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Regular Article

Development of Caco-2 cells co-expressing CYP3A4 and NADPH-cytochrome P450 reductase using a human artificial chromosome for the prediction of intestinal extraction ratio of CYP3A4 substrates

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ABSTRACT

The Caco-2 cells co-expressing cytochrome P450 (CYP) 3A4 and NADPH-cytochrome P450 reductase (CPR) were developed using a human artificial chromosome (HAC) vector. The CYP3A4 and CPR genes were cloned into the HAC vector in CHO cells using the Cre-loxP system, and the microcell-mediated chromosome transfer technique was used to transfer the CYP3A4-CPR-HAC vector to Caco-2 cells. After seeding onto semipermeable culture inserts, the CYP3A4-CPR-HAC/Caco-2 cells were found to form tight monolayers, similar to the parental cells, as demonstrated by the high transepithelial electrical resistance (TEER) value and comparable permeability of non-CYP3A4 substrates between parent and CYP3A4-CPR-HAC/Caco-2 cell monolayers. The metabolic activity of CYP3A4 (midazolam 1′-hydroxylase activity) in the CYP3A4-CPR-HAC/Caco-2 cells was constant from 22 to 35 passages, indicating that HAC vectors conferred sufficient and sustained CYP3A4 activity to CYP3A4-CPR-HAC/Caco-2 cells. The strong relationship between the metabolic extraction ratios (ER) obtained from the CYP3A4-CPR-HAC/Caco-2 cells and calculated intestinal extraction ratios in humans (*Eg*) from reported intestinal availability (*Fg*) was found for 17 substrates of CYP3A4 ($r^2 = 0.84$). The present study suggests that the CYP3A4-CPR-HAC/Caco-2 cell monolayer can serve as an *in vitro* tool that facilitates the prediction of intestinal extraction ratio (or availability) in humans.

1. Introduction

Oral administration is one of the most common and convenient routes for drug delivery into systemic circulation or to target organs. Before reaching systemic circulation, orally administered drugs are absorbed at the intestine, and pass the intestinal and hepatic metabolism. To select or prioritize highly bioavailable candidates for clinical development, it is often important to accurately predict these first pass effects in humans. Until now, many robust and well-accepted approaches to the predictions of oral absorption and hepatic metabolism in humans have been reported; for example, the evaluations of permeability across the epithelial cell monolayer for oral absorption [1,2], or *in vitro* metabolism in liver microsomes or hepatocytes for hepatic metabolism [3,4]. On

Abbreviations: ABT, 1-aminobenzotriazole; CL_{int}, intrinsic clearance; CPR, NADPH-cytochrome P450 reductase; Ct, threshold cycle; CYP, cytochrome P450; *Eg*, intestinal extraction ratio in humans; ER, extraction ratio; *Fa*, fraction absorbed; *Fg*, intestinal availability; FISH, fluorescence in situ hybridization; GFP, green fluorescent protein; HAC, human artificial chromosome; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MMCT, microcell-mediated chromosome transfer; PAC, P1 artificial chromosome; P_{app}, apparent permeability coefficient; PCR, polymerase chain reaction; P-gp, P-glycoprotein; PPIA, peptidylprolyl isomerase A; RT-PCR, reverse transcription-polymerase chain reaction; TEER, transepithelial electrical resistance; TM, transport medium.

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the other hand, various attempts using intestinal or liver microsomes have been reported for quantitative prediction of intestinal metabolism [5–9], but no widely accepted strategies have been established yet.

As another approach to the evaluation of intestinal metabolism of compounds, CYP3A4-expressed epithelial cells, such as Caco-2, MDCK, and LLC-PK1 cells, were developed using plasmid vectors [10], virus vectors [11,12], and induction with 1,25-dihydroxyvitamin-D_3 [13]. These parent cell lines have widely been used for the assessment of membrane permeability and intestinal absorbability; however, they originally lack CYP3A4 expression. Although these studies confirmed that the epithelial cells expressing CYP3A4 possess certain levels of metabolic activity for the compounds during the transport across their monolayer, there have been no results addressing the quantitative predictability of these CYP3A4-expressing cells for the human intestinal extraction ratio (*Eg*) or availability (*Fg*). The difficulty in predicting *Eg* values possibly stems from the following reasons: 1) Insufficient metabolic activity prohibits accurate prediction [12,14]. 2) Loss of CYP3A4 expression during passage makes it difficult to conduct reproducible assays [10,11,15]. As a gene delivery vector, a human chromosome 21-derived human artificial chromosome (HAC) have demonstrated the compelling advantage of more sustained expressions over conventional plasmid and viral vectors, owing to their long-term stable episomal maintenance in host cells, without integration into the host genome [16,17]. Therefore, the development of epithelial cells expressing CYP3A4 using the HAC vector has the potential to overcome the aforementioned problems for the prediction of intestinal availability for CYP3A4 substrates in humans.

