Enzyme activity analysis of CYP2C18 with exon 5 skipped[1]

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KEY WORDS human CYP2C18 protein; tolbutamide; ifosfamide

ABSTRACT

AIM: To study the enzyme activity of CYP2C18 variant with exon 5 skipped. **METHODS:** A full length *CYP2C18* cDNA X1 and an exon 5 skipped variant *CYP2C18* X2 were separately subcloned into mammalian expression vector pREP9 to transfect HepG2 cells. The expression of CYP2C18 mRNA in transgenic cells and human liver tissues were determined by RT-PCR. The enzyme activity of CYP2C18 to oxidate tolbutamide in postmitochondrial supernate (S9) fraction was determined by HPLC. The cytotoxicity of ifosfamide to transgenic cells was evaluated by MTT test. **RESULTS:** HepG2-CYP2C18 X1 cells showed strong expression of the full length CYP2C18 mRNA. On the other hand, HepG2-CYP2C18 X2 cells had only infinitesimal expression of the exon-skipped CYP2C18 as well as the full length CYP2C18, while non-transfected HepG2 cell only demonstrated an infinitesimal expression of the full length CYP2C18. The expression of CYP2C18 exons 2 to 7 was also analyzed by RT-PCR in 7 extratumoral liver tissues. Among them, 3 samples expressed only wild type mRNA, whereas 4 samples expressed both wild type and alternative splicing products. The tolbutamide hydroxylase activity of CYP2C18 was tested, and it was shown that HepG2-2C18 X1 cells had higher enzyme activity than those of HepG2-2C18 X2 and HepG2 cells. The relative survival of HepG2-CYP2C18 X1 cells was lower than that of HepG2 cells with 1, 2, and 4 mmol/L ifosfamide treatments. In contrast, the relative survival of HepG2-CYP2C18 X2 cell was the same as that of HepG2 cell in 0.5 and 1 mmol/L of ifosfamide, but lower than that of HepG2 cell in 2 and 4 mmol/L of ifosfamide. **CONCLUSION:** CYP2C18 X1 could metabolize tolbutamide and ifosfamide efficiently. The exon 5-skipped CYP2C18 X2 could not metabolize tolbutamide, and could not metabolize ifosfamide effectively at low concentrations.

INTRODUCTION

Pre-mRNA splicing involves the precise removal of introns from pre-mRNA such that exons are linked together to form the mature RNA with intact translation reading frames. Splicing requires exon recognition, followed by accurate cleavage and rejoining, which are determined by the invariant GU and AG intronic dinucleotides at the 5′ (donor) and 3′ (acceptor) exon-intron junctions, respectively[1]. Human genes typically contain multiple introns, and in many cases the exons can be joined in more than one way to generate multiple mRNAs that encode distinct protein isoforms. This process, called alternative splicing, is a major mechanism for modulating the expression of cellular and viral genes and enables a single gene to increase its coding capacity, allowing the synthesis of several structurally

[1] Project Supported by the State Key Basic Research and Development Program (No 2002CB512901), National Natural Science Foundation of China (No 39770868), and Natural Science Foundation of Zhejiang Province (No 397490).
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Received 2003-10-08 Accepted 2004-04-01

and functionally distinct protein isoforms[2,3].

CYP2C18 is a major CYP2C in the skin[4] and the lung judged by its mRNA levels[5]. Its mRNA was also found in extrahepatic tissues such as brain, uterus, mammary gland, kidney, and duodenum[6]. Transcripts of *CYP2C18* that have skipped exon 4, exon 5, exon 4-6, or exon 4-7 were identified in epidermis[5]. The splicing process in higher eukaryote is characterized by the precise excision of introns that can be longer than 50000 bases and the joining of exons that are rarely over 300 bases. However, the mechanisms that regulate the splicing process and the generation of alternatively spliced mRNA products are still poorly understood[7].

