JPP Journal of Pharmacy and Pharmacology

JPP 2003, 55: 675–681

Received September 20, 2002
Accepted January 31, 2003
DOI 10.1211/002235702964
ISSN 0022-3573

Effects of continuous exposure to digoxin on MDR1 function and expression in Caco-2 cells

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Abstract

The Caco-2 cell line has been used widely for studying intestinal permeability and several transport functions, and express the multidrug resistance transporter MDR1/P-glycoprotein. Previously, the transient exposure to digoxin for 24 h was found to induce *MDR1* mRNA in Caco-2 cells. Here, a digoxin-tolerant Caco-2 subline (Caco/DX) was newly established by the continuous exposure of Caco-2 cells to digoxin, and the effects of continuous exposure to digoxin on MDR1 were examined. The 50% growth inhibitory concentration (IC_{50}) values for digoxin in Caco-2 and Caco/DX cells were 17.2 and 81.4 nM, respectively. The IC_{50} values for paclitaxel, an MDR1 substrate, were 1.0 and 547 nM, respectively, whereas the cytotoxicity of 5-fluorouracil was comparable in both cells. The uptake and efflux of Rhodamine123, an MDR1 substrate, in Caco/DX cells were significantly less and greater, respectively, than those in Caco-2 cells, and these transports were affected by the addition of ciclosporin. The expression of *MDR1* mRNA in Caco/DX cells was approximately 2- and 1.7-fold compared with Caco-2 cells and Caco-2 cells treated with 100 nM digoxin for 24 h, respectively. On the other hand, *MRP1* mRNA in Caco/DX cells was unchanged. These observations confirmed that the continuous exposure to digoxin, as well as the transient exposure, induced MDR1 in Caco-2 cells.

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Funding: This work was supported in part by a Grant-in-Aid for Young Scientists (B) (No. 13771445) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by a Frontier Research Program from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Introduction

The multidrug resistance transporter MDR1/P-glycoprotein was first discovered by its ability to confer multidrug resistance against a variety of structurally unrelated anticancer drugs on tumour cells (Gottesman & Pastan 1993). MDR1 is a glycosylated membrane protein of 1280 amino acids (170 kDa), which consists of two similar regions containing six putative transmembrane segments and two intracellular binding sites for ATP, and acts as an efflux pump to remove anticancer drugs from cells. The magnitude of resistance to anticancer drugs was shown to be dependent on the level of MDR1 expression (Bradshaw & Arceci 1998; Ueda et al 1999). In addition to MDR1, other ATP-binding cassette (ABC) transporters are also involved in efflux pump or multidrug resistance. Among these, multidrug resistance-associated protein 1 (MRP1) was shown to be similar to MDR1-mediated resistance to a range of structurally and functionally unrelated drugs (Hipfner et al 1999; Leslie et al 2001).

On the other hand, it was shown that MDR1 was expressed in the apical side of normal tissues including the liver, kidneys, intestine and brain (Thiebaut et al 1987), and it is known to export unnecessary or toxic exogenous substances or metabolites out of the body. MDR1 plays important roles in the pharmacokinetics of pharmacological agents, in for example their absorption and excretion (Borst et al 1993; Kusuhara et al 1998; Tanigawara 2000). Therefore, an understanding of the factors regulating its function and expression is important to know how they alter pharmacokinetic profiles or treatment efficacy.

To date, it has been reported that some types of cellular stress increase MDR1 expression (Bates et al 1989; Chin et al 1990; Chaudhary & Roninson 1993; Kim et al 1998). We demonstrated that the transient exposure to digoxin, a cardiac glycoside, for 24 h induced *MDR1* mRNA expression in the human colon adenocarcinoma cell line Caco-2 (Takara et al 2002b), which is used widely to study intestinal permeability and

transport functions. Digoxin has been identified as a substrate of MDR1 in-vitro and in-vivo (Tanigawara et al 1992; Schinkel et al 1996, 1997). In clinical situations, digoxin is mainly prescribed for patients with congestive heart failure, and moreover, its repeated and long-term oral administration is usually performed. However, the effects of long-term exposure to digoxin on the function and expression of MDR1 in the intestine are unclear in-vitro and in-vivo.

