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Decreased expression of P-glycoprotein during differentiation in the human intestinal cell line Caco-2

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Abstract

The expression profile of the multidrug resistance (*MDR*) *1* gene product P-glycoprotein (Pgp) was examined during culture using Caco-2 cells as an *in vitro* model. Levels of MDR1 and cyclooxygenase 2 mRNA expression in Caco-2 cells were the highest on day 3 and decreased with days in culture, but the level of cyclooxygenase 1 was stable throughout the culture period. The stability of MDR1 mRNA was 7-fold higher on day 3 than on day 9, and the run-on assay suggested the transcription rate of the *MDR1* gene on day 3 tended to be higher than on day 9. In addition, the expression of Pgp was comparable with that of MDR1 mRNA, but was inversely correlated with villin expression. The Pgp-mediated tacrolimus transport was the highest on day 1 and the lowest on day 11. These results suggested that the changeable mRNA stability rather than transcription rate of MDR1 contributed to its up-regulation during cell proliferation and down-regulation after post-confluent differentiation in Caco-2 cells. Therefore, the temporal induction and subsequent down-regulation of the enterocyte Pgp could affect bioavailability of several drugs during the regeneration of the intestinal wall.

Keywords: Multidrug resistance; Immunosuppressant; Transporter; Small intestine; Transplantation; Tacrolimus

1. Introduction

The *MDR* gene product Pgp is highly expressed in the plasma membranes of several types of tumor cells and acts as an ATP-driven efflux pump of various antitumor agents [1]. However, Pgp is also expressed in the plasma membrane in several normal tissues, such as the brain, liver, kidney, and small intestine [2]. In the small intestine, Pgp is localized in the brush-border membranes and mediates extrusion of structurally unrelated drugs [3], including the immunosuppressants tacrolimus and cyclosporine A [4]. In addition, the tissue distribution and oral bioavailability of various xenobiotics, including tacrolimus, were markedly enhanced in Pgp-deficient mice [5,6]. Therefore, Pgp is considered to act as an absorptive barrier for orally administered drugs.

The expression of hepatic Pgp was rapidly up-regulated during regeneration within 24 hr after partial hepatectomy [7]. Recently, we found that the level of MDR1 mRNA expression in the small intestine was enhanced during regeneration of the graft intestine after rejection in a recipient of living-donor small bowel transplantation [8]. In this case, a high dose of tacrolimus was required to elevate blood levels to within the therapeutic range, because the absorption rate of tacrolimus was suggested to be reduced by the enhanced expression of the intestinal absorptive barrier Pgp. Therefore, we hypothesized that the expression level of enterocyte Pgp was higher in the proliferation than that in the quiescent state, and the transporter acted as a xenobiotic efflux pump preventing cytotoxicity during tissue regeneration after injury.

In this study, we examined whether the changes in Pgp expression were related to cell proliferation and whether the expression level corresponded to the activity as an efflux pump using Caco-2 cells as an *in vitro* model.

2. Materials and methods

2.1. Materials

[*methyl*-^{3}H]Thymidine (3070 GBq/mmol), [5,6-^{3}H]uridine (1.41 TBq/mmol), and [α-^{32}P]UTP (111 TBq/mmol) were obtained from Amersham Biotech and dihydro-

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Abbreviations: MDR, multidrug resistance; Pgp, P-glycoprotein; COX, cyclooxygenase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PCR, polymerase chain reaction.

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[^{3}H]FK506 (3219 GBq/mmol) was obtained from PerkinElmer Life Science Products. All other chemicals were of the highest purity available.

2.2. Cell culture and proliferation

Caco-2 cells obtained from the American Type Culture Collection (ATCC HTB-37) were cultured in complete medium consisting of Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum (Whittaker Bioproducts) and 1% nonessential amino acid (Invitrogen) in an atmosphere of 5% CO_2/95% air at 37°. Caco-2 cells were seeded on 35-mm plastic culture dishes at a density of 2.0×10^5 cells in 2 mL of complete culture medium throughout the experiments. In the present study, the Caco-2 cells were used between passages 35 and 49.

Cell proliferation was assessed by measuring cell number and DNA synthesis. DNA synthesis was determined by [^{3}H]thymidine incorporation according to the method of Halline *et al*. [9], with some modifications. Caco-2 cells were incubated with complete medium supplemented with [^{3}H]thymidine (92.5 kBq/mL) at 37° in an atmosphere containing 5% CO_2/95% air for 4 hr. After washing, the cells were denatured in 5% trichloroacetic acid for 15 min. The cells were solubilized in 0.5 N NaOH, and neutralized by addition of 1 N HCl. The precipitate was centrifuged at 10,000 *g* for 15 min at 4°, and the pellet was resuspended in 0.5 N HCl and hydrolyzed at 95° for 15 min. Aliquots were placed in a liquid scintillation counter, and small aliquots were analyzed for DNA content by spectrometry. Cell proliferation is expressed as dpm/μg DNA.

