Modeling of drug-mediated CYP3A4 induction by using human iPS cell-derived enterocyte-like cells

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A R T I C L E   I N F O
Article history:
Received 2 March 2016
Accepted 5 March 2016
Available online 8 March 2016

Keywords:
Human iPS cells
Enterocyte-like cells
CYP3A4 induction
Nuclear receptor

A B S T R A C T
Many drugs have potential to induce the expression of drug-metabolizing enzymes, particularly cytochrome P450 3A4 (CYP3A4), in small intestinal enterocytes. Therefore, a model that can accurately evaluate drug-mediated CYP3A4 induction is urgently needed. In this study, we overlaid Matrigel on the human induced pluripotent stem cells-derived enterocyte-like cells (hiPS-ELCs) to generate the mature hiPS-ELCs that could be applied to drug-mediated CYP3A4 induction test. By overlaying Matrigel in the maturation process of enterocyte-like cells, the gene expression levels of intestinal markers (VILLIN, sucrase-isomaltase, intestine-specific homeobox, caulud type homeobox 2, and intestinal fatty acid-binding protein) were enhanced suggesting that the enterocyte-like cells were matured by Matrigel overlay. The percentage of VILLIN-positive cells in the hiPS-ELCs found to be approximately 55.6%. To examine the CYP3A4 induction potential, the hiPS-ELCs were treated with various drugs. Treatment with dexamethasone, phenobarbital, rifampicin, or 1α,25-dihydroxyvitamin D3 resulted in 5.8-fold, 13.4-fold, 9.8-fold, or 95.0-fold induction of CYP3A4 expression relative to that in the untreated controls, respectively. These results suggest that our hiPS-ELCs would be a useful model for CYP3A4 induction test.

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1. Introduction
Cytochrome P450 3A4 (CYP3A4) is a dominant drug-metabolizing enzyme in the enterocytes. Because CYP3A4 contributes to the first-pass metabolism of many commercial drugs, it is important to investigate the CYP3A4-mediated intestinal metabolism in order to improve oral drug bioavailability [1,2]. It is known that the CYP3A4 expression in enterocytes can be induced by various drugs, such as dexamethasone (DEX), phenobarbital (PB), rifampicin (rif), and 1α,25-dihydroxyvitamin D3 (VD3) [3,4]. The induction of CYP3A4 expression in the enterocytes by such drugs might affect the pharmacokinetics of concomitant drugs administered orally. Therefore, a model that could evaluate drug-mediated CYP3A4 induction in the enterocytes would be useful for drug discovery.

Caco-2 cells (a human colon carcinoma cell line) are widely used as a monolayer model of human intestinal absorption of drugs [5,6]. However, it is difficult to evaluate drug-mediated CYP3A4 induction by using a Caco-2 cell monolayer model because of the low expression levels of CYP3A4 and pregnane X receptor (PXR, a nuclear receptor that is necessary for CYP3A4 induction) in Caco-2 cells [7]. Although LS180 cells (a human colon carcinoma cell line) are positive for PXR [8,9], the gene expression profiles of other nuclear receptors, such as glucocorticoid receptor (GR), are

Abbreviations: CDX2, caudal type homeobox 2; CYP3A4, cytochrome P450 3A4; DEX, dexamethasone; EGF, epidermal growth factor; FGF2, fibroblast growth factor 2; FXR, farnesoid X receptor; GR, glucocorticoid receptor; hiPS-ELCs, human induced pluripotent stem cell-derived enterocyte-like cells; IFABP, intestinal fatty acid-binding protein; ISX, intestine-specific homeobox; PB, phenobarbital; PXR, pregnane X receptor; RIF, rifampicin; SHP, small heterodimer partner; SI, sucrase-isomaltase; VDR, vitamin D receptor; VD3, 1α,25-dihydroxyvitamin D3.

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different from that of human enterocytes [10]. To the best of our knowledge, an in vitro model that can accurately evaluate drug-mediated CYP3A4 induction in human enterocytes has not been established.

Recently, several groups have reported that intestinal tissues and organoids, which consist of all four intestinal cell types (paneth cells, goblet cells, enterocytes, and enteroendocrine cells), could be differentiated from human pluripotent stem cells in vitro [11–13]. However, because these intestinal tissues and organoids are generated under 3-dimensional culture conditions, it would be difficult to generate a monolayer model using these intestinal tissues and organoids. Therefore, it might be difficult to apply these intestinal tissues and organoids to drug absorption and metabolism studies. Although some researchers have demonstrated that enterocyte-like cells (ELCs) could be generated under 2-dimensional culture conditions from human pluripotent stem cells, their drug-mediated CYP3A4 induction potency has not been well characterized [14–18].

