Heterologous expression of human cytochrome P450 2E1 in HepG2 cell line

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INTRODUCTION

Cytochrome P450 2E1 (CYP2E1) is the only member of the CYP2E subfamily in humans. Approximately 7% of the liver CYP content consists of CYP2E1, although individual variation of the level of hepatic CYP2E1 expression can be existed by an order of magnitude. CYP2E1 is also expressed in a number of extrahepatic tissues including the lungs[1] and brain[2]. CYP2E1 takes part in the biotransformation of ethanol, acetone, and many small-molecule substrates such as halogenated hydrocarbons (1,1,1-trichloroethane, 1,2-dichloropropane, carbon tetrachloride, chloroform, ethylene dibromide, ethylene dichloride, halothane, methylchloride, methylene dichloride, vinylchloride and trichloroethylene, most of which are hepatotoxic), acetaldehyde, benzene, and styrene. It is known for its ability to metabolize volatile anesthetics such as halothane, enflurane, isoflurane, and sevoflurane, acetaminophen, phenacetin and chlorzoxazone. Another group of CYP2E1 substrates are nitroamines. CYP2E1 is involved in chemical activation of many carcinogens, procarcinogens, and toxicants[3,5].

Genetically engineered mammalian cells expressing CYP subtypes have provided new tools for investigations of the metabolism and CYP-mediated metabolic activation of chemicals. The stable expression system of CYP in cells has made it possible to evaluate the relative risk of a chemical in toxicological testing in vitro[6,7]. Human CYP1A1[8,9, CYP2B6[10], CYP2A6[9, CYP3A4[10], CYP2C9[11], CYP2C18 and a phase II metabolism enzyme UDP-glucuronosyltransferase, UGT1A9[12] have been stably expressed in Chinese hamster lung CHL cells in our laboratory. Among the human hepatic cell lines, HepG2 derived from a human liver tumor has been characterized to retain many xenobiotic-metabolizing activities as compared to fibroblasts. Therefore, HepG2 cell is useful in prediction of the metabolism and cytotoxicity of chemicals in human liver[14]. But it does not produce significant amounts of CYP[15,16]. Yoshitomi et al[17] have established in HepG2 cells stable expression of a series of human CYP subtypes, such as CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4.

In this study, human CYP2E1 cDNA was amplified by reverse transcription-polymerase chain reaction (RT-PCR), and a transgenic cell line HepG2-CYP2E1 stably expressing CYP2E1 was established to assess the metabolic and toxicological characteristics of CYP2E1.

MATERIALS AND METHODS

Materials

Restriction endonucleases, Moloney murine leukemia virus (M-MuLV) reverse transcriptase were supplied by MBI Fermentas AB, Lithuania. PCR primers, DNA sequence primers, random hexamer primers, and dNTPs were synthesized or supplied by Shanghai Sangon Biotechnology Corp. Expand fidelity PCR system and NADPH were from Roche Molecular Biochemicals. DNA sequencing kit was purchased from Perkin-Elmer Co. The TRIZol reagent, G418, Dulbecco’s modified Eagle’s medium (DMEM) and newborn...
bovine calf sera were from Gibco. Diethyl pyrocarbonate (DEPC), MTI, and N-nitrosodimethamine (NDEA) were purchased from Sigma Chemical Co. T4 DNA ligase and pGEM-T vector system were from Promega. 4-nitrophenol and p-nitroacetol were from Tokyo Kasei Kogyo Co. Ltd, Japan. Other chemical reagents used were of analytical purity from the commercial sources.

**Methods**

Cloning of human CYP2E1 cDNA from human liver  Total RNA was extracted from a surgical specimen of human liver with TRIzol reagent according to the manufacturer’s instructions. RT-PCR amplifications were described before, using Expand fidelity PCR system[11]. Two specific 27 mer oligonucleotide PCR primers were amplified according to the mRNA sequence of CYP2E1 reported by Song et al[1](GenBank access No. J02625). The sense primer corresponding to base position -8 to 19 was 5’-AGGTGATCATGTGCCTGCAGGTA-3’, with a restriction site of Kpn I (underlined). The anti-sense primer, corresponding to the base position 1 507 to 1 534, was 5’-ACATAATGAAAGCTTGGTGGAAAGCCG-3’, with a restriction site of Hind III (underlined). The anticipated PCR product was 1 542 bp in length. PCR was performed at 94°C for 2 min, then 35 cycles at 94°C for 60 s, at 62°C for 60 s, at 72°C for 2 min, and extension at 72°C for 10 min. An aliquot (10 µL) from PCR was subjected to electrophoresis in a 1% agarose gel stained with ethidium bromide.