2.3. Competitive polymerase chain reaction (PCR)

mRNA expression was quantified by competitive PCR as described previously [8]. The total RNA fraction of Caco-2 cells was extracted using RNeasy spin columns (Qiagen Gmbh). Aliquots of 1 μg of total RNA were reverse transcribed using Superscript™II reverse transcriptase (Invitrogen). The single-stranded DNA was used for subsequent competitive PCR. The primer sets and PCR conditions specific for MDR1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were as described previously [8]. The primers and PCR conditions specific for cyclooxygenase (COX) 1 and COX2 were as described by Newton *et al*. [10].

Separation and quantification of the amplified PCR products were performed according to the method of Siebert and Larrick [11]. Following competitive PCR for GAPDH with the same batch of single-stranded DNA used to detect the cellular mRNA of MDR1, COX1, and COX2, the densitometry data were normalized for each batch of RNA by correcting the amount of GAPDH as an internal control.

2.4. Measurement of RNA stability and run-on assays

The sensitivity to transcriptional inhibition by actinomycin D of Caco-2 cells was tested according to the methods of Muller *et al*. [12]. Caco-2 cells were plated in 35-mm dishes and incubated at 37° in fresh medium containing various concentrations of actinomycin D (0.1–50 μg/mL) and 74 kBq [5,6-^{3}H]uridine for 3 hr. After [5,6-^{3}H]uridine labeling, cells were washed and harvested, and the incorporation of [5,6-^{3}H]uridine expressed as trichloroacetic acid-precipitable counts/min was determined.

For RNA stability studies, cells were incubated at 37° for various periods in the presence of actinomycin D (10 μg/mL). Total RNA was then extracted as described above, and each MDR1 or GAPDH expression level was determined by competitive PCR with competitor DNA of 100 zmol/assay for MDR1 at day 3, 25 zmol/assay for MDR1 at day 9, and 500 zmol/assay for GAPDH, respectively. The data were expressed as the band density ratio of mRNA to competitor.

The isolation of nuclei and run-on assays were performed according to the methods of Suruga *et al*. [13]. Briefly, the nuclei of Caco-2 cells (1×10^7 nuclei/100 μL) were incubated for 60 min at 30° with [α-^{32}P]UTP (3.7 MBq/reaction), and the labeled RNA was isolated and measured by scintillation counting. The RNA samples (≥1,000,000 cpm) were hybridized to Hybond N+ nylon membranes containing 2 μg of fixed cDNA probes; the full-length MDR1 cDNA (kindly provided by Dr. K. Ueda, Kyoto University) and the gel-purified PCR products amplified with the primer set specific for GAPDH as described above.

2.5. Western blotting

The crude membrane fractions from the Caco-2 cells were isolated as previously described [14]. The protein expression levels of MDR1 and villin, which was used as a marker of the differentiation of the Caco-2 cells [15], in each crude plasma membrane fraction (50 μg/lane) were evaluated by Western blotting. Western blotting using C219 monoclonal antibody (CIS Bio International) for MDR1 was performed as described [16], and a polyclonal antibody for villin (Santa Cruz Biotechnology, Inc.) was used according to the manufacturer's instructions.

2.6. Drug transport study

The accumulation of dihydro-[^{3}H]FK506 was measured in cells grown on 35-mm dishes. After removal of the culture medium, cells were washed once with Dulbecco's PBS, and preincubated for 30 min in PBS containing 5 mM D-glucose supplemented with or without 100 μM unlabeled verapamil, a potent inhibitor of Pgp [17]. After preincubation, the cells were incubated with dihydro-[^{3}H]FK506 (2 nM, 6.4 kBq/mL) in the presence or absence of 100 μM verapamil at 37° for 1 hr. After incubation, the drug solution was aspirated

off, and the cells were washed once with ice-cold PBS containing 3% BSA and twice with ice-cold BSA-free PBS. The cells were solubilized in 0.5 N NaOH, and the cell-associated radioactivity was determined in ACSII (Amersham Biotech) by liquid scintillation counting. The protein content of the cells was determined by the method of Bradford [18], using a Bio-Rad protein assay kit (Bio-Rad) with bovine γ-globulin as the standard. The total accumulation of dihydro-[^{3}H]FK506 was determined for the radiolabeled drug alone. The Pgp-depressed accumulation of dihydro-[^{3}H]FK506 was determined in the presence of 100 μM verapamil. Then, the Pgp-mediated efflux of the drug was determined from the difference between the values for total and Pgp-depressed accumulation [19].

