Gene Expression Profiles of ABC Transporters and Cytochrome P450 3A in Caco-2 and Human Colorectal Cancer Cell Lines

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Purpose. The mRNA levels of MDR1 (P-glycoprotein), multidrug resistance-associated proteins (MRP1, MRP2), cytochrome P450 3A (CYP3A) and villin in human colorectal cell lines (HCT-15, LoVo, DLD-1, HCT-116 and SW620) were quantitatively compared with those in Caco-2 cells.

Methods. The mRNA levels were determined by real time quantitative polymerase chain reaction and expressed as the relative concentrations of MDR1 mRNA to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

Results. MDR1 mRNA was expressed in HCT-15 LoVo and DLD-1 cells at similar or lower level to Caco-2. The expression of MRP1 mRNA in the cell lines tested was comparable with Caco-2. MRP2 mRNA was detected only in HCT-116 and SW620 at significantly lower level than Caco-2. CYP3A mRNA was detected in HCT-15, LoVo, DLD-1 and SW620 at similar level to Caco-2.

Conclusions. HCT-15 LoVo and DLD-1 cells express proteins important for regulating the intestinal absorption of drugs, i.e., MDR1, MRP1 and CYP3A, whereas HCT-116 and SW620 cells were not acceptable for evaluation of absorption properties of drug candidates.

KEY WORDS: MDR1; multidrug resistance-associated protein (MRP); villin; *in vitro* screening model.

INTRODUCTION

It has become necessary to rapidly assess the pharmacokinetics and toxicologic properties of large numbers of candidate drugs produced through combinatorial chemistry and high throughput screening for pharmacologic activity. During the last decade, Caco-2 cells have been widely accepted as the most useful *in vitro* model for the rapid screening of intestinal drug absorption (1). The Caco-2 cell line is capable of morphologic and biochemical differentiation *in vitro* to form intestinal epithelium under normal culture conditions, although it was obtained from human colorectal cancer (1–6).

In treating the small intestine, MDR1 (P-glycoprotein) and cytochrome P450 3A4 (CYP3A4) have recently attracted a great deal of attention because of their barrier function against xenobiotics (3,5,7). The contribution of the multidrug resistance-associated protein (MRP) family to drug extrusion also has been demonstrated using Caco-2 cells (8). The cells express these metabolizing enzymes and ATP-binding cassette (ABC) transporters and have been used for evaluation of transport mechanisms. However, their expression levels were low (1,3–5), and a number of studies have been performed to produce monolayer-forming subclones by transfection or induction (9–11). In our previous study, levels of MDR1, MRP1, MRP2, and CYP3A mRNA in Caco-2 cells obtained from several laboratories in Japan were compared with those in human duodenal enterocytes, normal colorectal tissues, and colorectal adenocarcinomas. Caco-2 cells were found to show highly variable mRNA expression profiles of these proteins, presumably depending on the culture and/or passage conditions, and they were more similar to those of human colorectal tissues than human duodenal enterocytes (12). This raised questions regarding whether Caco-2 is the most suitable basic cell line for the development of *in vitro* screening models to predict the oral absorption of drugs.

In the present study, the effects of culture period on the levels of MDR1, CYP3A, and villin mRNA expression in Caco-2 cells were evaluated by real-time quantitative reverse transcription-polymerase chain reaction (12). The mRNA levels of MDR1, MRP1, MRP2, CYP3A, and villin (a constitutively expressed and enterocyte-specific protein) in the human colorectal cancer cell lines HCT-15, LoVo, DLD-1, HCT-116, and SW620 also were compared quantitatively with those in Caco-2 cells.

MATERIALS AND METHODS

Cells and Cell Culture

Caco-2 cells (passage 47) were obtained from the RIKEN Cell Bank (RIKEN RCB0988, Saitama, Japan). HCT-15 (passage 43), LoVo (passage 36), DLD-1 (passage 27), HCT-116 (unknown) and SW620 (passage 99) were purchased from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Caco-2 cells were grown in a culture medium consisting of Dulbecco's modified Eagle's medium (Invitrogen Corp., Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA), 0.1 mM MEM nonessential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). HCT-15 and DLD-1 cells were grown in RPMI1640 medium (Invitrogen) with 10% FBS. LoVo, HCT-116, and SW620 cells were grown in Ham's modified F-12 medium (ICN Biomedicals, Aurora, OH, USA) with 10% FBS, McCoy 5A modified medium (ICN Biomedicals) with 10% FBS, and L-15 modified medium (ICN Biomedicals) with 10% FBS, respectively. Caco-2, HCT-15, LoVo, DLD-1, HCT-116, and SW620 cells seeded at a cell density of 1.0×10^5, 1.7×10^6, 1.6×10^6, 1.2×10^6, 1.4×10^5 and 3.3×10^6 cells, respectively, in 10 ml of complete culture medium in 25 cm^2 culture flasks (Nunclon™ flasks, Nalge Nunc International, Rochester, NY, USA) were grown in an atmosphere of 95% air and 5%

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CO_2 at 37°C, and subcultured every 5–7 days using a mixture of 0.02% EDTA and 0.05% trypsin (Invitrogen).

