Caco-2 was obtained from American Type Culture Collection (Rockville, MD). Caco-2 cells (47–52 passage) were maintained in culture medium consisting of Dulbecco's modified Eagle's medium (D-MEM; Cat. No. 12800-017, Gibco BRL, Life Technologies, Inc., NY) supplemented with 10% heat-inactivated FBS (Lot No. 09017, BioWhittaker, Walkersville, MD), 100 U/mL penicillin G, 100 μg/L streptomycin sulfate and 0.1 mM non-essential amino acids (Gibco BRL). Cells (2×10^4 cells/cm^2) were seeded on plastic culture dishes (100 mm in diameter), grown in a humidified atmosphere of 5% CO_2-95% air at 37°C, and subcultured with 0.05% trypsin-0.02% EDTA (Gibco BRL).

Growth inhibitory effects of paclitaxel. The growth inhibitory effects of paclitaxel were assessed in Caco-2 cells by WST-1 (tetrazolium salts) colorimetric assay (Dojindo Laboratories) (13). Caco-2 cells (1×10^6 cells/60 mm dish/5 mL) were seeded and pretreated with or without digoxin at the indicated concentrations for 48 h in a humidified atmosphere of 5% CO_2-95% air at 37°C. After trypsinization, Caco-2 cells (5,000 cells/well) were seeded again into 96-well plates (Cat. No. 3596, Corning Inc.) in 100 μL of culture medium without any drugs on Day 0. The culture medium was exchanged for that containing paclitaxel at various concentrations without digoxin on day 1. After incubation for 72 h at 37°C (on Day 4), the culture medium was exchanged for 110 μL of that containing WST-1 reagent solution (10 μL WST-1 solution and 100 μL the culture medium), and 3 h later, the absorbance was determined at 450 nm with a reference wavelength of 630 nm using a microplate reader (Spectra Fluor; Tecan Switzerland, Switzerland).

The 50% growth inhibitory concentration (IC_{50}) of paclitaxel in Caco-2 cells was calculated according to the sigmoid inhibitory effect model, $E = E_{max} \times [1 - C^{\gamma}/(C^{\gamma} + IC_{50}^{\gamma})]$, using the nonlinear least-squares fitting method (Solver, Microsoft Excel 2001 for Macintosh). E and E_{max} represent the surviving fraction (% of control) and its maximum, respectively, and C and γ represent the drug concentration in the medium (nM) and the sigmoidicity factor, respectively.

Uptake and efflux of Rhodamine123. In the uptake experiments, Caco-2 cells (5×10^4 cells) were seeded into 24-well plates (Cat. No. 3526, Corning Inc.) in 1 mL/well of culture medium. The culture medium was exchanged for the fresh culture medium every 2 days and plates were incubated for 10 days in a humidified atmosphere of 5% CO_2-95% air at 37°C. Then, the culture medium was exchanged for the fresh culture medium with or without digoxin at the indicated concentrations, and further incubated for 48 h in a humidified atmosphere of 5% CO_2-95% air at 37°C. After pretreatment with digoxin, cells were washed three times with warmed Hanks' balanced salt solution (HBSS; 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.5 mM $MgCl_2$, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 5.6 mM glucose, 0.06 mM phenol red, and 25 mM HEPES). The uptake experiments were started by addition of fresh HBSS containing 3 μM Rhodamine123, and further incubation for the indicated times at 37°C. Uptake experiments were stopped by aspiration of HBSS from the wells, followed by washing three times with ice-cold phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM $NaHPO_4$).

In the efflux experiments, Caco-2 cells were cultured in the same manner as described for the uptake experiments. The cells were pretreated with or without digoxin for 48 h, and then cells were washed three times with warmed HBSS and incubated in fresh HBSS containing 10 μM Rhodamine123 for 60 min (Loading time). After loading, HBSS was immediately removed from the wells and cells were quickly washed three times with HBSS. Efflux experiments were started by addition of fresh warmed HBSS, 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 stopping the uptake or efflux experiments, cells were solubilized with 1 mL of 0.3 M NaOH, and aliquots (500 μL) were neutralized by 500 μL of 0.3 M HCl. Aliquots (200 μL) of the neutralize cell-solubilized solution were transferred to 96-well black plates (Cat. No. 3915, Corning, Inc.) and the fluorescence intensity of Rhodamine123 was measured at an excitation wavelength of 485 nm and emission wavelength of 535 nm using a Spectra Fluor (Tecan). Protein content was determined by the Lowry method (14), and bovine serum albumin was used as the standard.