2.7. Statistical analysis

Statistical analysis was performed by Student's *t*-test. Provability values of <0.05 were considered significant

3. Results

3.1. Expression of MDR1 mRNA in Caco-2 cells during culture

Figure 1 shows the [^{3}H]thymidine incorporation and cell number during culture. [^{3}H]Thymidine incorporation was greatest in pre-confluent cells on day 3, when the cell number was still low. The incorporation decreased upon reaching confluence. In contrast, the number of Caco-2 cells increased linearly on reaching confluence (Fig. 1). These observations indicated that DNA synthesis was the most active and the cells showed the highest rate of proliferation on day 3.

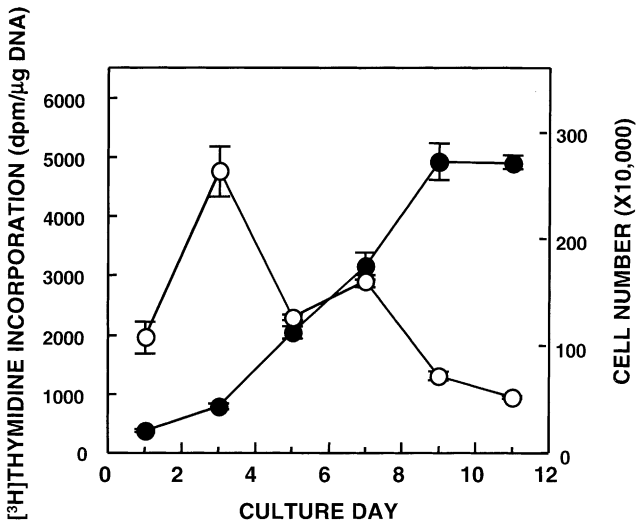

Fig. 1. Proliferation in Caco-2 cells. Cell proliferation was evaluated based on [^{3}H]thymidine incorporation (○) and cell number (●). The amount of [^{3}H]thymidine incorporated into the DNA was determined by measuring radioactivity. Data are shown as dpm/μg DNA. Each point represents the mean ± SE of three experiments.

Next, we quantified the mRNA expression levels of MDR1 in Caco-2 cells by competitive PCR every other day for 11 days after seeding using the mRNA expression level of GAPDH as an internal control. Figure 2A shows typical results of electrophoresis of the amplified PCR products for each gene in the pre-confluent and post-confluent states. For the positive control of the constitutive and changeable mRNA expression during the pre-confluent and post-confluent states, we chose two mRNAs transcribed from *COX1* and *COX2* to compare the expression profile of MDR1 mRNA in Caco-2 cells. The results of quantification of mRNA expression are shown in Fig. 2B. The mRNA expression levels of MDR1 were the highest on day 3 after seeding. On days 9 and 11, the mRNA expression of MDR1 was decreased to 20% of that on day 3. Similarly, the mRNA expression level of COX2 was the highest on day 3, and decreased rapidly on day 5. In contrast, the mRNA expression of COX1 was maintained at a low level throughout the culture periods.

3.2. Experiments on the stability and transcription rate of the MDR1 mRNA

The peak in expression of MDR1 mRNA on day 3 in Caco-2 cells could be due to a modification of the stability of transcripts and/or to a change in the transcription rate of the *MDR1* gene. To investigate the two mechanisms, transcript stability and run-on transcription assays of MDR1 were performed. Because the sensitivity of cells on days 3 and 9 to actinomycin D was unknown, a pilot experiment was performed to determine the concentrations of actinomycin D necessary to adequately inhibit transcription in Caco-2 cells. Both cells at days 3 and 9 were about equally sensitive to transcriptional inhibition by actinomycin D (Fig. 3A). From these results, 10 μg/mL actinomycin D was used in the stability experiments.

As shown in Fig. 3B, the disappearance of MDR1 mRNA with time was then measured by competitive PCR with a competitor concentration of 100 zmol/assay for the RNA from cells on day 3 and 25 zmol/assay for the RNA from cells on day 9. The stability of MDR1 mRNA on day 3 was significantly higher than that on day 9 with half-lives of 21.0 ± 2.5 hr and 3.3 ± 0.2 hr (mean ± SE of three independent cultures, $P < 0.01$), respectively. However, the disappearance of the GAPDH mRNA/μg total RNA with time was not observed, and the stability of the mRNA was very similar between days 3 and 9 (Fig. 3C). The run-on assay indicated that the rate of *MDR1* gene transcription appeared to be a little higher in nuclei from pre-confluent cells on day 3 than in nuclei from post-confluent cells on day 9 ($P = 0.046$) (Fig. 4).