In this study, we overlaid Matrigel on the human induced pluripotent stem cell-derived enterocyte-like cells (hiPS-ELCs) to generate the mature hiPS-ELCs that could be applied to drug-mediated CYP3A4 induction tests. To investigate whether the overlaying Matrigel matured the hiPS-ELCs, we analyzed the expression levels of intestinal markers. Moreover, we performed a drug-mediated CYP3A4 induction test in the hiPS-ELCs, Caco-2, and LS180 cells.

2. Materials and methods

2.1. Human iPS cells culture

A human iPS cell line, Tic (provided by Dr. A. Umezawa, National Center for Child Health and Development), was maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts (Millipore) with ReproStem medium (ReproCELL) supplemented with 10 ng/ml fibroblast growth factor 2 (FGF2, KATAYAMA CHEMICAL INDUSTRIES).

2.2. In vitro differentiation

Before the initiation of enterocyte differentiation, human iPS cells were dissociated into clumps by using dispase (Roche) and plated onto growth factor reduced BD Matrigel Basement Membrane Matrix (BD Biosciences). These cells were cultured in the MEF-conditioned medium for 2–3 days. The differentiation protocol for the induction of definitive endoderm cells was described previously [19]. Briefly, for the definitive endoderm differentiation, human iPS cells were cultured for 4 days in L-Wnt-3A-expressing cell-conditioned RPMI1640 medium (Sigma) containing 100 ng/ml Activin A (R&D Systems), 1 × GlutaMAX, 0.2% fetal bovine serum (FBS), and 1 × B27 Supplement Minus Vitamin A (Life Technologies). For the induction of intestinal cells, the definitive endoderm cells were cultured for 4 days in the intestinal differentiation medium (DMEM-High Glucose medium (Invitrogen) containing 10% Knockout Serum Replacement (KSR, Invitrogen), 1% Non-Essential Amino Acid Solution (NEAA, Invitrogen), Penicillin-Streptomycin (P/S), 1 × GlutaMAX, and 100 µM β-mercaptoethanol) supplemented with 5 µM 6-Bromoiridurin-3′-oxime (BIO, Calbiochem) and 10 µM N-[3,5-difluorophenyl) acetyl]-L-alanyl-2-phenyl-1, 1-dimethylylethyl ester-glycine (DAPT, Peptide Institute). And then, these cells were cultured for 12 days in the intestinal differentiation medium supplemented with 1 µM BIO and 2.5 µM DAPT. For the induction of enterocyte-like cells, the intestine-like cells were cultured for 5 days in L-Wnt-3A-expressing cell-conditioned intestinal differentiation medium containing 0.1 µM BIO, 1 µM DAPT, 2 µM SB431542 (Wako), and 250 ng/ml epidermal growth factor (EGF, R&D systems). And then, these cells were overlaid with thin growth factor reduced Matrigel (50 µg/cm²), and cultured for 5 days in the intestinal differentiation medium containing 250 ng/ml EGF.

2.3. Caco-2 and LS-180 cell culture and differentiation

Caco-2 cells (ATCC, HTB-37) were cultured with Minimal Essential Medium (MEM, Sigma) containing 10% FBS, 1% NEAA, 2 mM l-glutamine, and P/S. For differentiation of Caco-2 cells, Caco-2 cells were cultured for 21 days after they reached confluence. LS-180 cells (ATCC, CL-187) were cultured with MEM containing 10% FBS, 1% NEAA, 2 mM l-glutamine, and P/S.

2.4. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from Caco-2 cells, LS-180 cells, and human iPS cells and their derivatives using ISOGENE (NIPPON GENE). cDNA was synthesized using 500 ng of total RNA with a Superscript VILO cDNA synthesis kit (Invitrogen). Human Small Intestine Total RNA (4 lots) was purchased from BioChain. Real-time RT-PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems) using a StepOnePlus real-time PCR system (Applied Biosystems). Relative quantification was performed against a standard curve and the values were normalized against the input determined for the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). PCR primers sequences were obtained from qPrimerDepot (http://primerdepot.nci.nih.gov/).