Reverse Transcription (RT) and Real Time Quantitative Polymerase Chain Reaction (PCR)

To evaluate the effects of culture period on the levels of MDR1, CYP3A, and villin mRNA expression in Caco-2 cells, cell pellets were prepared 6, 14, 21, and 28 days after seeding of 1.0×10^5 cells in 10 ml of complete culture medium in 25 cm^2 culture flasks. The levels of MDR1, MRP1, MRP2, CYP3A, and villin mRNA in the human colorectal cancer cell lines of HCT-15, LoVo, DLD-1, HCT-116, and SW620 were compared quantitatively with those in Caco-2 cells. Caco-2 cell pellets were prepared 6 days after seeding. Cell pellets of HCT-15, LoVo, DLD-1, HCT-116 and SW620 cells were prepared 7 days after seeding of 1.7×10^6, 1.6×10^6, 1.2×10^6, 1.4×10^5 and 3.3×10^6 cells in 10 ml of complete culture medium in 25 cm^2 culture flasks. Total RNA was extracted using an RNeasy Mini kit (Quiagen, Hilden, Germany) and an RNase-Free DNase Set (Quiagen). RT and real-time quantitative PCR were performed as described previously (12). Primers and TaqMan probes were designed using the Primer Express 1.0 program (Applied Biosystems, Foster City, CA, USA). The primers and TaqMan probes for MDR1, MRP1, MRP2, and CYP3A mRNA were reported previously (12), and those for villin mRNA were as follows: forward primer (5′-TGA CCC TGA GAC CCC CAT C-3′), reverse primer (5′-TCA GCA GTG ATC TGG CTC CA-3′), and TaqMan Probe (5′-TTG TGG TGA AGC AGG GAC ACG AGC-3′). Primers and the TaqMan probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Applied Biosystems (TaqMan GAPDH Control Reagent Kit). As the authentic standard in each run of the assay, Caco-2 cells from the RIKEN Cell Bank were used for MDR1, MRP1, MRP2, villin, and GAPDH mRNA. Of the CYP3A subfamily, CYP3A4 is the most abundant in the small intestine, but in the present colorectal cancer cell lines, it is unknown whether the DNA fragment for CYP3A4, CYP3A5, CYP3A7, CYP3A43, or CYP3AP was amplified by the primer sets used. We confirmed the amplification of specific DNA fragment for CYP3A4 in the intestinal biopsy sample obtained from a representative subject by direct sequencing, and used this biopsy sample as the authentic standard for CYP3A (12). The levels of MDR1, MRP1, MRP2, CYP3A, and villin mRNA are expressed as concentrations relative to GAPDH mRNA.

Statistical Analysis

Values are given as the means ± standard error of more than three dishes. Statistical comparisons were performed by one-way analysis of variance followed by Sheffé's test. P values of less than 0.05 (two-tailed) were considered significant.

RESULTS AND DISCUSSION

Caco-2 cells have been used as an *in vitro* model for screening of intestinal drug absorption. The ABC transporters and drug metabolizing enzymes located in the epithelial cells of the small intestine, including MDR1, MRPs, and CYP3A4, have attracted a great deal of attention as molecular barriers for absorption (7,8,13). In our previous investigation, it was found that the levels of MDR1, MRP1, MRP2, and CYP3A mRNA in Caco-2 cells showed relatively large variability depending on culture and/or passage conditions, and were markedly lower than those in human duodenal enterocytes (12). Caco-2 cells can differentiate morphologically and biochemically *in vitro* to form intestinal epithelium under normal culture conditions, and probably undergo marked changes in expression profile (1–6). In the present study, the effects of culture period on the levels of MDR1, CYP3A, and villin mRNA expression in Caco-2 cells were evaluated. The absolute levels of GAPDH mRNA expression were significantly changed in Caco-2 cells 6, 14, 21, and 28 days after seeding: 7.55 ± 0.22, 12.45 ± 0.87, 2.77 ± 0.40, and 0.91 ± 0.10, respectively. This result was consistent with that of the report (8). As shown in Fig. 1, the relative concentration of MDR1 mRNA to GAPDH mRNA was 0.61 ± 0.03 on day 6 and significantly decreased by 85% on day 14, and thereafter remained almost constant up to day 28. Recently, MDR1 has