3.3. Detection of Pgp

To examine whether the expression profile of MDR1 mRNA was comparable with that of the protein, we

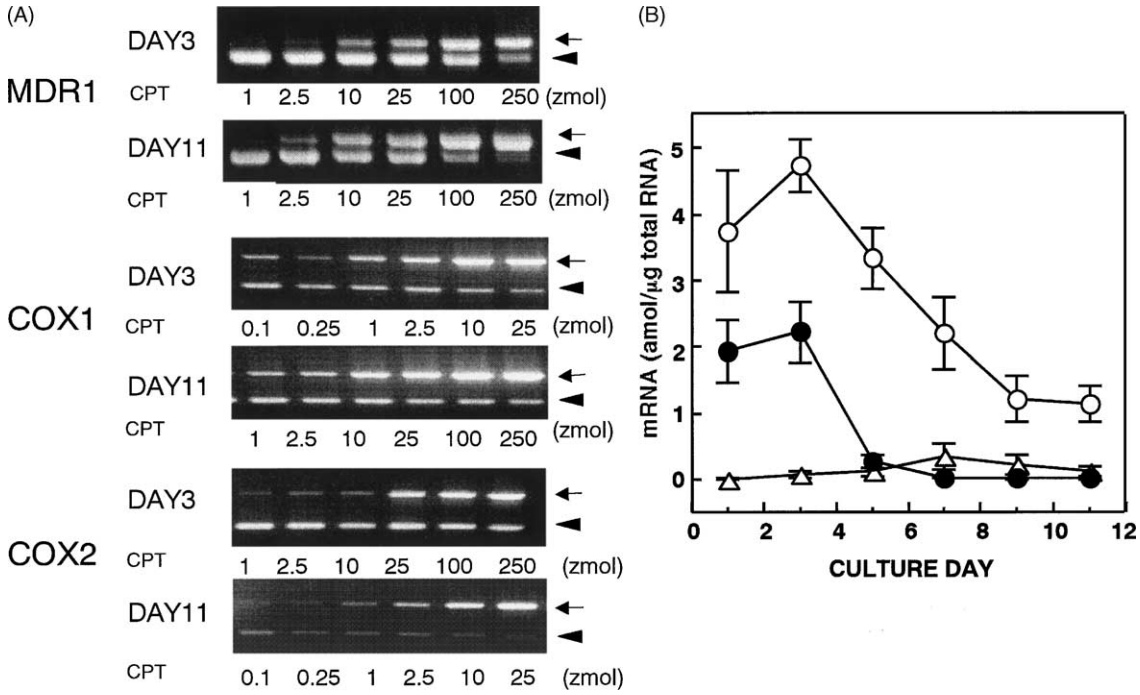

Fig. 2. Quantification of mRNA expression in Caco-2 cells. Total RNA extracted from Caco-2 cells was reverse transcribed. (A) Representative results of competitive PCR specific for MDR1, COX1, and COX2 on days 3 and 11 were shown, respectively. The arrow and arrowhead indicate the bands derived from competitor DNA and single-stranded DNA of Caco-2 cells, respectively. The number under each PCR-band shows the amount of competitor DNA applied in the PCR reactions. CPT, competitor; zmol, 1×10^{-21} mol. (B) MDR1 (○), COX1 (△), and COX2 (●) mRNA expressions were quantified by competitive PCR using specific primers and competitors for each mRNA. No significant differences were observed in GAPDH expression throughout the culture period. Each point represents the mean ± SE of three separate experiments within three passages. Each experiment was performed in duplicate.

performed Western blot analysis using C219 monoclonal antibody as the primary antibody for Pgp. Figure 5 shows the expression profile of Pgp in comparison with that of villin. Although a band of about 70 kDa was equally observed between days 1 and 11, no other signal corresponding to another ABC transporter was detected in the blot using C219 monoclonal antibody. The expression of Pgp was high on days 1 and 3 and decreased with time. In contrast, the expression of villin increased with time.

3.4. Transport experiment

Since Pgp is considered to have a role in the detoxication of xenobiotics, it is necessary to clarify whether Pgp also acts as an efflux pump in pre-confluent cells. We examined the Pgp-mediated dihydro-[^{3}H]FK506 transport in Caco-2 cells as a function of time in culture, using verapamil as an inhibitor of the transporter. As shown in Fig. 6A, the accumulation of dihydro-[^{3}H]FK506 in the Caco-2 cells peaked on day 1 and decreased with time in culture. In addition, the efflux was also the greatest on day 1 (Fig. 6B).