RT-PCR analysis of MDR1 mRNA. Caco-2 cells (1×10^6 cells) were seeded on plastic culture dishes (60 mm in diameter) in 5 mL of culture medium. The culture medium was exchanged for fresh culture medium every 2 days and cells were incubated for 10 days in a humidified atmosphere of 5% CO_2-95% air at 37°C. Then, the culture medium was exchanged for that with or without digoxin at the indicated concentrations, and further incubated at 37°C for 24 h. The level of *MDR1* mRNA in Caco-2 cells was measured by RT-PCR. Total RNA was isolated using an RNeasy mini kit (Qiagen Inc., CA), and aliquots (0.1 μg) of the RNA were used for reverse transcription using an RNA PCR kit (AMV) ver. 2.1 (Takara Shuzo Co., Ltd., Shiga, Japan). PCR primers for amplification of *MDR1* cDNA and β2-microglobulin (β2m) cDNA were synthesized by Genset K. K. (Kyoto, Japan). Primers MDR1-F (5′-CCC ATC ATT GCA ATA GCA GG-3′) and MDR1-R (5′-GTT CAA ACT TCT GCT CCT GA-3′) were used to amplify MDR1 (15). Primers β2m-F (5′-ACC CCC ACT GAA AAA GAT GA-3′) and β2m-R (5′-ATC TTC AAA CCT CCA TGA TG-3′) were used to amplify β2m (15). β2m was used as a control to check the integrity of RNA preparations, as well as to demonstrate that the effects of the drug on *MDR1* mRNA expression were not due to nonspecific effects on transcription. PCR amplification of cDNA was performed in a total reaction volume of 25 μL using an RNA PCR kit (AMV) ver. 2.1 (Takara). PCR amplification was initiated by one cycle of 94°C for 2 min followed by 30 sequential cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min in a thermal cycler (iCycler Thermal Cycler, Bio-Rad Laboratories, Inc., CA). PCR products were separated on Tris-acetate-EDTA 3% agarose gels containing 100 ng/mL ethidium bromide, and photographed under UV illumination at 312 nm (TFX-20.MC, Ieda Chemicals Co. Ltd., Tokyo, Japan) with a Polaroid camera (DS-300, Funakoshi Co. Ltd., Tokyo, Japan). Band densities were measured using the computer program NIH Image ver. 1.62 (National Institutes of Health, Bethesda, MD), and the ratio of band density (*MDR1*/β2m) was calculated.

Statistical analysis. Comparisons between two and among more than three groups were performed by Student's unpaired *t*-test and non-repeated one-way analysis of variance (ANOVA) followed by Scheffe's test, respectively. The *p* values of less than 0.05 (two-tailed) were considered significant.

RESULTS

Cytotoxicity of paclitaxel. The growth inhibition curve of the effect of paclitaxel on non-treated Caco-2 cells was shifted to a higher concentration range by pretreatment with 1 μM digoxin (Fig. 1). Table 1 summarizes the effects of pretreatment with digoxin on the 50% growth inhibitory concentration (IC_{50}) of paclitaxel in Caco-2 cells. The IC_{50} values for paclitaxel in digoxin-treated Caco-2 cells were increased in a digoxin concentration-dependent manner. However, pretreatment with 1 μM digoxin decreased the IC_{50} value of paclitaxel in Caco-2 cells, as compared with that of 100 nM digoxin.

Uptake of Rhodamine123 by Caco-2 cells. The accumulation of Rhodamine123 in Caco-2 cells increased in a time-dependent manner (Fig. 2). The uptake of Rhodamine123 was significantly reduced by pretreat-

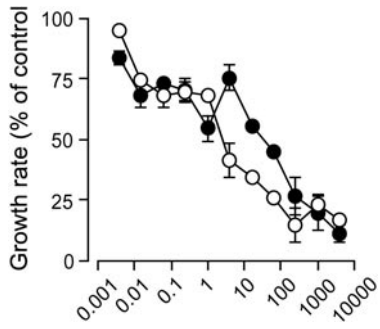

FIG. 1. Growth inhibitory curves of paclitaxel in Caco-2 cells pretreated with or without digoxin. Cells were pretreated without (○) or with (●) 1 μM digoxin for 48 h at 37°C, and then the cytotoxicity of paclitaxel was evaluated. Each point represents the mean ± SE of at least three experiments.