To investigate how the continuous exposure to digoxin affects MDR1 or MRP1, another protein involved in efflux transport, Caco-2 cells were exposed to digoxin for three months and established the novel digoxin-tolerant Caco-2 cells, designated as Caco/DX cells. Using the Caco/DX cells, the effects of the continuous exposure to 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* and *MRP1* mRNAs, respectively.

Materials and Methods

Chemicals

Digoxin was purchased from Aldrich Chemical Co. (Milwaukee, WI). 5-Fluorouracil, paclitaxel and ciclosporin were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) and 1-methoxy-5-methylphenazinium methylsulfate (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.

Establishment of digoxin-tolerant Caco-2 subline

The human colon adenocarcinoma cell line Caco-2 cells (47–52 passages) were grown in complete culture medium consisting of Dulbecco's modified Eagle's medium (DMEM; Cat. No. 12800-017, Invitrogen, Co., Carlsbad, CA) supplemented with 10% heat-inactivated foetal bovine serum (Lot. No. 09017, BioWhittaker, Walkersville, MD), 100 U mL^{-1} benzylpenicillin (penicillin G), 100 $\mu g\,L^{-1}$ streptomycin sulfate and 0.1 mM non-essential amino acids (Invitrogen). Cells were seeded at a density of 5×10^5 cells per 60-mm dish (diam.) in 5 mL complete culture medium. The cells were selected for digoxin tolerance by continuous exposure to digoxin. The concentration of digoxin to which cells were exposed was 100 nM (approximately 80 ng mL^{-1}) from the first. Digoxin at this concentration was moderately cytotoxic for Caco-2 cells, and induced *MDR1* mRNA by its exposure for 24 h (Takara et al 2002b). To clone a subline that could tolerate 100 nM digoxin, cells were cultured in complete culture medium with 100 nM digoxin for approximately three months. The clone, designated as Caco/DX cells, was maintained in complete culture medium containing 100 nM digoxin.

Growth inhibition assay

The growth inhibitory effects of digoxin and anticancer drugs were assessed in Caco-2 and Caco/DX cells using a WST-1 assay (Iida et al 2001; Takara et al 2002a, 2002b). Both cell lines (5000 cells/well) were seeded into 96-well plates (Corning Inc., NY) in 100 μL culture medium without any drugs on day 0. The culture medium was exchanged for one containing test drugs at various concentrations on day 1. After 72-h incubation at 37 °C (on day 4), the culture medium was exchanged for 110 μL medium containing WST-1 solution (10 μL WST-1 solution and 100 μL culture medium). The absorbance was determined at 450 nm 3 h later, with a reference wavelength of 630 nm using a microplate reader (Spectra Fluor; Tecan Switzerland, Switzerland). The 50% growth inhibitory concentrations (IC_{50}) were calculated according to the sigmoid inhibitory effect model, $E = E_{max} \times (1 - C^{\gamma}/(C^{\gamma} + IC_{50}{}^{\gamma}))$, using the non-linear 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

Uptake and efflux of Rhodamine123 were determined as described by Takara et al (2002b). In the uptake experiments, cells (5×10^4 cells) were seeded into 24-well plates (Corning Inc.) in 1 mL/well culture medium. The culture medium was exchanged every two days for fresh culture medium without digoxin (Caco-2) or with 100 nM digoxin (Caco/DX), and then cells were precultured for 10 days in a humidified atmosphere of 5% CO_2–95% air at 37 °C. Both cell lines were washed twice with warmed Hanks' balanced salt solution (HBSS), and the uptake experiments were started by addition of fresh HBSS containing 3 μM Rhodamine123, and further incubated for the indicated times at 37 °C.

In the efflux experiments, Caco-2 and Caco/DX cells were cultured in the same manner as described for the uptake experiments. 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 washed rapidly three times with HBSS. Efflux experiments were started by addition of fresh warmed HBSS, and further incubated for the indicated times at 37 °C.

