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Serum derived from ulcerative colitis mouse changes the metabolism of the fluorescent substrate by P450 depending on the degree of disease progression

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ABSTRACT

Ulcerative colitis (UC) is characterized by erosions of the intestinal mucosa. The number of patients with UC has recently been increasing rapidly. Since the diagnosis of UC is complex and difficult, a simple, rapid, noninvasive technique for diagnosing UC is needed urgently. The expression of cytochrome P450 (P450 or CYP) species in mouse liver is known to be changed dependent on the disease. Various components such as P450 substrates and P450 metabolites in the blood may possibly change with the UC-specific way in mouse. In this study, in order to evaluate UC-specific components in UC mouse serum, we analyzed the influence of serum derived from UC mice on the results of fluorescent P450 inhibition assays based on 12 human P450 enzymes, such as CYP1A1, CYP2C8, CYP2E1,CYP3A4, CYP1A2, CYP2D6, CYP2A13, CYP2B6, CYP2C9, CYP2C18, CYP2C19, and CYP3A5. At first, in order to induce UC, mice received 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) dissolved in their drinking water for 7 days. Next, these 12 human P450 enzymes were expressed in *E. coli* cells. Then, P450 fluorescent competition reaction was performed using these P450 enzymes and serum of UC mice. We found that the metabolism of fluorescent substrates by CYP2B6, CYP2C19, CYP2E1, and CYP1A1 in the presence of serum obtained from DSS-treated mice was activated by 42%, 37%, 37%, and 23%, respectively, relative to that associated with sera from control mice. A receiver operating characteristic (ROC) curve analysis was carried out with the 31 samples of UC mice and healthy mice. Area under the ROC curve (AUC) value was calculated from ROC curve. AUC value of CYP2E1 and CYP2C19 showed 0.921 and 0.892, respectively. Therefore, it was shown that CYP2C19 and CYP2E1 could be used as biomarkers for evaluating ulcerative colitis. From these results, it is suggested that these simple fluorescent P450 inhibition assays have potential as a new diagnostic procedure for UC in mouse. This study is the first report on a simple non-invasive method for evaluating UC using P450 enzyme and serum interaction.

1. Introduction

The inflammatory bowel disease known as ulcerative colitis (UC) is characterized by erosions of the intestinal mucosa. The number of patients with UC has recently been increasing rapidly [1]. Since the diagnosis of UC is complex and difficult [2,3], involving the integration of clinical signs and symptoms with findings from histology, radiology, and endoscopy [4], a simple, rapid, noninvasive technique for diagnosing UC is needed urgently.

Nuclear factor κB (NF-κB) is induced at the onset of inflammatory disease and regulates the expression of cytochrome P450 (P450, CYP) enzymes [5]. Different molecular species of P450 are distributed between the endoplasmic reticulum and mitochondria of animal cells. P450 is widely distributed among eukaryotic and prokaryotic organisms, and participates in the metabolism of various endogenous and exogenous compounds. Fifty microsomal P450s and 7 mitochondrial P450s have been identified in human [6]. Compared with healthy controls, mice with 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS)-induced UC showed increased expression of several inflammation-related cytokines and altered hepatic expression of several CYP species [7,8]. Therefore, we hypothesized that various components such as P450 substrates and metabolites in serum could be a unique biomarker

Abbreviations: BOMCC, 7-benzyloxy-methyloxy-3-cyanocoumarin; CYP, cytochrome P450; DAI, disease activity index; DBOMF, dibenzyl-methyl-fluorescein; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; EOMCC, 7-ethoxy-methyloxy-3-cyanocoumarin; IL, interleukin; KC, keratinocyte chemoattractant; NF-κB, nuclear factor κB; P450, cytochrome P450; TNF, tumor necrosis factor; UC, ulcerative colitis

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for mouse UC.