Previously, we have identified an exon 5 skipped cDNA of *CYP2C18* in human extratumoral liver tissue[8]. To study the exon skipping phenomenon and its impacts on CYP2C18 enzyme activities, the wild type *CYP2C18* cDNA and the exon 5 skipped *CYP2C18* cDNA were separately subcloned into eukaryotic expression vector pREP9 to transfect HepG2 cells. The expression of CYP2C18 mRNA in HepG2 cells, transgenic HepG2 cells, and 7 extratumoral liver tissues were analyzed by RT-PCR. Their tolbutamide hydroxylase activity and the cytotoxicity of ifosfamide on these cells were also evaluated.

MATERIALS AND METHODS

Materials Restriction endonucleases and Moloney murine leukemia virus (M-MuLV) reverse transcriptase were purchased from MBI Fermentas AB, Lithuania. PCR primers, random hexamer primers, *Taq* DNA polymerase, and dNTPs were synthesized or supplied by Shanghai Sangon Biotechnology Co. The TRIzol reagent, G418, Dulbecco's modified Eagle's medium (DMEM) and newborn bovine calf sera were obtained from Gibco. NADPH was obtained from Roche Molecular Biochemicals. Diethyl pyrocarbonate (DEPC), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), tolbutamide, and hydroxytolbutamide were purchased from Sigma Chemical Company. T4 DNA ligase was from Promega. Ifosfamide was produced by Jiangsu Hengrui Medicine Co Ltd. Other chemical reagents used were all of analytical purity from the commercial sources. Extratumoral liver tissue samples were collected from patients undergoing hepatocellular carcinoma resection from the affiliated hospitals of Zhejiang University School of Medicine and stored at -70 ºC.

Construction of the pREP9 based expression plasmid for *CYP2C18* X1 and X2 The *Nhe* I/*Xho* I fragment of wild type and exon 5 skipped *CYP2C18* cDNA (Named X1 and X2, respectively) recombined in pGEM vector were subcloned to mammalian expression vector pREP9 (Invitrogen). The recombinants were used to transform *E coli* Top 10, and the transfectants were screened by ampicillin resistance and further identified by restriction mapping.

Transfection of HepG2 cells Hepatoma HepG2 cells were transfected with the resultant recombinant plasmids, pREP9-CYP2C18 X1 and pREP9-CYP2C18 X2, respectively, using a modified calcium phosphate method and screened with G418[9]. Two transgenic cell lines named HepG2-CYP2C18 X1 and X2 were established.

Detection of CYP2C18 mRNA expression by RT-PCR Total RNA was prepared from G418-resistant clones by TRIzol reagent. RT-PCR was performed as described before[10], using primers CYP2C18 m1 and CYP2C18 m2 (200 nmol/L), whose sequence were described previously[8]. Beta-actin was used as internal control. The sense and anti-sense primers (20 nmol/L) used for beta-actin (GenBank accession No NM_001101) were 5´- TCC CTG GAG AAG AGC TAC GA-3´ (776-795) and 5´-CAA GAA AGG GTG TAA CGC AAC-3´ (1217-1237), respectively. The PCR was performed at 94 ºC for 2 min, then 35 cycles of 94 ºC for 45 s, 55 ºC for 45 s, 72 ºC for 60 s, and finally 72 ºC for 7 min. An aliquot (10 mL) from the PCR was subjected to electrophoresis in a 1.2 % agarose gel. The anticipated beta-actin PCR product was 464 bp in length, and those of CYP2C18 X1 and X2 were 1004 bp and 827 bp, respectively.

Preparation of postmitochondrial supernate (S9) of HepG2-CYP2C18 The preparation of S9 fraction was conducted as described before[8]. The protein concentration in S9 was determined by Lowry's method, with bovine serum albumin as standard.

Tolbutamide hydroxylase assays The tolbutamide hydroxylase activity of S9 was determined by HPLC as described before[10,8]. A Shim-pack CLC-ODS column (15 cm×0.6 cm ID) was used. The mobile phase was constituted with 0.05 % phosphoric acid (pH 2.6) and acetonitrile (6:4, v:v) with a flow rate of 1 mL/min.