After the uptake or efflux reaction was stopped by aspiration of HBSS from the wells, cells were washed three times with ice-cold phosphate buffered saline (PBS). Cells were then lysed by 1 mL 0.3 M NaOH, and 500 μL samples were neutralized by 500 μL 0.3 M HCl. Samples (200 μL) of the neutralized soluble fraction were

transferred to 96-well black plates (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 (Lowry et al 1951), and bovine serum albumin was used as the standard.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis of *MDR1* and *MRP1* mRNA

Caco-2 and Caco/DX cells (1×10^6 cells) were seeded on plastic culture dishes (60-mm diam.) in 5 mL culture medium. Cells were precultured as described in the above Rhodamine123 transport experiments, and then the culture medium was exchanged for medium without digoxin (Caco-2) or medium with 100 nM digoxin (Caco/DX). The cells were incubated for a further 24 h in a humidified atmosphere of 5% CO_2–95% air at 37 °C. In the case of transient exposure to digoxin, Caco-2 cells were precultured in the same manner, the culture medium was exchanged for one with 100 nM digoxin, and incubated for a further 24 h in a humidified atmosphere of 5% CO_2–95% air at 37 °C.

Total RNA from the cells in three separate dishes was isolated using an RNeasy mini kit (Qiagen Inc., CA), and samples (0.1 μg) of the RNA were used for reverse transcription with an RNA PCR kit (AMV) ver. 2.1 (Takara Shuzo Co., Ltd, Shiga, Japan). PCR primers for amplification of *MDR1, MRP1* and β2-microglobulin (β2 m) 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 (Kim et al 1995; Takara et al 2002b). Primers MRP1-F (5′-ATC AAG ACC GCT GTC ATT GG-3′) and MRP1-R (5′-GAG CAA GGA TGA CTT GCA GG-3′) were used to amplify MRP1 (Kim et al 1995; Takara et al 2002b). Primers β2 m-F (5′-ACC CCC ACT GAA AAA GAT GA-3′) and β2 m-R (5′-ATC TTC AAA CCT CCA TGA TG-3′) were used to amplify β2 m (Kim et al 1995; Takara et al 2002b). 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^{-1} ethidium bromide, the 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* or *MRP1*/β2 m) 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 followed by Fisher's protected least significant difference (Fisher's PLSD), respectively; *P* values of less than 0.05 (two-tailed) were considered significant.

Results

Growth inhibitory effects of digoxin and anticancer drugs

Figure 1 shows the growth inhibitory curves of digoxin on Caco-2 and Caco/DX cells. The growth inhibitory curve of digoxin in Caco/DX cells was shifted to the higher concentration range compared with Caco-2 cells. Table 1 summarizes the 50% growth inhibitory concentrations (IC_{50}) in Caco-2 and Caco/DX cells. The IC_{50} values of digoxin in Caco-2 and Caco/DX cells were 17.2 and 81.4 nM, respectively, and the relative resistance in the latter was approximately 5.0-fold. The IC_{50} values of paclitaxel, a substrate for MDR1, in Caco-2 and Caco/DX cells were 1.0 and 547 nM, respectively, and the relative resistance was 547-fold. In contrast, the IC_{50} value of 5-fluorouracil in Caco/DX cells was comparable with that in Caco-2 cells.

Uptake and efflux of Rhodamine123

Figure 2 shows the time course of Rhodamine123 uptake in Caco-2 and Caco/DX cells. The uptake of Rhodamine123 in Caco-2 and Caco/DX cells was increased in a time-dependent manner, and its uptake after 60 min in Caco/DX cells was significantly less compared with Caco-2 cells, while during the first 30 min it was significantly larger.

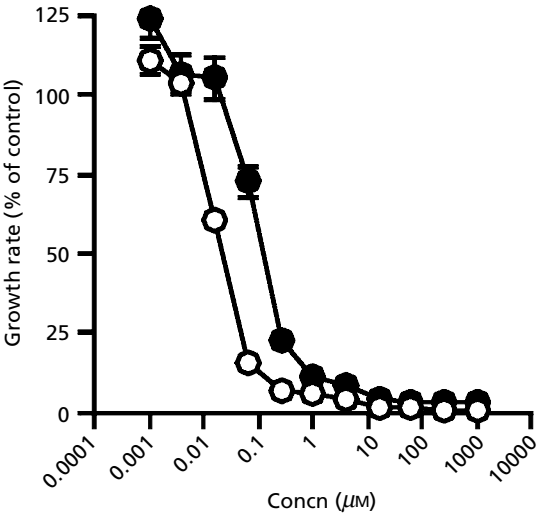

Figure 1 Growth inhibitory curves of digoxin in Caco-2 (○) and Caco/DX (●) cells. The cytotoxicity of digoxin at the indicated concentrations was evaluated by WST-1 assay. Each point represents the mean ± s.e. of eight experiments.