2.5. Flow cytometry

Single-cell suspensions of the hiPS-ELCs were treated with 1 × Permeabilization Buffer (-Bioscience), and then incubated with the primary antibody, followed by the secondary antibody (Table 1). Flow cytometry analysis was performed using a FACS LSR Fortessa flow cytometer (BD Biosciences).

2.6. CYP3A4 induction test

The hiPS-ELCs, Caco-2, and LS180 cells were treated with 250 µM dexamethasone (DEX, Wako), 1 mM phenobarbital (PB, Wako), 20 µM rifampicin (RIF, Wako), or 100 nM 1α,25-dihydroxyvitamin D3 (VD3, Cayman) for 48 h. Controls were treated with DMSO (final concentration 0.1%).

Table 1

<table>
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<th>Name of antibody</th>
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Fig. 1. Efficient enterocyte differentiation from human iPS cells. (A) The procedure for differentiation of human iPS cells (Tic) into enterocyte-like cells (ELC) via definitive endoderm cells is presented schematically. Details are described in the Materials and Methods section. (B) The gene expression levels of VILLIN, sucrase-isomaltase (SI), intestine-specific homeobox (ISX), caudal type homeobox 2 (CDX2), and intestinal fatty acid-binding protein (IFABP) in undifferentiated human iPS cells (iPSCs), hiPS-ELCs (ELC), Matrigel overlaid hiPS-ELCs (ELC-MG), LS180 cells (LS180), Caco-2 cells (Caco-2), and adult small intestine (Small Intestine) were examined by real-time RT-PCR. On the y axis, the gene expression levels in the adult small intestine were taken as 1.0. (C) To evaluate enterocyte differentiation efficiency, the percentages of SI-positive, VILLIN-positive, and CDX2-positive cells were measured by FACS analysis. All data are represented as means ± SD (n = 3). *p < 0.05, **p < 0.01.
phenobarbital (PB), 20 μM taken as 1.0. All data are represented as means ± SD (n ≥ 3). *p < 0.05, **p < 0.01 (compared with DMSO-treated cells).

2.7. Statistical analysis

Statistical analysis was performed using the unpaired two-tailed Student’s t test.

3. Results

3.1. Efficient enterocyte differentiation from human iPS cells

To generate the hiPS-ELCs, human induced pluripotent stem (iPS) cells were differentiated by sequential treatment with various cytokines and compounds such as Activin A, BIO, DAPT, SB431542, EGF, and Wnt-3A according to our previous report [20]. To promote their maturation, the hiPS-ELCs were overlaid with Matrigel [21]. The percentages of SI-positive, VILLIN-positive, and CDX2-positive cells in the hiPS-ELCs were examined by FACS analysis, and found to be approximately 25%, 55%, and 90%, respectively (Fig. 1C). From these results, ELCs were efficiently generated from human iPS cells by using our enterocyte differentiation protocol.

3.2. CYP3A4 induction potential of the hiPS-ELCs, Caco-2, and LS180 cells

To examine the drug-mediated CYP3A4 induction potency of the hiPS-ELCs, the hiPS-ELCs were treated with DEX, PB, RIF, or VD3, all of which are known as CYP3A4 inducers [21]. Treatment with DEX, PB, RIF, or VD3 resulted in 5.8-fold, 13.4-fold, 9.8-fold, or 95.0-fold induction of CYP3A4 expression relative to that in the untreated controls, respectively (Fig. 2A). Because Caco-2 cells are the most widely used enterocyte model, we also examined the CYP3A4 induction potency in Caco-2 cells. Consistent with the previous reports [8,9], we found that CYP3A4 expression was induced only by VD3 treatment (Fig. 2B). In addition, we examined the CYP3A4 induction potency in LS180 cells. The CYP3A4 expression levels were increased by PB, RIF, or VD3 treatment, but not by DEX treatment (Fig. 2C). From these results, hiPS-ELC would be a more suitable model for CYP3A4 induction testing than Caco-2 and LS180 cells.

3.3. Gene expression analysis of nuclear factors

It is known that drug-mediated CYP3A4 induction is mainly regulated by nuclear receptors [22], such as PXR, GR, farnesoid X receptor (FXR), small heterodimer partner (SHP), and vitamin D receptor (VDR). For example, it is known that CYP3A4 induction by RIF treatment is mediated by PXR [23] and CYP3A4 induction by VD3 treatment is mediated by VDR [4]. These facts suggest that nuclear receptors play an important role in drug-mediated CYP3A4 induction. Therefore, we analyzed the gene expression levels of intestinal nuclear receptors (PXR, GR, FXR, SHP, and VDR) in the hiPS-ELCs (Fig. 3). The gene expression levels of PXR, GR, FXR, and SHP in the hiPS-ELCs were significantly higher than those in Caco-2 and LS180 cells. On the other hand, there was no significant difference in the VDR expression level between the hiPS-ELCs and LS180 cells. These results suggest that the gene expression pattern of intestinal nuclear receptors in the hiPS-ELCs is more similar to that in the adult small intestine than are the patterns of intestinal nuclear receptor expression in the Caco-2 and LS180 cells (Fig. 4).