4. Discussion

There have been several reports concerning the up-regulation or induction of Pgp expression in the regenerating liver [7,20]. However, there have been no previous reports on the regulation of Pgp expression in enterocytes during tissue regeneration. Recently, we suggested that the enhanced Pgp expression in small intestinal biopsy specimens was responsible for the reduction of the bioavailability of tacrolimus in a living-donor small bowel recipient during regeneration after rejection [8]. Therefore, we hypothesized that the level of Pgp expression was enhanced in enterocytes as well as hepatocytes during the proliferation, and the highly expressed Pgp acted as an absorptive barrier to orally administered drugs. In the present study, we examined the relation between Pgp expression and cell proliferation using a cultured human intestinal cell line, Caco-2, as an *in vitro* model.

The expression profile of MDR1 was related with the cell proliferative activity at both the mRNA and protein levels (Figs. 1, 2, and 5). In addition, the half-life of the MDR1 mRNA in Caco-2 cells on day 3 was approximately 7-fold longer than that on day 9 (Fig. 3B), and the transcriptional rate of MDR1 mRNA in the Caco-2 cells on day 3 was a little higher than on day 9 (Fig. 4). These results indicated that the high expression level of MDR1 mRNA during the proliferation of Caco-2 cells was mainly regulated by the mRNA stability rather than its transcription rate. In contrast, the expression of villin was correlated with the number of cells (Figs. 1 and 5). Hodin *et al.* [21] reported that the villin mRNA expression was transcriptionally regulated during post-confluent differentiation in Caco-2 cells. These results indicated that the expression of