Table 1 IC_{50} values for various types of drugs in Caco-2 and Caco/DX cells.

Drug	Caco-2 cells		Caco/DX cells		Relative resistance[b]
	IC_{50}	n[a]	IC_{50}	n	
Digoxin	17.2 ± 0.7 nM	8	81.4 ± 7.7 nM	8	4.7
Paclitaxel	1.0 ± 0.3 nM	3	547 ± 338 nM	3	547
5-Fluorouracil	12.3 ± 4.5 μM	7	9.0 ± 3.1 μM	8	0.7

Each IC_{50} value represents the mean ± s.e. [a]Number of independent experiments. [b]Relative resistance: the IC_{50} value for drugs in Caco/DX cells was divided by that in Caco-2 cells.

Figure 3 shows the time course of Rhodamine123 efflux from Caco-2 and Caco/DX cells. The efflux of Rhodamine123 from Caco-2 cells increased in a time-dependent manner, and its residual amount in Caco-2 cells was approximately 60% at 90 min. The efflux of Rhodamine123 from Caco/DX cells was also increased in a time-dependent manner, and the residual amount was significantly less than that in Caco-2 cells. Moreover, this decrease in the residual amount was significantly restored by addition of 10 μM ciclosporin, and this restoration was comparable with that in Caco-2 cells (Figure 4).

MDR1 and *MRP1* mRNA expression

MDR1 and *MRP1* mRNA expression in Caco-2 and Caco/DX cells were investigated using RT-PCR (Figures 5 and 6). The level of expression of *MDR1* mRNA in Caco/DX cells from the three separate dishes was approximately 2-fold compared with Caco-2 cells ($P < 0.01$), and this was also significantly higher compared with Caco-2 cells treated with 100 nM digoxin for 24 h. On the other hand, the expression of *MRP1* mRNA in Caco/DX cells from the three separate dishes was equivalent to that in

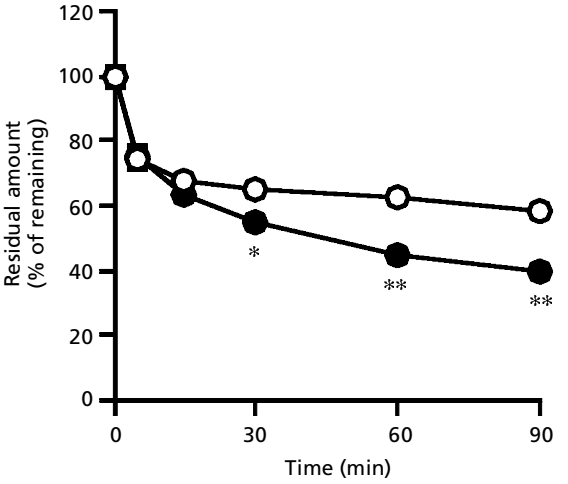

Figure 3 Time courses of Rhodamine123 efflux from Caco-2 (○) and Caco/DX (●) cells. Both cell lines were exposed to 10 μM Rhodamine123 for 60 min at 37 °C, and its efflux was evaluated for the indicated times at 37 °C. Each point represents the mean ± s.e. of four experiments. $*P < 0.05$, $**P < 0.01$ compared with Caco-2 cells at the corresponding time points, respectively.

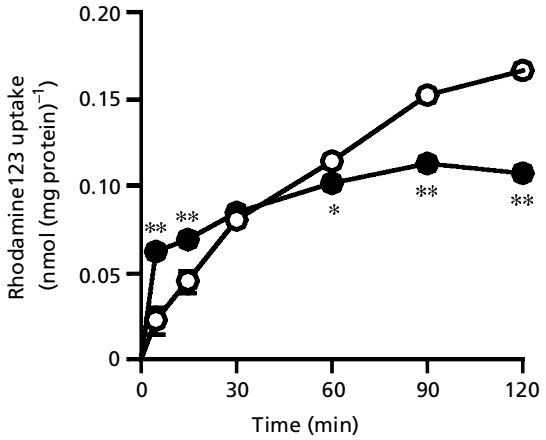

Figure 2 Time courses of Rhodamine123 uptake in Caco-2 (○) and Caco/DX (●) cells. Both cell lines were exposed to 3 μM Rhodamine123 for the indicated times at 37 °C. Each point represents the mean ± s.e. of three or four experiments. $*P < 0.05$, $**P < 0.01$ compared with Caco-2 cells at the corresponding time points, respectively.