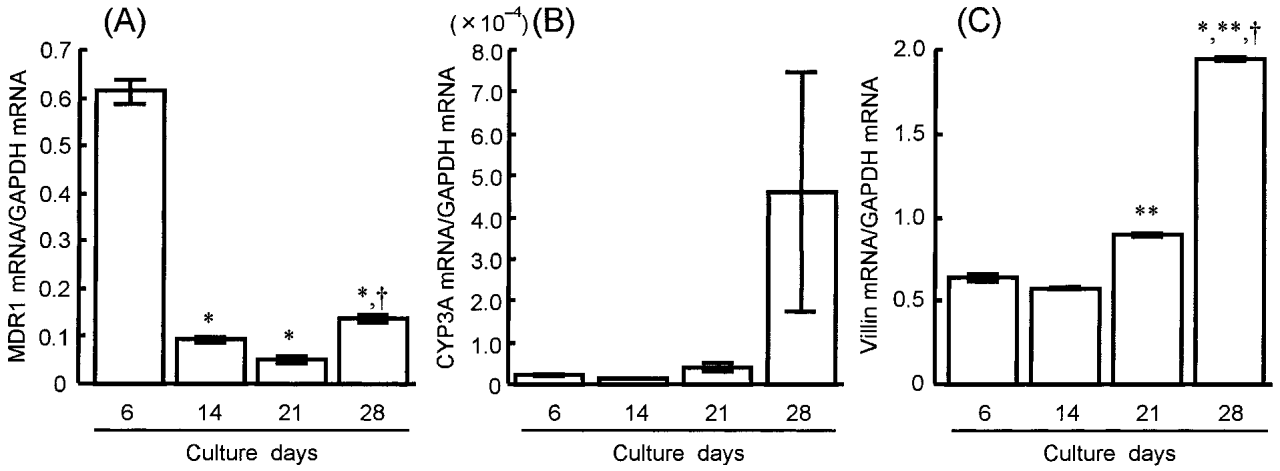

Fig. 1. Relative concentrations of mRNAs for MDR1 (A), CYP3A (B) and villin (C) in Caco-2 cells 6, 14, 21, and 28 days after seeding. Concentrations of MDR1, CYP3A, and villin mRNA relative to GAPDH mRNA were determined by real-time quantitative PCR. GAPDH was selected as an endogenous RNA control to normalize for differences in the amount of total RNA. Each bar represents the average and standard error of the respective relative concentration. *Statistically significant difference ($p < 0.05$) compared with day 6. **Statistically significant difference ($p < 0.05$) compared with day 14. †Statistically significant difference ($p < 0.05$) compared with day 21.