Cytotoxicity assay The cytotoxicity of ifosfamide to HepG2, HepG2-CYP2C18 X1 and X2 cells were examined using MTT test[11,12]. Briefly, cells were seeded

into 96-well cell culture plate at a density of 3×10^3 cells/well and incubated overnight. The medium was discarded, and new medium containing 0, 0.5, 1, 2, 4, 8, or 16 mmol/L of ifosfamide were added to respective wells. Each concentration was tested for 4 times. 72 h later, the medium was discarded and 20 mL of MTT in PBS (50 g/L) was added to each well. The MTT was discarded 4 h later and 100 mL dimethylsulfoxide was added. After formazan was dissolved, the absorbance at 570 nm was read on a microtiter plate reader, with 630 nm as reference. Relative survival was represented as the absorbance of treated sample/absorbance of control culture without ifosfamide. The data was analyzed by Student's *t*-test.

RESULTS

Establishment of transgenic cell lines and their expression of CYP2C18 HepG2-CYP2C18 X1 and HepG2-CYP2C18 X2 cells were established by transfecting HepG2 cells with the recombinant plasmids pREP9-CYP2C18 X1 and pREP9-CYP2C18 X2. HepG2-CYP2C18 X1 cells strongly expressed the full length CYP2C18. On the other hand, HepG2-CYP2C18 X2 cells only had infinitesimal expression of exon skipped CYP2C18 as well as the full length CYP2C18, while HepG2 cells showed infinitesimal expression of the full length CYP2C18 (Fig 1).

The expression pattern of CYP2C18 in human extratumoral liver tissues To determine the prevalence of exon 5 skipped CYP2C18 in human liver tissue, transcripts of CYP2C18 exon 2 to 7 were examined with RT-PCR in 7 extratumoral liver tissues. It was found that among the seven samples, three samples expressed wild type mRNA only, while 4 samples expressed both wild type and skipped mRNA (Fig 2), suggesting that this type of alternative splicing occurs quite frequently in human.

CYP2C18 had tolbutamide hydroxylase activities The ability of the S9 fraction of the established cell lines to metabolize tolbutamide to hydroxytolbutamide was determined by HPLC. A representative chromatogram of the reaction extracts was shown in Fig 3. HepG2-CYP2C18 X1 cells had the highest tolbutamide hydroxylase activity (0.63 ± 0.08 μmol·min^{-1}·g^{-1}), which was much higher than those of HepG2 (0.14 ± 0.03 μmol·min^{-1}·g^{-1}) and HepG2-CYP2C18 X2 cells (0.14 ± 0.07 μmol·min^{-1}·g^{-1}). Statistical analysis revealed a significant difference between HepG2-CYP2C18 X1 and HepG2, as well as HepG2-CYP2C18 X1 and HepG2-CYP2C18 X2 cells ($P<0.01$, $n=3$).

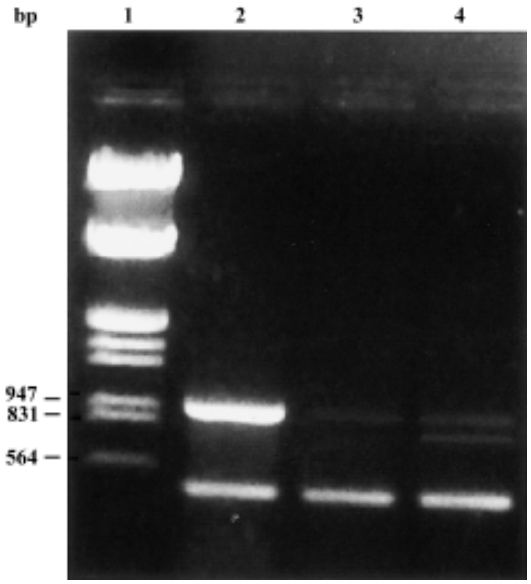

Fig 1. RT-PCR identification of the expression of CYP2C18 mRNA in transgenic HepG2 cells. The expression of full length CYP2C18 was identified as the presence of a 1004 bp fragment, while the presence of an 827 bp fragment indicated the expression of exon 5 skipped CYP2C18. Beta-actin was used as internal control (464 bp). Lane 1: Marker (λ/*Eco*R I and *Hin*d III); Lane 2: HepG2-CYP2C18 X1 cells; Lane 3: HepG2 cells; Lane 4: HepG2-CYP2C18 X2 cells.