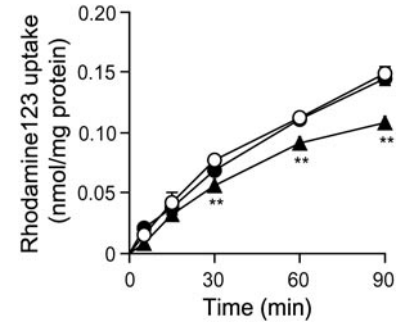

FIG. 2. Time courses of Rhodamine123 accumulation in Caco-2 cells pretreated with or without digoxin. Cells were pretreated without (○) or with 1 nM (●) or 1 μM (▲) digoxin for 48 h at 37°C, and then the accumulation of 10 μM Rhodamine123 was evaluated. Each point represents the mean ± SE of three experiments. $^{**}P < 0.01$ significantly different from the non-treated groups at the corresponding time points.

ment with 1 μM digoxin as compared with that in non-treated Caco-2 cells, although pretreatment with 1 nM digoxin showed no effect.

Efflux of Rhodamine123 from Caco-2 cells. The residual amount of Rhodamine123 in Caco-2 cells decreased in a time-dependent manner, and was about 60% at 90 min (Fig. 3). This decrease was enhanced by pretreatment with 1 μM digoxin, although no effects were seen at 1 nM digoxin. Moreover, this decrease in the residual amount was significantly restored by addition of 10 μM cyclosporin, and this restoration was comparable to that in non-treated Caco-2 cells (Fig. 4).

Expression of MDR1 mRNA in Caco-2 cells. The expression of *MDR1* mRNA in Caco-2 cells increased in a digoxin concentration-dependent manner (Fig. 5). The expression of *MDR1* mRNA was hardly affected by treatment with 1 nM digoxin, but a significant increase of ca. 4-fold was observed in Caco-2 cells treated with 1 μM digoxin.

DISCUSSION

Various types of anticancer drugs have been shown to induce MDR1 *in vitro* and *in vivo* (10, 11). Recently, it was reported that drugs other than anticancer drugs also induced MDR1 (5, 6, 12, 16, 17) regardless of whether they were substrates for MDR1. On the other hand, the pharmacokinetics of digoxin were shown to be dependent on not only the expression of MDR1 in the kidney but also in the small intestine (5–8). Therefore, it is important to understand the factors that affect the function and expression of MDR1 in intestine. Here, the human colon carcinoma cell line Caco-2 was selected as a model of intestinal epithelial cells, as these cells have been widely used for studying intestinal permeability and several transport functions, including the transport of various drugs. Moreover, these cells endogenously express MDR1. In this study, the effects of digoxin on sensitivity to paclitaxel, an MDR1 substrate, and function and expression of MDR1 were examined.

The growth inhibitory curve of the non-treated Caco-2 cells by paclitaxel was shifted to a higher con-

TABLE 1

The IC_{50} Values for Paclitaxel in Caco-2 Cells Pretreated with or without Digoxin

	IC_{50} (nM)	Relative resistance[a]
Non-treated Caco-2 cells	1.02 ± 0.34	—
1 nM digoxin	1.75 ± 1.01	1.7
10 nM digoxin	52.0 ± 47.6	50.9
100 nM digoxin	341 ± 196	344
1 μM digoxin	150 ± 33	148

Note. The values are the means ± S.E. of three or four independent experiments.

[a] The relative resistance = IC_{50} values in the digoxin-treated Caco-2 cells/IC_{50} values in non-treated Caco-2 cells.

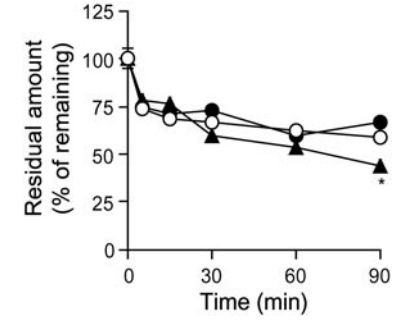

FIG. 3. Time courses of Rhodamine123 efflux from Caco-2 cells pretreated with or without digoxin. Cells were pretreated without (○) or with 1 nM (●) or 1 μM (▲) digoxin for 48 h at 37°C, and then the efflux of 10 μM Rhodamine123 was evaluated. Each point represents the mean ± SE of three or six experiments. $^{*}P < 0.05$ significantly different from the non-treated groups at the corresponding time points.

centration range by pretreatment with 1 μM digoxin (Fig. 1), and the IC_{50} values for paclitaxel increased in a digoxin concentration-dependent manner (Table 1). The accumulation of Rhodamine123 after 30 min in Caco-2 cells pretreated with 1 μM digoxin for 48 h was significantly reduced (Fig. 2), and the efflux of Rhodamine123 from Caco-2 cells was significantly enhanced at 90 min (Fig. 3). This increase in efflux was restored by addition of 10 μM cyclosporin (Fig. 4). Moreover, the expression of *MDR1* mRNA in Caco-2 cells was increased in a concentration-dependent manner, with a ca. 4-fold increase by treatment with 1 μM digoxin (Fig. 5). These observations confirmed that digoxin up-regulated MDR1 in Caco-2 cells.

