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Molecular and functional characterization of flavin-containing monooxygenases in cynomolgus macaque

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ABSTRACT

Flavin-containing monooxygenases (FMOs), drug-metabolizing enzymes essential for the metabolism of endogenous biochemicals and foreign compounds, have been characterized in human (including FMO1-5 and FMO6P), but remain to be investigated in cynomolgus macaque. In this study, cDNAs of cynomolgus FMO1-5 and FMO6 were isolated and characterized. Amino acid sequences of cynomolgus FMO1-5, respectively, shared high sequence identities (94–98%) and were closely clustered in a phylogenetic tree, with human FMO1-5. Eight different transcripts, due to alternative splicing, were isolated for cynomolgus *FMO6*, which is highly identical (~96%) to human *FMO6P*. Among the 10 tissue types analyzed, cynomolgus *FMO1*, *FMO2*, *FMO4*, and *FMO6* were most abundantly expressed in kidney, while cynomolgus *FMO3* and *FMO5* were most abundantly expressed in liver. In kidney and liver, the most abundantly expressed cynomolgus *FMO* genes were *FMO1* and *FMO3*, respectively. Cynomolgus FMO1, FMO2, FMO3, and FMO5 metabolized benzydamine, and FMO1/FMO3 and FMO3 also metabolized methimazole and trimethylamine, respectively. Rates of benzydamine *N*-oxygenation (catalyzed by FMO3) varied (approximately 20-fold) among the 28 cynomolgus livers and were significantly correlated with FMO3 protein expression, indicating that the inter-animal variations in benzydamine *N*-oxygenation might be partly accounted for by the variable FMO3 expression. Cynomolgus FMO6 metabolized benzydamine only slightly, but minimal expression of *FMO6* in all tissue precludes the importance of FMO6 in drug metabolism, unlike cynomolgus FMO1, FMO2, FMO3, and FMO5 which were all functional. Abundant expression of *FMO1* and *FMO3* in kidney and liver, respectively, suggest their importance in drug metabolism in cynomolgus macaque, similar to human.

1. Introduction

Flavin-containing monooxygenase (FMO) is a family of NADPH- and FAD-dependent enzymes important for oxidative metabolism of a wide variety of nitrogen-, sulfur-, or phosphorous-containing xenobiotics, such as drugs, pesticides, and industrial chemicals [1]. Among five human *FMO* genes (*FMO1-5*) identified, *FMO1*, *FMO2*, *FMO3*, and *FMO4* are present in the gene cluster and *FMO5* is located outside this cluster, in the genome [2]. *FMO* gene expression patterns are tissue-, species-, and temporal-specific, which could influence drug metabolism and toxicity. Human *FMO1* is predominantly expressed in kidney, while human *FMO3* is predominantly expressed in liver [3]. Mouse *Fmo1* and *Fmo3* are expressed in liver, but expression of *Fmo3* is limited to females [4,5]. In contrast, human *FMO4* and *FMO5* show widespread expression patterns in various tissues [6].

Human *FMO2* is expressed preferentially in lung, but it is also expressed in skeletal muscle, kidney, and prostate [2]. However, Caucasians and Asians are homozygous for the *FMO2*2A* allele, which contains a nonsense mutation at codon 472, resulting in non-functional, truncated proteins even if translated, due to the generation of a premature termination codon [7]. In contrast, some African Americans (26%) are homozygous or heterozygous for the *FMO2*1* allele, which encodes the full-length catalytically active protein [7,8]. Human *FMO6* gives rise to several different transcripts due to alternative splicing, but none of them appear to encode a functional protein [9], and thus, is likely a pseudogene.

Although FMO1 and FMO3 catalyze common FMO substrates, such as benzydamine [10] and tazatotene [11], they also show distinct substrate specificities, including methionine [12] and trimethylamine [13]. In the case of the latter, loss-of-function mutations in *FMO3* are responsible for the disorder called trimethylamine uria (TMAU) or fish-odor syndrome [14,15]. In

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the affected individuals, the malodorous free amine, trimethylamine (TMA), derived from dietary precursors (e.g. choline and lecithin), cannot be metabolized into the odorless *N*-oxide, resulting in excessive amounts of TMA in breath, sweat, and urine [16,17].