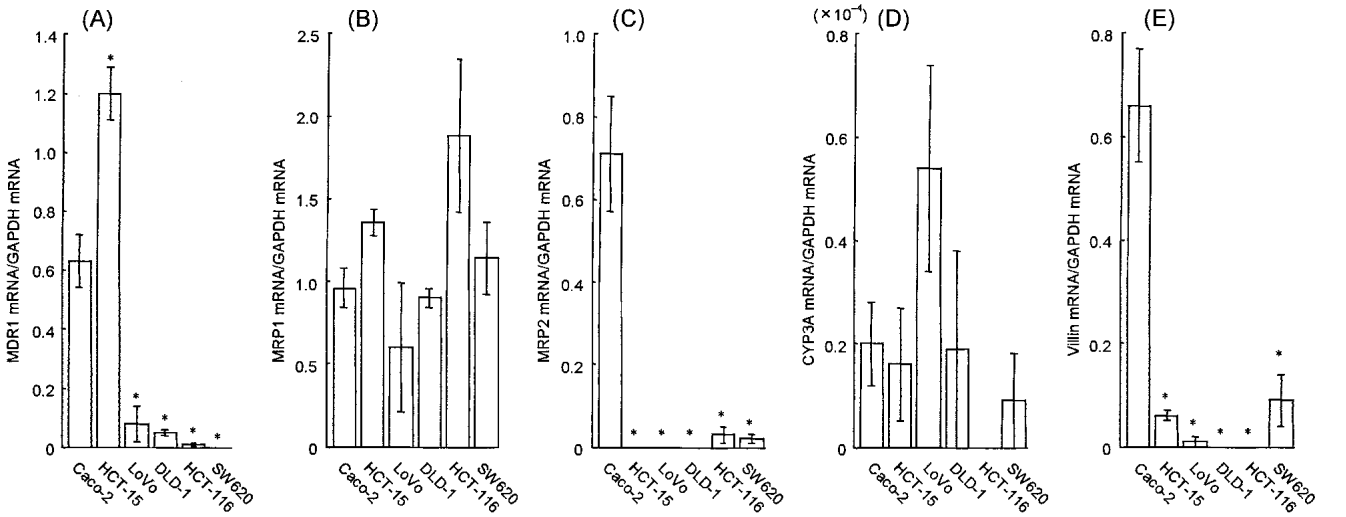

Fig. 2. Relative concentrations of mRNAs for MDR1 (A), MRP1 (B), MRP2 (C), CYP3A (D), and villin (E) in Caco-2, HCT-15, LoVo, DLD-1, HCT-116, and SW620 cells. Concentrations of MDR1, MRP1, MRP2, CYP3A, and villin mRNA relative to GAPDH mRNA were determined by real-time quantitative PCR. GAPDH was selected as an endogenous RNA control to normalize for differences in the amount of total RNA. Each bar represents the average and standard error of the respective relative concentration. *Statistically significant difference ($p < 0.05$) compared with Caco-2 cells

been suggested to play an important role in cell apoptosis and differentiation (14), and this alterations in expression profile might reflect differentiation to form microvillous structures. CYP3A mRNA level in Caco-2 cells increased during culture and reached about 20-fold higher than baseline ($p < 0.05$), i.e., 0.23 ± 0.01 on day 6 and 4.46 ± 2.59 on day 28, although the level was far lower than its standard level in the duodenal enterocytes (12). The relative concentration of villin mRNA in Caco-2 cells on day 6 was 0.64 ± 0.03, which then significantly increased to 1.94 ± 0.01 at day 28, presumably reflecting maturation of the microvillous structure.

Recently, a number of studies using Caco-2 cells as the basic cell line have attempted to produce monolayer-forming subclones by transfection or induction, thereby allowing more accurate prediction of intestinal drug absorption and the mechanisms of action of drugs (9–11,15). In the present study, to search for a basic cell line more suitable for the development of *in vitro* screening models than Caco-2, the gene expression profiles of the above proteins in various human colorectal cancer cell lines were examined. Figure 2 shows the relative concentrations of MDR1, MRP1, MRP2, CYP3A, and villin mRNA in human colorectal cancer cells. The relative concentrations of MDR1 mRNA in the Caco-2, HCT-15, LoVo, DLD-1, HCT-116, and SW620 cells were 0.63 ± 0.09, 1.20 ± 0.09, 0.08 ± 0.06, 0.05 ± 0.01, 0.01 ± 0.01 and 0.00 ± 0.00, respectively, and the highest value was found in the HCT-15 cells. The MRP1 mRNA was detected in all colorectal cancer cells in the present study, and there was no significant difference in its levels of expression between Caco-2 and the other cell lines examined. In contrast, MRP2 mRNA was detected in HCT-116 and SW620 cells but at levels significantly lower than that in Caco-2 cells (0.03 ± 0.02, 0.02 ± 0.01, and 0.71 ± 0.14, respectively). HCT-15, LoVo, and DLD-1 cells had no detectable levels of MRP2 mRNA. HCT-116 cells had no detectable CYP3A mRNA, and the other colorectal cancer cell lines had barely detectable levels of CYP3A mRNA. Although which CYP3A subfamily the DNA fragment was amplified for in these cell lines is not clear, the levels were no more than one thousandth of that in the small intestine. Villin mRNA also was evaluated in these cell lines and showed relative concentrations of 0.66 ± 0.11, 0.06 ± 0.01, 0.01 ± 0.01, 0.00 ± 0.00, 0.00 ± 0.00, and 0.09 ± 0.05 in Caco-2, HCT-15, LoVo, DLD-1, HCT-116, and SW620, respectively.

In summary, Caco-2, HCT-15, LoVo and DLD-1 cells express proteins important for regulating the intestinal absorption of drugs, i.e., MDR1, MRP1, and CYP3A, whereas DLD-1, HCT-116 and SW620 cells were not acceptable for evaluation of absorption properties of drug candidates. It is important to check the expression levels of these proteins when using Caco-2 cells, as they were susceptible to down-regulation or upregulation, presumably depending on the culture and/or passage conditions. The levels of expression of these molecules in Caco-2 cells were markedly lower than those in human duodenal enterocytes. Development of *in vitro* monolayer-forming subclones of Caco-2 cells with higher levels of these important proteins is required to allow more-accurate estimation of intestinal drug absorption.

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