Analysis of the structure and function of the human P450 enzyme was made possible by a heterologous expression system expressing P450 in yeast [9]. Currently, various P450 expression systems have been developed, such as *E. coli*, insect cells and human cell culture [10]. Dong et al. developed co-expression system of human P450 and reductase in *E. coli* [11]. By using this co-expression method in *E. coli*, unlike the expression system of human P450 enzyme in yeast, we have been able to obtain a large number of active mammalian P450 enzymes. However, it is necessary to optimize the appropriate N-terminal amino acid sequence of each P450 enzyme for the expression of human P450 species into *E. coli* cells [12].

In the current study, we used fluorescent P450 inhibition assays to evaluate UC-specific components in mouse serum. Fluorescent P450 inhibition assays are known to be highly sensitive techniques for evaluating the effects of various compounds, including drugs, industrial chemicals, and food components, on P450 activity [13–16]. Using similar assays, we revealed the mouse UC-specific fluorescence pattern associated with 12 predominant drug-metabolizing human P450 enzymes, such as CYP1A1, CYP2C8, CYP2E1, CYP3A4, CYP1A2, CYP2D6, CYP2A13, CYP2B6, CYP2C9, CYP2C18, CYP2C19, and CYP3A5. Our results suggest these simple fluorescent P450 inhibition assays have potential as a simple, rapid, noninvasive technique for diagnosing UC mouse.

2. Materials and methods

2.1. Chemicals and reagents

Sodium chloride, ethylenediaminetetraacetic acid (EDTA), ampicillin, isoflurane and Streptozotocin (STZ) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, acetonitrile, glycerin (analytical grade), tryptone, yeast extract, isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Nacalai Tesque (Kyoto, Japan). Glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PDH), and nicotinamide adenine dinucleotide phosphate· 4 H_2O (NADPH) were purchased from Oriental Yeast Company (Tokyo, Japan). Dextran sulfate sodium (DSS, MW 36,000–50,000) was purchased from MP Biomedicals (Aurora, OH). Aminolevulinic acid was purchased from Cosmo Energy Holdings (Tokyo, Japan). Bovine serum albumin was purchased from Sigma-Aldrich (Tokyo, Japan). Vivid dibenzyl-methyl-fluorescein (DBOMF) Substrate, Vivid 7-benzyloxy-methyloxy-3-cyanocoumarin (BOMCC) Substrate, Vivid 7-ethoxy-methyloxy-3-cyanocoumarin (EOMCC) Substrate, and Nunc 384-well plates (catalog no. 460518) were purchased from Thermo Fisher Scientific (Tokyo, Japan). Nucleospin RNA Mini Kit was purchased from Macherey-Nagel (Düren, Germany). PrimeScript RT Reagent Kit (Perfect Real Time) was purchased from Takara Bio Inc. (Otsu, Japan). Protein Assay Kit was purchased from Bio-Rad Laboratories (Hercules, CA).

The 1-M KPB buffer consisted of 0.17 M potassium dihydrogen phosphate and 0.72 M dipotassium hydrogen phosphate. Stock buffer, used for the resuspension of the *Escherichia coli* (*E.coli*) membrane fractions, consisted of 20% glycerin, 100 mM KPB, and 1 mM EDTA. Mice received DSS in their drinking water (final concentration, 3%). The 5 × buffer for the NADPH regeneration system [17] is comprised of 100 mM KPB, 10 mM G6P, 2 mM NADPH, and 2 units/mL G6PDH. LB medium consisted of 1% tryptone, 0.5% yeast extract, and 1% NaCl. TB medium contained 1.2% tryptone, 0.8% yeast extract, 0.4% glycerin, and 100 mM KPB.

2.2. Animals

Female C57BL/6JJ mice (age, 8 weeks) were purchased from Japan SLC (Shizuoka, Japan). The mice were kept at a room temperature of 23 ± 2 °C with 50% ± 10% relative humidity on a 12:12-h light:dark cycle (lights on, 08:00 to 20:00). This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Kobe University.