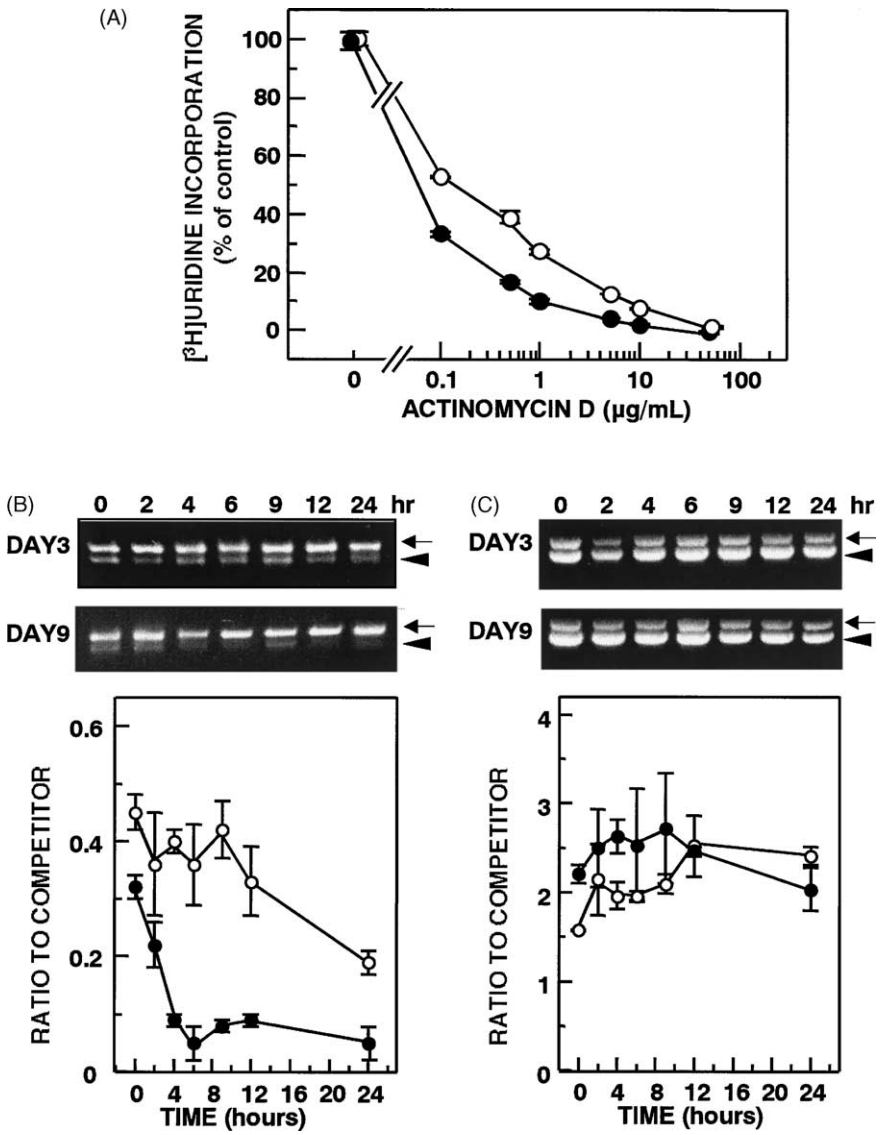

Fig. 3. Sensitivity of transcription to inhibition by actinomycin D in Caco-2 cells (A) and stability of MDR1 mRNA (B) and GAPDH mRNA (C). (A) The incorporation of [5,6-^{3}H]uridine into acid-insoluble counts/min was assessed in actinomycin D-treated Caco-2 cells on day 3 (○) or on day 9 (●) as described under "Section 2". Values are expressed as a percentage of controls labeled in the absence of actinomycin D. (B) and (C) The stability of MDR1 mRNA (B) and GAPDH mRNA (C) derived from actinomycin D-treated (10 μg/mL) Caco-2 cells was determined as outlined under "Section 2". Representative results of competitive PCR analyses of RNAs isolated from cells treated with actinomycin D for various times are shown at the top of each panel. The single-stranded DNAs from cells on day 3 (○) or day 9 (●) were used for subsequent competitive PCR with competitor DNA at 100 zmol/assay for MDR1 on day 3, 25 zmol/assay for MDR1 on day 9, and 500 zmol/assay for GAPDH, respectively. The arrow and arrowhead indicate the bands derived from competitor DNA and single-stranded DNA of Caco-2 cells, respectively. The results of densitometric scans of these photographs are shown as graphs at the bottom of each panel. Data were expressed as the densitometric ratio of the bands between derived from the single-stranded DNA and the competitor DNA in Caco-2 cells. Each value represents the mean ± SE of three experiments.

enterocyte Pgp was mainly enhanced in Caco-2 cells by increased mRNA stability in the proliferating state, and was reduced accompanying the cell differentiation. To our knowledge, this is the first report that the high expression level of Pgp in a cell line serving as a model for intestinal cells during proliferation is down-regulated with differentiation.

In our previous study, the up-regulated enterocyte Pgp was suggested to prevent the absorption of orally administered tacrolimus [8]. In the Caco-2 cells, we also found that the efflux of tacrolimus was greater in the pre-confluent cells than in the post-confluent cells (Fig. 6B). These data suggested that the up-regulation of MDR1 observed in mRNA level as well as protein level was reflected in the enhanced Pgp-mediated tacrolimus transport activity. Although the efflux activity of dihydro-[^{3}H]FK506 in the Caco-2 cells peaked on day 1, the accumulation of dihydro-[^{3}H]FK506 was also the highest on day 1. In addition, Pgp was found to be distributed in the cytoplasm as well as in the apical membrane in the proliferating Caco-2 cells by confocal microscopy (data not shown). These data suggested the possibility of the accumulation of

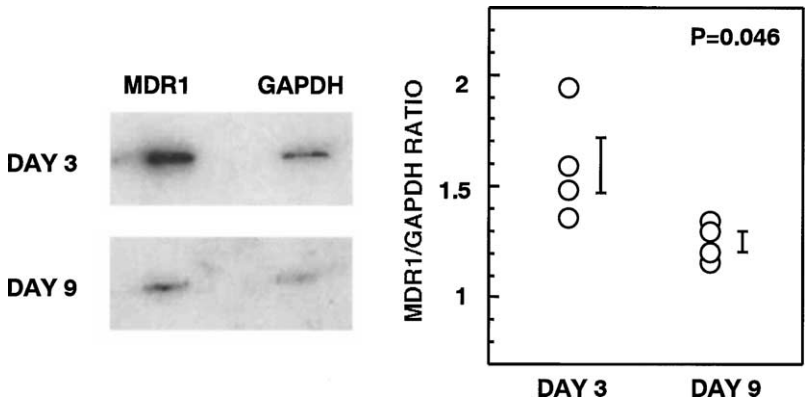

Fig. 4. Relative transcription rates of the *MDR1* gene in Caco-2 cells at days 3 and 9 of culture. Cells at day 3 or 9 of culture were harvested, nuclei were isolated, and the transcription rate was determined by run-on assays. After incubation of nuclei in the presence of [α-^{32}P]UTP, labeled RNA was purified and hybridized to dot-blots containing 2 μg of MDR1 cDNA or the purified PCR product of GAPDH as described under "Section 2". A representative autoradiograph is shown at the left. The quantification of signals was performed by densitometric analysis. Results were expressed as the densitometric ratio of MDR1 to GAPDH on day 3 or 9. Each bar represents the mean ± SE of four experiments.

tacrolimus in the cytoplasmic organelles. Molinari *et al.* [22] demonstrated that doxorubicin was accumulated in the Pgp-positive Golgi apparatus and was prevented to reach into the nuclei, and the accumulation of doxorubicin in the cytoplasm was enhanced in the presence of cyclosporine A with the functional inhibition of Pgp in the melanoma cell lines. Shapiro *et al.* [23] also showed as the drug sequestration site in the drug resistance tumor cells Pgp-containing cytoplasmic vesicles, in which the Pgp was oriented so that drugs are transported and concentrated in the interior of the vesicles. These data and our findings suggested that the Pgp-positive cytoplasmic organelles functioned partly as an accumulation site to prevent the accumulation of drugs or xenobiotics into the nuclei. Therefore, the intracellular Pgp might play roles in not only the intrinsic drug resistance but the detoxication by accumulating xenobiotics into the cytoplasmic organelles in addition to the efflux activity at the plasma membrane.