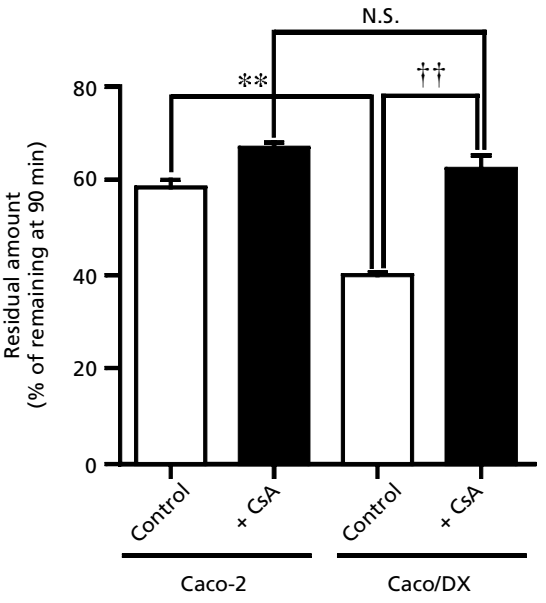

Figure 4 Effects of ciclosporin on Rhodamine123 efflux from Caco-2 and Caco/DX cells. Both cell lines were exposed to 10 μM Rhodamine123 for 60 min at 37 °C, and its efflux was evaluated at 90 min in the absence (□) or presence (■) of 10 μM ciclosporin (CsA). Each column represents the mean ± s.e. of four experiments. $**P < 0.01$ compared with control in Caco-2 cells. $††P < 0.01$ compared with control in Caco/DX cells. N.S., not significant.

Caco-2 cells, and this was also comparable with that in Caco-2 cells treated with 100 nM digoxin for 24 h.

Discussion

In this study, Caco/DX cells were newly established by continuous exposure of the human colon adenocarcinoma Caco-2 cell line to digoxin, which is a substrate for MDR1 (Tanigawara et al 1992; Schinkel et al 1996, 1997). As digoxin has recently been reported to be a cytotoxic agent using human primary tumour and tumour cell lines (Johansson et al 2001; Yeh et al 2001), the sensitivity of Caco/DX cells to digoxin was first compared with that of Caco-2 cells (Table 1). The IC50 values for digoxin in Caco-2 and Caco/DX cells were 17.2 and 81.4 nM, respectively, and the relative resistance, i.e. the ratio of IC50 between Caco-2 and Caco/DX cells, was approximately 5.0-fold. This indicated that the sensitivity to digoxin in Caco-2 cells was reduced by continuous exposure to digoxin. Thus, Caco/DX cells were considered to be a novel Caco-2 subline with tolerance to digoxin.

Next, the characterization of Caco/DX cells was performed by assessing the growth inhibition by anticancer drugs, the uptake and efflux of Rhodamine123, which is a substrate for MDR1, and the level of *MDR1* mRNA expression using RT-PCR. The IC50 values for paclitaxel, a substrate for MDR1 (Takara et al 2002a), in Caco-2 and Caco/DX cells were 1.0 and 547 nM, respectively (Table 1), and the relative resistance of the latter was 547-fold. In contrast, the cytotoxicity of 5-fluorouracil, which is not a substrate for MDR1 (Takara et al 2002a), in Caco/DX cells was comparable with Caco-2 cells. The uptake of Rhodamine123 after 60 min in Caco/DX cells was significantly less than in Caco-2 cells, while during the first 30 min it was significantly greater (Figure 2). Although this reason remains unclear, the membrane permeability of Rhodamine123 may be accelerated by continuous exposure of Caco-2 cells to digoxin. The decreased Rhodamine123 uptake in Caco/DX cells was also restored in the presence of 10 μM ciclosporin, a representative substrate for MDR1 (unpublished data). Moreover, the residual amount of Rhodamine123 after its efflux from Caco/DX cells for 90 min was approximately 40% (Figure 3), and it was significantly lower than that in Caco-2 cells (approximately 60%). These were restored to the equivalent level in both cell lines by addition of 10 μM ciclosporin (Figure 4). The level of *MDR1* mRNA expression in Caco/DX cells was approximately 2-fold compared with Caco-2 cells, suggesting induction of MDR1 in Caco/DX cells (Figure 5), and this was significantly higher than that in Caco-2 cells treated with 100 nM digoxin for 24 h. Furthermore, the increased MDR1 expression in Caco/DX cells was stable in the presence of 100 nM digoxin, however its stability in the absence of digoxin was not examined. Collectively, these observations confirmed that continuous exposure to digoxin induced MDR1 in Caco-2 cells, similarly to transient exposure (Takara et al 2002b), and MDR1 induced by digoxin also acted as an efflux pump, which sent intracellular substrate out of the cells.