Construction of recombinant pGEM-CYP2E1 and sequencing of CYP2E1 cDNA  The PCR products were ligated with a pGEM-T vector, and transformed to E. coli DH5α. CYP2E1 cDNA cloned in pGEM-T was sequenced by Perkin-Elmer-ABI Prism 310 automated DNA sequencer with primers of beta-actin

Preparation of postmitochondrial supernate (S9) of HepG2 cell line  The procedure of preparation of S9 fraction was described before[11]. The protein in S9 was determined by the Lowry’s method, with bovine serum albumin as standard.

4-nitrophenol hydroxylase assays[20–22]  CYP2E1 4-nitrophenol hydroxylase activity of S9 was determined by spectrophotometry, 0.5 mL incubations contained 0.25 mg S9 protein, 0.2 mmol·L⁻¹ 4-nitrophenol in 0.1 mmol·L⁻¹ potassium phosphate buffer (pH 6.8), reactions were initiated with 1 mmol·L⁻¹ NADPH and carried out in air at 37°C for 60 min. Reactions were terminated by adding 0.25 mL of 0.6 mmol·L⁻¹ perchloric acid and centrifugate at 10 000×g for 5 min to remove protein. The supernatant was mixed with 1/10 volume of 10 mol·L⁻¹ NaOH. The absorbance of clarified supernatants was measured at 510 nm, and the amount of product was quantitated using a standard curve generated by adding known amounts of p-nitroacetol to incubations without NADPH.

Cytotoxicity assay[23, 24]  HepG2 and HepG2-CYP2E1 cells were seeded in 96 well cell culture plates at a density of 1×10⁴ cells each well and incubated overnight. The medium was discarded, and a new medium containing NDEA 0, 1, 10, 100 mmol·L⁻¹ was added to respective wells, with 6 duplications at each concentration. 72 h later, the medium was discarded and 20 µL of 50 g·L⁻¹ MTT in PBS was added to each well. The MTT was discarded 4 h later and 100 µL dimethylsulfoxide was added. After formazan was dissolved, the absorbance was read at 570 nm as reference on the microtiter plate reader. Relative survival was represented as the relative toxicity to the control culture without NDEA. The significance of difference of relative survival between HepG2-CYP2E1 and HepG2 cells was analyzed by Student’s t test. Micronucleus test[25–28]  1×10⁴ HepG2 and HepG2-CYP2E1 cells were seeded in each 100 mL culture bottle and cultured overnight. The medium was discarded and 0, 5, 10, 20 mol·L⁻¹ NDEA in 4 mL serum-free medium was applied to respective bottles and incubated for 4 h. Subsequently the cells were washed twice in PBS and incubated for 20 h with 4 mL completed medium containing 3 mg·L⁻¹ of cytochalasin B. Then the cells were washed twice in PBS and harvested in 2 mL PBS and fixed with 0.4 mL fixed solution (methanol: acetic acid 3:1) for 30 min. The cells were centrifugated, the PBS was discarded and pelleted cells were refixed with 2 mL of fixed solution for 30 min. The cells were centrifugated, refixed and centrifugated again, and then dropped onto slides, dried in the air and stained with 10 % Giemsa solution. The scoring criteria for binucleated cells and micronucleus (MN) were based on a report of HUman Micronucleus project[27]. The frequency of MN formation was expressed as per thousand of binucleated cells with MN. The significance of difference of MN rate between HepG2-CYP2E1 and HepG2 cells was analyzed by checking the tables for determining the statistical significance of mutation frequencies[29].