4. Discussion

In this study, we succeeded in promoting enterocyte differentiation from human iPS cells by overlaying Matrigel. Moreover, we found that hiPS-ELCs have drug-mediated CYP3A4 induction potency (Fig. 2A). The hiPS-ELCs were matured by Matrigel overlay (Fig. 1B). It is known that various cytokines and extracellular matrices are included in Matrigel, such as insulin-like growth factor-1 (IGF-1) and laminin [24]. It was reported that IGF-1 promotes the proliferation of the small intestinal epithelium [25], while laminin promotes differentiation and maturation in Caco-2 cells [26,27]. It might be possible that IGF-1 and laminin play important roles in the proliferation and maturation of hiPS-ELCs. In addition, we succeeded in reducing the culture period (30 days) by Matrigel overlay as compared with our previous report (34 days) [20]. Such a reduction in the culture period could decrease costs which is an important requirement for the generation of hiPS-ELCs. The percentages of SI- and VILLIN-positive cells in the hiPS-ELCs were approximately 25% and 55%, respectively, although the...
percentage of CDX2-positive cells in the hiPS-ELCs was approximately 90% (Fig. 1C). It is known that CDX2 is a marker of immature cells in the intestinal lineage [11], while SI and VILLIN are markers of mature cells in the small intestinal epithelium [28]. Therefore, the percentages of intestinal markers in the hiPS-ELCs differed largely from each other.
The hiPS-ELCs expressed nuclear receptors, such as PXR, GR, and VDR (Fig. 3). In contrast, Caco-2 cells expressed only VDR, and did not express PXR or GR (Fig. 3). These nuclear receptors are necessary for the CYP3A4 induction by drugs such as DEX (a GR agonist), PB (a PXR agonist), RIF (a PXR agonist) and VD3 (a VDR agonist). Consistently, DEX, PB and RIF treatments did not induce CYP3A4 expression in Caco-2 cells (Fig. 2B). On the other hand, LS180 cells expressed PXR and VDR, but not GR. Therefore, CYP3A4 expression in LS180 cells was induced by PB, RIF and VD3 but not by DEX (Fig. 2C). In contrast to Caco-2 and LS180 cells, the hiPS-ELCs expressed CYP3A4 (21) (2006) 414–423. These results suggest that hiPS-ELCs are a more appropriate model for drug-mediated CYP3A4 induction than Caco-2 and LS180 cells (Fig. 4). However, the fold-changes of CYP3A4 expression induced by PB, RIF, and VD3 treatment in hiPS-HLCs were still lower than those in LS180 cells. The drug-mediated CYP3A4 induction potency of hiPS-HLCs would be further enhanced by improving the enterocyte differentiation method.

In this study, we have succeeded in generating the mature hiPS-ELCs for use in the drug-mediated CYP3A4 induction test. Because CYP3A4-mediated small intestinal metabolism plays an important role in oral drug bioavailability, our hiPS-ELC model would be useful for generating drugs that have a low risk of unexpected drug–drug interactions. We believe that our hiPS-ELC model would thus be useful for discovering safer and more selective drugs.

Conflict of interest statement

The authors declare no competing financial interests.

Acknowledgments

We thank Ms. Yasuko Hagihara, Ms. Natsumi Mimura, and Ms. Ayaka Sakamoto for their excellent technical support. We also thank Dr. Eiri Ono (K-CONNEX, Kyoto University) for his drawing of Fig. 4. This research is supported by the grants (15mk0101011h0102) from Japan Agency for Medical Research and development (AMED) and the Mochida Memorial Foundation for Medical and Pharmaceutical Research. This research is also supported by the Keihanshin Consortium for Fostering the Next Generation of Global Leaders in Research (K-CONNEX), established by Human Resource Development Program for Science and Technology, MEXT. YN was supported by a Grant-in-Aid for the Japan Society for the Promotion of Science Fellows.

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