COX1 is constitutively expressed whereas COX2 is an inducible [24]. In some gastrointestinal cancer cells highly expressing COX2, including Caco-2 cells, the enzyme was considered to participate in cell proliferation [25,26]. In addition, Erickson *et al.* [27] demonstrated that the COX2-selective inhibitor SC-58125, but not the COX1-selective inhibitor VSA, inhibited [^{3}H]thymidine incorporation in the rat intestinal cell line IEC-18. Since Pgp is overex-

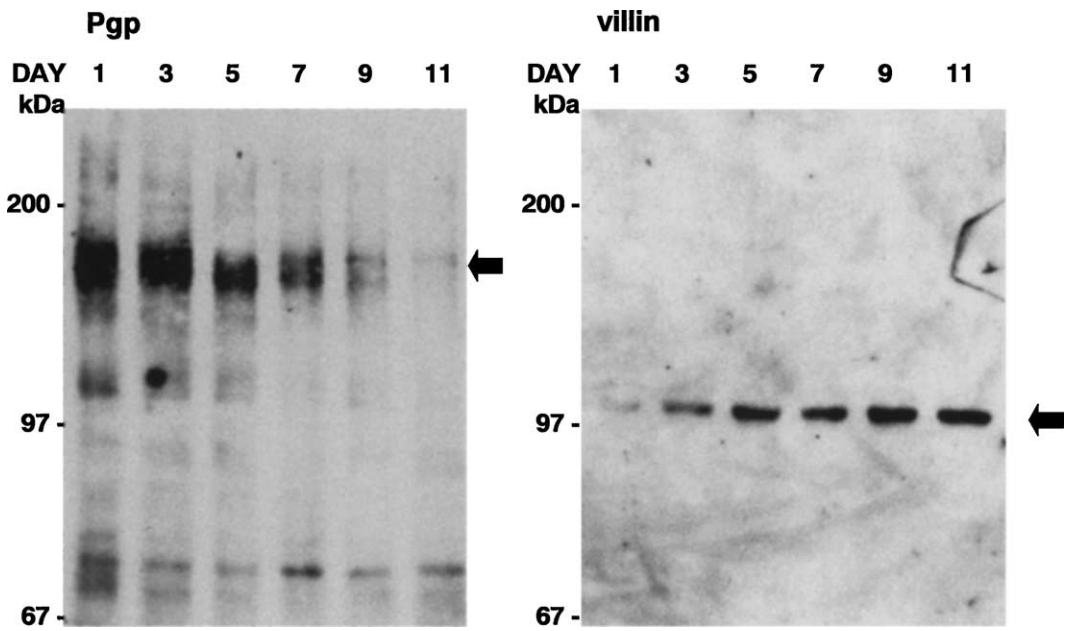

Fig. 5. P-glycoprotein and villin expression in Caco-2 cells. Aliquots of 50 μg of the crude plasma membrane fractions isolated from Caco-2 cells were separated by SDS–polyacrylamide gel electrophoresis. Pgp expression was detected using C219 monoclonal antibody. The blot was stripped and used for detection of villin expression using a polyclonal antibody for villin.