**RESULTS**

Construction of recombinants of pGEM-CYP2E1  The recombinant of pGEM-CYP2E1 was constructed with the human CYP2E1 cDNA inserted into the cloning site of pGEM-T vector. Selection and identification of the recombinant were carried out by Kpn I/Hind III endonuclease digestion and agarose gel electrophoresis (Figure 1). The cloned cDNA segment was sequenced completely. In comparison with the cDNA sequence reported by Song et al (GenBank access No. J02625), there was one base difference in cloned cDNA, 105 T→C, while the encoding amino acid was 35G. But there was no sequence difference as compared with that reported by Umeno et al[20] (GenBank access No. J02843).

Construction of recombinants of pREP9-CYP2E1  The Kpn I/Hind III fragment (1.54 kb) containing complete CYP2E1 cDNA was subcloned into the Kpn I/Hind III site
of mammalian expression vector pREP9. Selection and identification of the recombinant were carried out by Kpn I/ Hind III endonuclease digestion and agarose gel electrophoresis (Figure 2). The resulting plasmid was designated as pREP9-CYP2E1 and contained the entire coding region, along with 8 bp of the 5’ and 52 bp of the 3’ untranslated region of CYP2E1 cDNA, respectively.

**Figure 1** Electrophoretic identification of recombinant of pGEM-CYP2E1. Lane 1: Markers (λ/Eco R I and Hind III), 2: PCR products of CYP2E1 (1.54 kb), 3: Recombinant of pGEM-CYP2E1 digested by Kpn I and Hind III, 4: pGEM-T vector.

**Figure 2** Electrophoretic identification of recombinant of pREP9-CYP2E1. Lane 1: Markers (λ/Hind III), 2: RT-PCR products of HepG2 cells showing only beta-actin 462 bp, 3: RT-PCR products of HepG2 cells showing only beta-actin 462 bp, 4: pREP9 vector.

**Figure 3** Identification of CYP2E1 mRNA expression in HepG2-CYP2E1 and HepG2 cells by RT-PCR with beta-actin as internal control. Lane 1: Markers (λ/EcoR I and Hind III), 2: RT-PCR products of HepG2 cells showing only beta-actin 462 bp, 3: RT-PCR products of HepG2-CYP2E1 cells showing 462 bp of beta-actin and 690 bp of CYP2E1.

**Establishment of transgenic cell line with CYP2E1 mRNA expression and 4-nitrophenol hydroxylase activity**

HepG2 cells were transfected with pREP9-CYP2E1, and selected with G418. The surviving clones were propagated and a cell line termed HepG2-CYP2E1 was established. The expression of CYP2E1 mRNA could be detected in HepG2-CYP2E1 cells but not in HepG2 cells by RT-PCR (Figure 3). The 4-nitrophenol hydroxylase activity in S9 of HepG2-CYP2E1 cells was found 0.162±0.025 nmol·min⁻¹·mg⁻¹ S9 protein (n=3), but not detectable in parent HepG2 cells.

**HepG2-CYP2E1 cells increased cytotoxicity and MN rate by NDEA**

Cells were exposed to various concentrations of NDEA. The relative survival rate of HepG2-CYP2E1 cells was lower than that of HepG2 cells in 10 and 100 mmol·L⁻¹ NDEA (P<0.05 and P<0.01, respectively), as shown in Figure 4. The MN rate of HepG2-CYP2E1 was higher than that of HepG2 cells in 10 and 20 mmol·L⁻¹ NDEA (P<0.05 and P<0.01 respectively) as shown in Figure 5.

**Figure 4** Cytotoxicity of NDEA against HepG2-CYP2E1 and HepG2 cells. Cells were exposed to various concentrations of NDEA. Relative survival rate was represented as the relative toxicity to the control culture without NDEA. The results presented were the average of six duplications (n=6). *P<0.05, **P<0.01 vs HepG2 cells.

**Figure 5** MN rates in HepG2-CYP2E1 and HepG2 cells induced by NDEA. Cells were exposed to various concentrations of NDEA. The data were expressed as per thousand of binucleated cells with MN. *P<0.05, **P<0.01 vs HepG2 cells.