Cynomolgus macaque (*Macaca fascicularis*) is an important primate species widely used in drug metabolism studies. Molecular and functional characteristics of cytochromes P450 (P450s), an important family of drug-metabolizing enzymes, are similar in cynomolgus macaque and human [18]; however, some differences do exist. For example, CYP2C76 is not orthologous to any human P450 and is partly responsible for differences in pitavastatin metabolism between cynomolgus macaque and human [19,20]. Moreover, *CYP1D1* and *CYP2G2*, pseudogenized in human, are expressed in cynomolgus macaque as functional genes [21,22], which might be responsible for species differences in drug metabolism. Therefore, identification and characterization of drug-metabolizing enzymes are essential for understanding the similarities and differences of drug metabolism between model animals and human. In the case of cynomolgus macaque, identification of FMO cDNAs has not been reported, yet.

In this study, we isolated cDNAs encoding orthologs of human FMO1-5 from cynomolgus livers. The analysis also included cynomolgus FMO6, which is highly identical to human FMO6P. The identified cynomolgus FMOs were analyzed for their molecular and functional characteristics by sequence and phylogenetic analyses, tissue expression patterns, and enzymatic analysis of proteins heterologously expressed in *Escherichia coli* (*E. coli*).

2. Materials and methods

2.1. Materials

Benzydamine and methimazole were purchased from Sigma–Aldrich (St. Louis, MO), and trimethylamine was purchased from Wako Pure Chemicals (Osaka, Japan). Oligonucleotides and fluorescent probes were synthesized by Greiner (Tokyo, Japan). Anti-human FMO3 antibody, and horseradish peroxidase-conjugated goat anti-rabbit IgG were purchased from BD Gentest (Woburn, MA). Anti-histidine tag antibody was purchased from BioDynamics Lab. (Tokyo, Japan). All other reagents were purchased from Sigma–Aldrich or Wako Pure Chemicals, unless otherwise specified.

2.2. Tissues and preparation of RNA and microsomes

Tissue samples (brain, lung, heart, liver, kidney, adrenal gland, jejunum, testis, ovary, and uterus) were collected from six cynomolgus macaques (three males and three females, 4–5 years of age, 3–5 kg). This study was reviewed and approved by the Institutional Animal Care and Use Committee of Shin Nippon Biomedical Laboratories, Ltd. From these samples, total RNA was extracted as described previously [19] and used for analysis of tissue expression patterns. Similarly, total RNA was extracted from liver samples from cynomolgus macaques (three males and three females from Cambodia, 4–5 years of age, 3–5 kg) and rhesus macaques (three males from China, 7 years of age, weighing 3–5 kg), and was used to isolate FMO cDNAs. Total RNAs previously extracted from livers of 22 prenatal and postnatal cynomolgus macaques (11, 15, or 19 weeks of gestation; 1, 6, 12, and 18 months postnatal; and 3 years of age) [23] were used to analyze gene expression. Microsomes were prepared from liver samples from 30 cynomolgus macaques (15 males and 15 females from Indochina or Indonesia, 4–9 years of age) and 2 rhesus macaques (males from China, 10–11 years of age) as described previously [24]. Human liver microsomes used in this study were the same samples prepared previously [25].