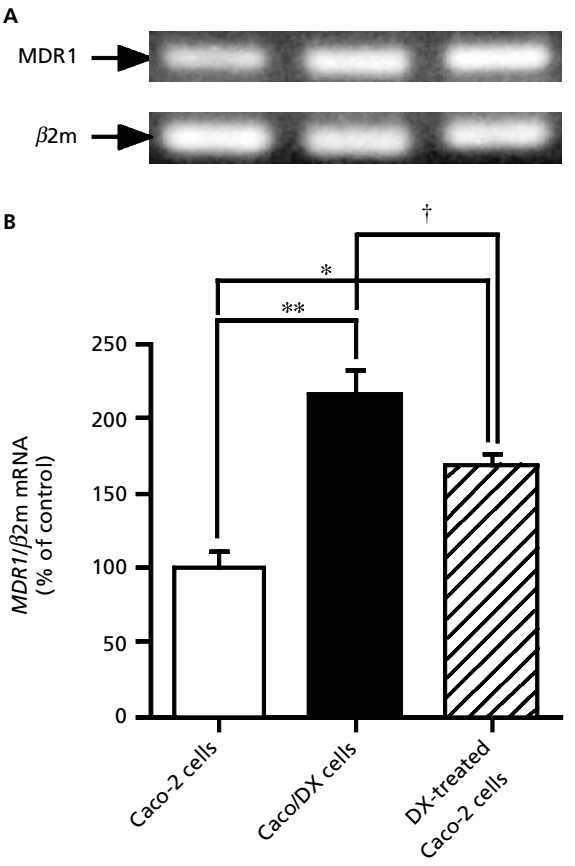

Figure 5 Expression of *MDR1* mRNA in Caco-2 and Caco/DX cells. Total RNA was extracted from Caco-2 and Caco/DX cells, and the levels of *MDR1* mRNA were measured by RT-PCR. A. Representative electrophoretograms of MDR1 and β2 m. Sizes of MDR1 and β2 m were 157 bp and 114 bp, respectively. B. Data were obtained after densitometric analysis of three independent electrophoretograms. Results are expressed as percentages of the *MDR1* mRNA levels in Caco-2 cells. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with Caco-2 cells, respectively. $\dagger P < 0.05$ compared with Caco/DX cells.

In addition to MDR1, many ABC transporters are involved in efflux pump or multidrug resistance. Among these, MRP1 was shown to be similar to MDR1-mediated resistance to a range of structurally and functionally unrelated drugs (Hipfner et al 1999; Leslie et al 2001). This study showed that *MRP1* mRNA was expressed in Caco-2 and in Caco/DX cells, but no induction by digoxin was observed in Caco/DX cells (Figure 6). Kopnin et al (1992) and Shen et al (1986) reported that cell lines selected for high levels of MDR1 often show *MDR1* gene amplification, and cells with low levels of resistance, despite increased levels of *MDR1* mRNA, do not usually show any *MDR1* gene amplification. On the other hand, a report using human lung tumour and normal cells found no *MRP1* gene amplification, despite its high level of expression in a number of lung tumours. This suggested that *MRP1* gene amplification in human tumours was not likely to be a common mechanism of MRP1 over-expression