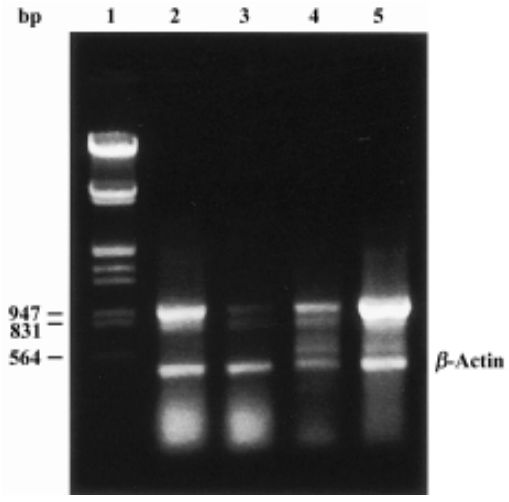

Fig 2. The expression pattern of CYP2C18 in representative human liver tissues determined by RT-PCR. The expression of full length CYP2C18 was identified as the presence of a 1004 bp fragment, while the presence of an 827 bp fragment indicated the expression of exon 5 skipped CYP2C18. Beta-actin was used as internal control (464 bp). Lane 1: Marker (λ/*Eco*R I and *Hin*d III); Lane 2-5: Representative liver tissue samples.

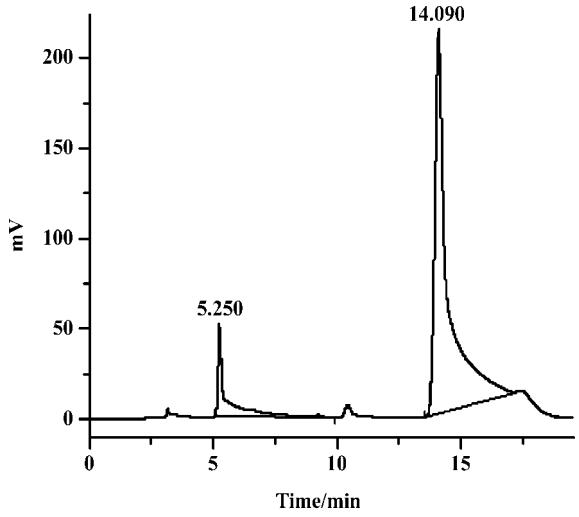

Fig 3. Chromatogram of the S9 reaction extracts for tolbutamide hydroxylase activities. Hydroxytolbutamide was monitored at the absorbance of 230 nm. The retention times for hydroxytolbutamide and tolbutamide were 5.25 and 14.09 min, respectively.

The cytotoxic effects of ifosfamide on HepG2 and transgenic cells The cytotoxicity of ifosfamide was assayed by MTT test. At higher concentrations (8 and 16 mmol/L) there were almost no cells survived (Fig 4). On the other hand, the relative survival of HepG2-CYP2C18 X1 cells was lower than those of HepG2 cells in 1, 2, 4 mmol/L of ifosfamide, suggesting that CYP2C18 was able to metabolize ifosfamide. In comparison, the relative survival of HepG2-CYP2C18 X2 cells was the same as that of HepG2 cells in 0.5, 1 mmol/L, but lower at 2, 4 mmol/L of ifosfamide (Fig 4), indicating that the exon 5 skipped CYP2C18 was less efficient to metabolize ifosfamide at lower concentrations.