In this study, we established an *in vitro* system that enabled us to predict *Fg* in humans. First, we developed Caco-2 cells that express both CYP3A4 and NADPH-cytochrome P450 reductase (CPR), the obligate electron donor to P450, using the HAC vector. Secondly, we investigated the relationship between the metabolic extraction ratios (ER) obtained from the CYP3A4-CPR-HAC/Caco-2 cells and calculated *in vivo Eg* values from the reported *Fg* values in humans for 17 substrates of CYP3A4. In addition, the stability of the metabolic activities in the transferred cells during serial passages was also examined.

2. Materials and methods

2.1. Materials

Caco-2 cells were obtained from DS Pharma Biomedical Co. (Osaka, Japan). Dulbecco's modified Eagle's medium, 0.25% trypsin-EDTA, Hanks' balanced salt solution, nonessential amino acids, penicillin-streptomycin, HEPES, sodium pyruvate, and GlutaMAX were obtained from Life Technologies (Carlsbad, CA). Fetal bovine serum was obtained from SAFC Biosciences (Lenexa, KS). Blasticidin S was purchased from Kaken Pharmaceutical (Tokyo, Japan). Twenty four-multiwell inserts (pore size: 1 μm) were purchased from Corning (Corning, NY). All compounds for the transport/metabolism assay were purchased from Sigma–Aldrich, LKT Laboratories (St. Paul, MN) and Tokyo Kasei Kogyo (Tokyo, Japan). All other chemicals and reagents were of analytical grade.

2.2. Cell culture

Parental Caco-2 cells were grown on culture flasks in Dulbecco's modified Eagle's medium with 4.5 g/L glucose, supplemented with 10% fetal bovine serum, 1 × nonessential amino acids, 1 × sodium pyruvate, 1 × GlutaMAX, 50 U/mL penicillin, and 50 μg/mL streptomycin. For CYP3A4-CPR-HAC/Caco-2 cells, the above medium was supplemented with 2 μg/mL blasticidin S. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Before reaching confluence, cells were treated with 0.25% trypsin-EDTA, and subcultured at a split ratio of 1:3, or seeded at 2.5×10^4 cells/well onto 24-well inserts. The culture medium was replaced once in the first week and every other day thereafter. Transepithelial electrical resistance (TEER) values were measured to check the integrity of the monolayer using Millicell-ERS (Millipore; Bedford, MA).

2.3. Construction of PAC vector

P1 artificial chromosome (PAC) vectors containing the CYP3A4 and CPR were constructed as described previously [18]. Briefly, fragments of CYP3A4 cDNA (approximately 1.5 Kb) and CPR cDNA (approximately 2.0 Kb) were amplified by reverse transcription-polymerase chain reaction (RT-PCR) using 35 cycles of 98 °C for 30 s, 60 °C for 1 min, and 68 °C for 2 min, using total RNA prepared from human primary hepatocytes (Tissue Transformation Technologies, Cat.# HA113200, Lot.045444210002-HH281) or HepG2 cells (ATCC), respectively. The primers used are listed in Supplementary Table S1. These cDNA were subcloned into CAG expression vector, and then the cassettes were cloned into a PAC vector, flanked by HS4 insulators, loxP and 3′-HPRT site, as described previously [18].

2.4. Construction of PAC-HAC vector

The CYP3A4-CPR-PAC vector was transfected into CHO (21HAC2) cells using Lipofectamine 2000 (Life Technologies) in accordance with manufacture's protocol. After 24 h, transfected cells were re-plated. Forty-eight hours after the transfection, 1 × hypoxanthine-aminopterin-thymidine (Sigma–Aldrich) was added to the culture medium. Individual colonies were isolated from day 13. After genomic polymerase chain reaction (PCR), RT-PCR, and fluorescence in situ hybridization (FISH) analyses, the candidate CHO clones for microcell-mediated chromosome transfer (MMCT) were selected. The CHO cells containing the HAC with the CYP3A4-CPR-PAC vector were utilized for the transfer of the HAC into the Caco-2 cells via MMCT, as described previously [16].

2.5. MMCT

MMCT is the most useful method to introduce a chromosome or a chromosome fragment into recipient cells. The CYP3A4-CPR-HAC/CHO cells were used as donor cells. In brief, microcells were separated from donor cells by centrifugation and fused with the Caco-2 cells in 45% polyethyleneglycol 1000 (Sigma–Aldrich) and 10% dimethylsulfoxide (Sigma–Aldrich). On the next day, fused cells were re-plated and 2 μg/mL blasticidin S was added to the culture medium. Individual colonies were isolated at one month after the selection.

2.6. Genomic PCR analyses

PCR analyses were carried out using standard methods. Genomic DNA was extracted from the CYP3A4-CPR-HAC/CHO cells and the CYP3A4-CPR-HAC/Caco-2 cells using a genomic DNA extraction kit (Gentra System, Minneapolis, MN). The genomic DNA from the CYP3A4-CPR-PAC vector and the CYP3A4-CPR-HAC/CHO cells were used as positive controls, while the genomic DNA from CHO (21HAC2) cells and parental Caco-2 cells were used as negative controls. The primers used are listed in Supplementary Table S1. Thermal cycling conditions were as follows: 98 °C for 5 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 90 s.