2.3. Preparation of E. coli membrane fractions expressing human P450s and P450 reductase

Vectors to express each of four species of human P450—pCW1A1, pCW2C8, pCW2E1, and pCW3A4—in *E. coli* were constructed as described previously [13]. Human liver cDNA library was obtained from Takara Bio Inc. (Otsu, Japan). To generate vectors to individually express another eight P450s—CYP1A2, CYP2D6, CYP2A13, CYP2B6, CYP2C9, CYP2C18, CYP2C19, and CYP3A5—in *E. coli*, cDNA fragments encoding these enzymes were cloned from human liver cDNA libraries by using PCR-based methods. The cycling conditions of PCR were as follows: 95 °C for 10 s,60 °C for 30 s followed by 30 cycles of 65 °C for 120 s and 95 °C for 30 s. The N-terminal sequences of the CYP1A2 and CYP2C9 cDNA fragments were modified to carry + and SalI sites by using the PCR primers; h1A2-F and h1A2-R for CYP1A2 and h2C9-F and h2C9-R for CYP2C9. It has been reported that the high-level expression of P450 cannot be achieved without modifications of the N-terminal amino acid sequence of P450 protein [10]. The N-terminal regions of the cDNA fragments corresponding to CYP2D6, CYP2A13, CYP2B6, CYP2C18, and CYP2C19 were deleted by using the following PCR primer sets: CYP2D6, h2D6-int-F and h2D6-R; CYP2A13, h2A13-int-F and h2A13-R; CYP2B6, h2B6-int-F and h2B6-R; CYP2C18, h2C18-int-F and h2C18-R; and CYP2C19, h2C19-int-F and h2C19-R. The full-length cDNA encoding CYP3A5 was cloned from a human liver cDNA library by using the PCR primers hCYP3A5-F and hCYP3A5-R-Sal during the first round of amplification and hCYP3A5-F-NdeOmpA and hCYP3A5-R-Sal during the second round; this amplification protocol added the bacterial signal peptide OmpA to the N-terminus of CYP3A5. The DNA sequence of each primer is shown in supplementary Table 1.

All amplified fragments were cloned into the pT7 site of the pT7Blue vector (Novagen, Madison, WI) and their sequences confirmed. The plasmids containing the cDNAs of CYP1A2, CYP2C9, and CYP3A5 were digested with NdeI and SalI, and the fragments obtained were subcloned into the SalI and NdeI sites of the pCW vector, which contains a cDNA copy of human NADPH-cytochrome P450 reductase [18]. The cDNA fragments corresponding to the N-terminally deleted CYP2D6, CYP2A13, CYP2B6, CYP2C18, and CYP2C19 were individually inserted between the SmaI and SalI sites of a previously modified pCW vector, thus adding 11 amino-acid residues, MAKKTSSKGKL, to the N-terminus of the P450 construct. Each of the resulting vector plasmids was used to transform *E. coli* JM109 cells, which were cultured in 5 mL of LB medium at 37 °C for 1 day; 3 mL of this overnight culture then was added to 300 mL of TB medium and incubated at 37 °C to an OD_{600} of 0.2–0.3. All liquid cultures were shaken at 150 rpm during culture. Once cultures achieved the appropriate density, IPTG (final concentration, 1 mM) and δ-aminolevulinic acid (final concentration at 0.5 mM) were added and the cultures incubated at 25 °C for 24 h, after which the cells were pelleted, suspended in 4 mL of stock buffer, and homogenized by using an ultrasonicator (Model VP-300 N, Taitec, Tokyo, Japan). The homogenate was centrifuged at 1000 × *g* for 20 min; the resulting supernatant then was centrifuged at 100,000 × *g* for 70 min. Each pellet (1 mL) was resuspended in 10 mL of stock buffer and stored at −80 °C.

Protein concentration in the membrane fraction was determined by Bradford's method using the Protein Assay Kit [19]. A standard curve was constructed from diluted bovine serum albumin (0.2–1.0 mg/mL). Reduced CO difference spectra were measured with a Hitachi UV-visible spectrophotometer U-3300 (Hitachi, Tokyo, Japan), according to the protocol published by Imaishi et al. [20]. The P450 hemoprotein contents in the membrane suspensions were determined with an extinction coefficient of 91.1 mM − 1 cm − 1 [21].