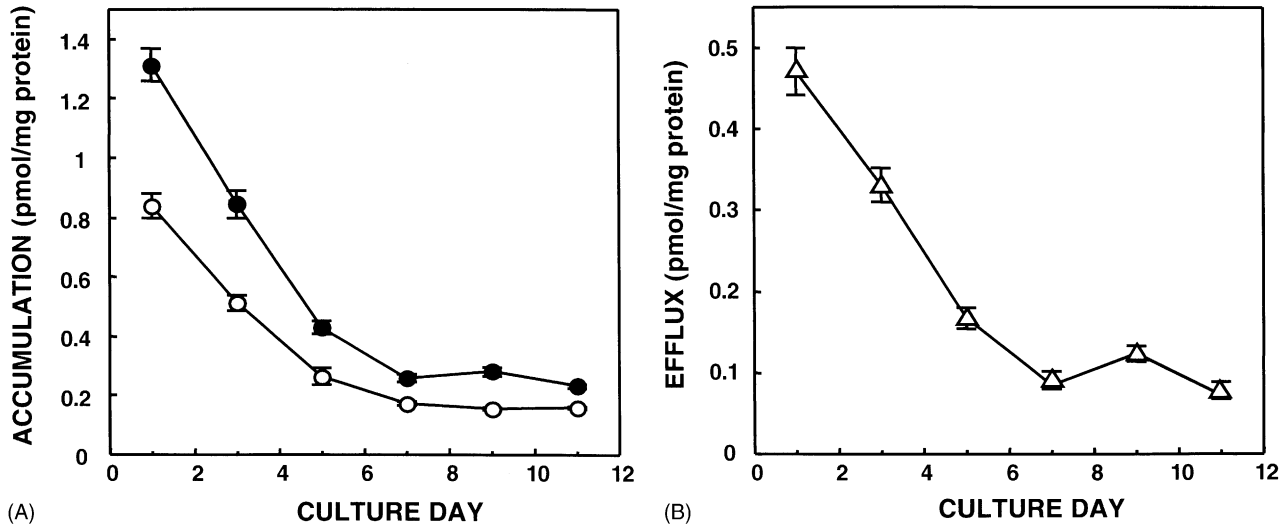

Fig. 6. P-glycoprotein-mediated dihydro-[^{3}H]FK506 transport in Caco-2 cells during culture. Caco-2 cells were incubated with [^{3}H]FK506 for 1 hr at 37° with or without 100 μM verapamil as a potent inhibitor. (A) Accumulation of dihydro-[^{3}H]FK506 with (●) or without (○) verapamil. (B) The efflux activity of dihydro-[^{3}H]FK506 *via* P-glycoprotein was determined by subtracting the accumulation of dihydro-[^{3}H]FK506 without verapamil from that with verapamil. Each point represents the mean ± SE of two to four separate experiments. Each experiment was performed in triplicate.

pressed in several cancer cell lines [28], the expression profile of COX2, but not COX1, would be correlated with that of Pgp. As expected, the mRNA expression levels of Pgp and COX2 were the highest on day 3, when the Caco-2 cells showed marked DNA synthesis activity (Figs. 1 and 2). However, the mRNA expression level of COX1 was little changed throughout the culture period. These data suggested that a similar regulatory mechanism(s), including mRNA stability and transcription rate, controls the expression of Pgp and COX2. Recently, Zieman *et al*. [29] demonstrated that structurally different COX inhibitors (indomethacin, meloxicam, NS-398) down-regulated not only the endogenous mdr1b mRNA expression but EGF-dependent overexpression in primary rat hepatocyte culture. This report concluded that the release of prostaglandins through activation of COX system participated in endogenous *mdr1b* gene regulation. Considering these reports and our present finding, although it is not clear whether *COX2* and *MDR1* gene expression is regulated by the same mechanism or associated mechanism, the COX2 expression profile is comparable with MDR1 in the intestine. The expression levels of intestinal Pgp and COX2 may be good markers for evaluating the state of enterocyte; i.e. proliferating/carcinogenic or differentiated/constitutive. Furthermore, characterization of the mechanism(s) of inducing the expression of both genes may provide new targets for treatment of gastrointestinal cancers.

In the small intestine, the expression of Pgp is detected mainly at the villi, in what are thought to be differentiated enterocytes [30], but not in the crypt where intestinal stem cells proliferate without differentiating. Enterocytes migrate from the proliferating crypt to the villus tip with differentiation [31]. However, the Pgp expression in the graft intestine was temporally enhanced during regeneration after rejection in a recipient of living-donor small bowel transplantation [8]. In the present study, we also found that the Pgp expression was correlated with the proliferative activity of Caco-2 cells. In addition, the mRNA stability and increased transcription rate appeared to result in the higher expression level of MDR1 mRNA in the Caco-2 cells on day 3 (Figs. 3 and 4). The present results and our previous findings suggested that MDR1 expression is greater in the proliferating enterocytes than in the differentiated cells, especially during regeneration. Fan *et al*. [32] demonstrated that the expression level of mdr1 mRNA and Pgp are inversely related to the cell density and corresponded to the proliferation, and the high level expression of mdr1 reduced the chemosensitivity for the anticancer drugs in the murine CT-26 colon carcinoma cell line. This report and our present results imply that there are some differences in the regulation and/or the mechanism(s) for the formation of intestine between the regeneration and the development of tissue, including the enterocyte Pgp expression profile.

In summary, we found that the intestinal down-regulation of Pgp was accompanied by cell differentiation in the Caco-2 cell line, and this regulation reflected the function of Pgp as an absorptive barrier in the detoxication process. The Caco-2 cells might be a good *in vitro* model for studying the regulation of Pgp during tissue regeneration.

Acknowledgments

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