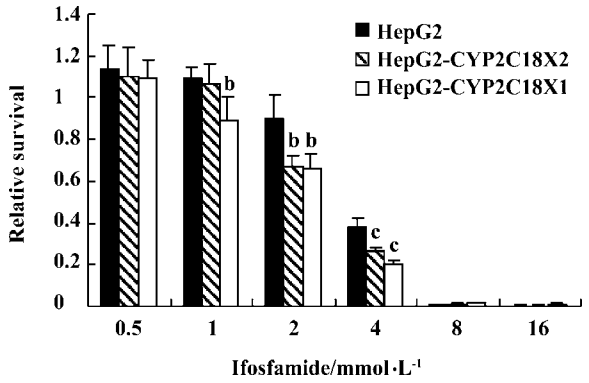

Fig 4. Cytotoxicity of ifosfamide against HepG2-CYP2C18 X1, HepG2-CYP2C18 X2 and HepG2 cells. Cells were exposed to various concentrations of ifosfamide. Relative survival was represented as the relative toxicity to the control culture without ifosfamide. *n*=4. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* HepG2 cells.

DISCUSSION

The *CYP2C18* cDNA with exon 5 skipped cloned from human liver was identical to that in epidermis as reported by Zaphiropoulos[5]. The splicing sites were a non-canonical GA-AG dinucleotide[13] located at the front of intron 4 and the last of intron 5. As reported in this study, exon skipped CYP2C18 mRNA was a common phenomenon in human liver, since there were 4 samples expressed exon skipped mRNA in 7 liver samples analyzed. CYP2C18 X1 could metabolize tolbutamide, with a relative activity of 0.63±0.08 μmol·min^{-1}·g^{-1} S9, which is relatively higher than that of CYP2C18 X1 transfected CHL cells (0.50±0.05 μmol·min^{-1}·g^{-1} S9)[8], but significant higher than that of HepG2-CYP2C18 X2 or HepG2 cells. In contrast, exon 5 skipped CYP2C18 could not metabolize tolbutamide.

The oxazaphosphorine prodrugs, cyclophosphamide and ifosfamide, are bioactivated in human liver microsome via a 4-hydroxylation reaction catalyzed by CYP2B6, 3A4[14], 2A6, 3A5, and three CYP2C enzymes (2C9, 2C18, 2C19)[15]. There was no surviving HepG2, HepG2-CYP2C18 X1 or X2 cells after exposure to 8 and 16 mmol/L ifosfamide. The relative survival of HepG2-CYP2C18 X1 cells was lower than those of HepG2 cells exposure to 1, 2, and 4 mmol/L of ifosfamide. The relative survival of HepG2-CYP2C18 X2 cells was the same as those of HepG2 cells in 0.5 and 1 mmol/L of ifosfamide but lower than those of HepG2 cells in 2 and 4 mmol/L of ifosfamide. Together, these data indicated that HepG2 cells could metabolically activate ifosfamide, while CYP2C18 X1 significantly increased the cytotoxicity of ifosfamide. Compared to CYP2C18 X1, the exon 5 skipped CYP2C18 X2 could only metabolically activate ifosfamide only at higher concentrations.

Although CYP2C18 is not included, based on the homology modeling of human CYP2 family[16], the deduced substrate recognition site (SRS) 3 for CYP2C18 should locate at amino acid residues 234-240. The exon 5 skipped CYP2C18 X2 lost 59 amino acid residues (from codon 215 to 273) which contains SRS 3, thus may affect its enzyme activity. Payne *et al*[17] had constructed a three-dimensional model to predict the CYP2C18 binding-sites residues and suggested that Arg 97 and Lys 105 are two key cationic residues for sub-

strate binding sites. Additional binding-site residues are Asn 204, Val 102, Phe 476, Thr 364, Pro 363, and Gly 111. Although the exon 5 skipped CYP2C18 retains these residues, it is known that mutations in residues outside the binding site can still disturb the optimal position of the backbone or side chains for key residues. This type of mutation can also alter the accessibility of the substrate to the binding site or even affect the catalytic reactions in some yet unknown mechanisms. By contrast, some mutations at the binding site may not significantly alter the enzyme function[17].

Taken together, we have shown that the expression of exon skipped CYP2C18 mRNA in human liver occured frequently. The full length CYP2C18 X1 could metabolize tolbutamide and ifosfamide, while the exon 5 skipped CYP2C18 X2 could not metabolize tolbutamide and lower concentrations of ifosfamide.

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