A concentration of 1 μM digoxin was not clinically achievable, as serum concentrations, are usually about 1 nM (0.25 mg, *p.o.*) to 30 nM (1 mg, *i.v.* infusion) (5, 6). When digoxin is administered orally, its concentration in the intestine is considered to be much higher than the serum concentration, presumably reaching the μM range. Thus, the repeated oral administration of digoxin in clinical use may up-regulate MDR1 in the intestine. Therefore, it was suggested that new types of drug-drug interactions would be caused by digoxin *via* the up-regulation of MDR1, and the resistance induced by digoxin might also be important in cancer chemotherapy. To date, however, there have been no reports concerning its clinically significance.

The mechanism of action of the increase in cardiac contractility by digoxin is the inhibition of Na^+-K^+ ATPase, which acts to increase the intracellular Na^+-Ca^{2+} exchange to increase intracellular calcium levels. This mechanism is similar to those of other cardiac glycosides including digitoxin and ouabain. Among these, Brouillard *et al.* reported that ouabain stimulated MDR1 expression *via* the inhibition of Na^+–K^+-ATPase (17). Thus, it was speculated that digoxin regulated MDR1 expression *via* a mechanism similar to that of ouabain. On the other hand, it was reported that some steroids bind the steroid and xenobiotic receptor (SXR) and regulate MDR1 expression (18). As digoxin includes a steroid structure in its molecular structure, it is also possible that it regulates MDR1 expression *via* binding to SXR receptors. Furthermore, digoxin might affect some transcription factors such as Sp1 (19, 20), YB-1 (21), HSF1 (22) and MEF1 (23), and regulate MDR1 expression. Additional studies on the mechanism of MDR1 up-regulation by digoxin are currently in progress in our laboratory.

In conclusion, the present findings indicated that digoxin up-regulated MDR1 in Caco-2 cells. These findings consequently suggested that digoxin causes a new types of drug-drug interaction *via* the up-regulation of MDR1 under clinical conditions.

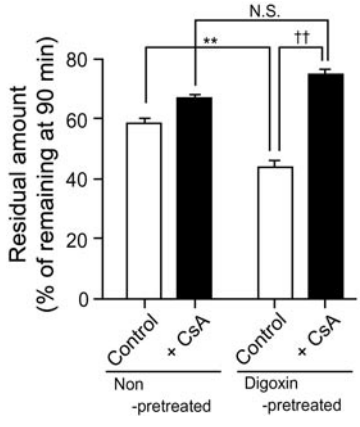

FIG. 4. Effects of cyclosporin on Rhodamine123 efflux from Caco-2 cells pretreated with or without digoxin. Cells were pretreated without or with 1 μM digoxin for 48 h at 37°C, and then the efflux of Rhodamine123 was evaluated at 90 min in the absence (□) and presence (■) of 10 μM cyclosporin (CsA). Each column represents the mean ± SE of three or four experiments. $^{**}P < 0.01$ significantly different from the control in non-treated Caco-2 cells. $^{††}P < 0.01$ significantly different from the control in DX-treated Caco-2 cells. N.S.; not significant.

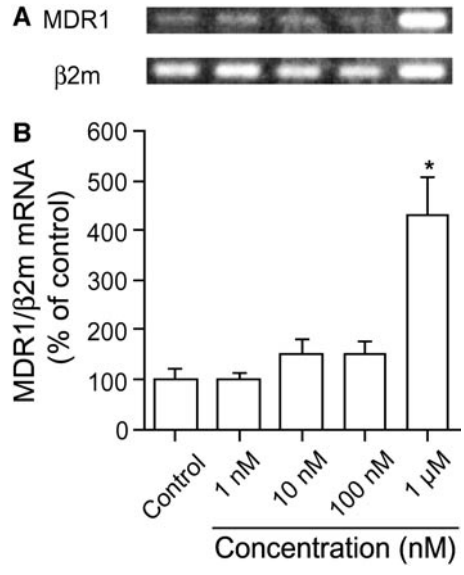

FIG. 5. Expression of *MDR1* mRNA in Caco-2 cells treated with or without digoxin. Cells were treated without or with the indicated concentrations of digoxin for 24 h at 37°C, and then total RNA was extracted. The levels of *MDR1* mRNA were measured using RT-PCR. (A) Representative electrophoretogram of MDR1 and β2m. Sizes of MDR1 and β2m were 167 and 120 bp, respectively. (B) Data were obtained after densitometric analysis of three independent electrophoretograms. Results are expressed as percentages of *MDR1*/β2m mRNA levels in the non-treated Caco-2 cells. $^{*}P < 0.05$ significantly different from the control.

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