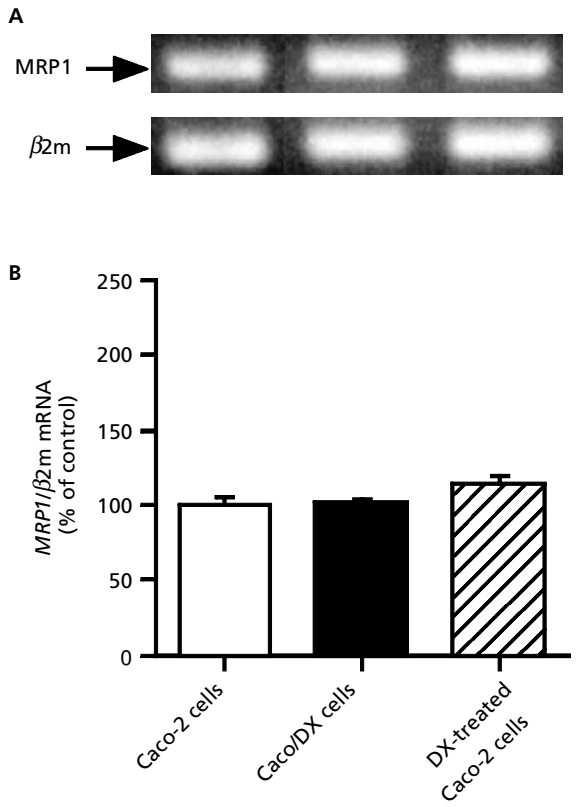

Figure 6 Expression of *MRP1* mRNA in Caco-2 and Caco/DX cells. Total RNA was extracted from Caco-2 and Caco/DX cells, and the levels of *MRP1* mRNA were measured by RT-PCR. A. Representative electrophoretograms of MRP1 and β2 m. Sizes of MRP1 and β2 m were 181 bp and 114 bp, respectively. B. Data were obtained after densitometric analysis of three independent electrophoretograms. Results are expressed as percentages of the *MRP1* mRNA levels in Caco-2 cells.

(Giaccone et al 1996). These observations suggested that the *MDR1* gene rather than the *MRP1* gene might be susceptible to amplification. Thus, continuous exposure to 100 nM digoxin was considered to predominantly induce expression of MDR1 but not MRP1.

Recently, Synold et al (2001) reported that MDR1 expression was regulated by the orphan nuclear receptor, steroid and xenobiotic receptor (SXR). Some steroids, rifampicin and paclitaxel but not docetaxel bind to SXR as ligands, and stimulate transcription of the *MDR1*. Moreover, ouabain was reported to up-regulate the MDR1 expression via the increase of cytosolic calcium concentration (Brouillard et al 2001). Digoxin includes a steroid structure in its molecular structure, being similar to that of ouabain. Considering these reports, digoxin might regulate MDR1 expression via SXR and/or a mechanism similar to that of ouabain. Therefore, Caco/DX cells may be a useful tool for clarifying the mechanism of the induction of MDR1 by digoxin.

It has been reported that MDR1 expression in the intestine influences the absorption capacity of drugs that are substrates for MDR1 in man (Greiner et al 1999; Johne et al 1999; Hoffmeyer et al 2000). The serum concentrations of digoxin in man are usually approximately 1 (0.25 mg, p.o.)–30 nM (1 mg, i.v. infusion) (Greiner et al 1999; Johne et al 1999). The concentration of digoxin used here (100 nM) was higher than its serum concentration. However, the concentration of digoxin in the intestine when administered orally is considered to reach at least 100 nM. Therefore, it was speculated that MDR1 expression in the intestine of patients with long-term oral administration of digoxin might be higher than that before its administration. The induction of MDR1 by long-term treatment with digoxin might substantially alter the pharmacokinetic profiles of both itself and of co-administered drugs that are substrates for MDR1. Moreover, this induction might be relevant to treatment efficacy and adverse effects of digoxin. It was consequently considered that the induction of MDR1 in the intestine by digoxin would be particularly important in clinical situations. However, at present there is no supportive clinical evidence, so in-vivo studies are essential to substantiate our fundamental findings in-vitro.

Conclusion

These observations confirmed that continuous exposure to digoxin, as well as the transient exposure, induced MDR1 in Caco-2 cells. This MDR1-over-expressing Caco-2 subline, Caco/DX, was a suitable system for examination of the mechanism of MDR1 induction by digoxin and the novel types of drug–drug interactions via up-regulation of MDR1.

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