**DISCUSSION**

Human CYP2E1 gene is located on the chromosome 10q24.3-qter. Up to date, seven CYP2E1 alleles have been identified (see: CYP2E1 alleles nomenclature at: http://www.imm.ki.se/CYPalleles/cyp2e1.htm). Only 3 alleles have nucleotide substitute, resulting in amino acid change. CYP2E1*1 is the wild type of human CYP2E1. CYP2E1*2 has a 1 168 G→A point mutation in exon 2 causing an R76H amino acid substitution, and CYP2E1*3 has a 10 059 G→A base substitution in exon 8 yielding a V389I amino acid exchange. The corresponding CYP2E1 cDNAs were expressed in COS-1 cells by Hu et al. [30]. The cellular levels of CYP2E1 mRNA, protein, and the rate of chlorozoxazone hydroxylation were monitored. CYP2E1*3 cDNA variant was indistinguishable from the wild type cDNA on all variables investigated, whereas CYP2E1*2 cDNA, although yielding similar amounts of mRNA, only caused 37 % of the protein expression and 36 % of the catalytic activity compared with the wild type cDNA. Complete screening by single-stranded conformation polymorphism of the three
populations revealed that these variant alleles were rare. Human CYP2E1 gene was functionally well conserved compared with other CYP enzymes active in drug metabolism, which suggested an important endogenous function in humans[80]. CYP2E1*4 has a 4 804 G→A point mutation in exon 4, resulting in V179I amino acid exchange. No significant difference in kinetic constants for chloroxzone hydroxylation between mutant and wild type was observed by expression of the wild type and mutated full length cDNAs in lymphoblastoid cells[31]. Our laboratory has once cloned a CYP2E1 cDNA (GenBank Access No. AF182276), which has two point mutations, i.e. 105 T→C, no amino change 35G, and 704G→T, and can result in V235A amino acid exchange. This cDNA was expressed in Chinese hamster lung CHL cells. We could not detect the N-nitrosodimethylamine demethylase activity in transgenic cells (data not shown). According to the homology modelling of human CYP2E1 based on the CYP2C5 crystal structure, the substrate recognition site (SRS) 1 was located at codon 100-118, SRS 2 at 200-211, SRS3 at 236-241, SRS4 at 291-305, SRS5 at 361-370, SRS6 at 470-480. The point mutations of CYP2E1*2, *3, *4 were not located on the SRS. The V235A amino acid exchange in our formerly cloned CYP2E1 was just at the front of SRS3. This might influence SRS3 and reduce the enzyme activity. Fortunately, this time we cloned a wild type CYP2E1.

It has been found that polymorphism of CYP2E1 gene is significant for inter-individual differences in toxicity of its substrates[33], and has some effect on the development of gastric cancer[44] and colorectal cancer[45]. The expression of CYP2E1 mRNA in HepG2 cells was validated by RT-PCR. The commonly used CYP2E1 probe substrates were chloroxzone[26,27] and 4-nitrophenol[38]. In this research, we used 4-nitrophenol 2-hydroxylase activity to evaluate the expression of CYP2E1, and the 4-nitrophenol hydroxylase activity of HepG2-CYP2E1 cells was found to be 0.162±0.025 nmol·min⁻¹·mg⁻¹ S9 protein, a little lower than those of HepG2-CYP2E1 E43 and E47 cells (0.19 and 0.34 nmol·min⁻¹·mg⁻¹ of microsome, respectively)[39], much lower than that of human liver (1.91±0.28 nmol·min⁻¹·mg⁻¹ of microsome)[40].

The most frequently used genotoxicity test in mammals is the micronucleus test, which provides a simple and rapid measurement of chromosome aberrations and is a scientific and regulatory assay accepted by supranational authorities such as the Organization for Economic Cooperation and Development (OECD), International Conference on Harmonization (ICH) and European Union (EU). NDEA could induce early experimental hepatocellular carcinomas[40] and esophageal neoplasms[40]. The metabolic activation of NDEA was mediated mainly by CYP2A6 and CYP2E1[41]. This study has shown that NDEA can decrease the relative survival rate of HepG2-CYP2E1 cells and increase the MN rate in binucleated cells as compared with HepG2 cells.

cDNA of human CYP2E1 was successfully cloned and a cell line, HepG2-CYP2E1, efficiently expressing mRNA and having the CYP2E1 enzymatic activities, was established. The cell line is useful for testing the cytotoxicity, mutagenicity and metabolism of xenobiotics and drugs, which may possibly be activated by CYP2E1.

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