2.3. Molecular cloning and sequencing

To isolate cynomolgus FMO cDNAs, reverse transcription (RT)-polymerase chain reaction (PCR) was performed using liver total RNA as described previously [19]. Briefly, the first-strand cDNA synthesis was carried out in a reaction containing 1 μg of total RNA, oligo (dT), and SuperScript III RT reverse transcriptase (Invitrogen, Tokyo, Japan) at 50 °C for 1 h. PCR reactions were performed using the RT product as the template with KOD-Plus-Neo DNA polymerase (Toyobo, Osaka, Japan) and a thermal cycler (Applied Biosystems, Tokyo, Japan) according to the manufacturer's protocols. PCR conditions included an initial denaturation at 94 °C for 2 min and 35 cycles at 98 °C for 10 s, 65 °C for 30 s, and 68 °C for 60 s, followed by a final extension at 68 °C for 7 min. The primers used were designed based on the macaque genome data, including 5′-GGCACCAGAAATTACAAGTACATAAAGAGAACA-3′ and 5′-CCATTTAGGAAATCTTCCACTTATAGCACAATC-3′ for FMO1, 5′-CCAAAAGGCAAAAACAAAGGAACTG-3′ and 5′-CAAAGTGTTATGCTGACTAGGACCATTGA-3′ for FMO2, 5′-ATGGGGAAGAAAGTGGCCATCA-3′ and 5′-GTGATTAGGTCAACCCAAGGAAAACAG-3′ for FMO3, 5′-AGCTGTCTAAACCTCCTACTCCTCAACTCA-3′ and 5′-CAGGTTCATCCTCGCCAAAGACTTAC-3′ for FMO4, 5′-CTGGAGATCATGACAAAGAAAAGAATTGC-3′ and 5′-AGTAGATTTCTGGGCAATATGAAACTGAGA-3′ for FMO5, and 5′-ATGAGCAAGAGGGTTGGCATCATC-3′ and 5′-TCTGAACTTTGAGACCTCAAAGACTTTCTC-3′ for FMO6. The amplified products were cloned into pCR4 vectors using Zero Blunt TOPO PCR Cloning Kit for Sequencing (Invitrogen) according to the manufacturer's protocol. The inserts were sequenced using ABI PRISM BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) with an ABI PRISM 3730 DNA Analyzer (Applied Biosystems).

2.4. Sequence analysis

Sequence data were analyzed with DNASIS Pro (Hitachi Software, Tokyo, Japan), the Genetyx system (Software Development, Tokyo, Japan), or Sequencher (Gene Codes Corporation, Ann Arbor, MI). Multiple alignment of amino acid sequences was performed using the ClustalW program. A phylogenetic tree was created by the neighbor-joining method. BLAST (National Center for Biotechnology Information) was used for the homology search. BLAT (UCSC Genome Bioinformatics) was used for analysis of the human and macaque (rhesus) genome data. Amino acid sequences found in GenBank were used in this analysis, including human FMO1 (NP_665683), FMO2 (NP_000837), FMO3 (NP_000838), FMO4 (NP_001503), and FMO5 (NP_714543); rhesus FMO2 (NP_001036242); dog FMO1 (NP_001003061) and FMO3 (NP_001003060); rat FMO1 (NP_036924), FMO2 (NP_653338), FMO3 (NP_445885), FMO4 (NP_653147), FMO5 (NP_653340), and FMO9 (NP_001102936); mouse Fmo1 (NP_034361), Fmo2 (NP_061369), Fmo3 (NP_032056), Fmo4 (NP_659127), Fmo5 (NP_001155237), Fmo6 (NP_001171509), Fmo9 (NP_766432), Fmo12 (NP_001157784), and Fmo13 (NP_001157778); and takifugu FMO (NP_001027928). The FMO amino acid sequences of cynomolgus (FMO1, FMO2, FMO3, FMO4, FMO5, and FMO6) and rhesus (FMO1 and FMO3) macaques were deduced from the cDNAs identified in this study. For analysis of gene structures, cynomolgus FMO1-6 cDNAs identified in this study, and the FMO cDNA sequences found in GenBank were used, including human FMO1 (NM_002021), FMO2 (NM_001460), FMO3 (NM_006894), FMO4 (NM_002022), FMO5 (NM_001461), and FMO6P (NR_002601); and rhesus FMO6 (XM_001094817).

2.5. Measurement of gene expression

Expression levels of cynomolgus *FMO1*, *FMO2*, *FMO3*, *FMO4*, *FMO5*, and *FMO6* were measured in brain, lung, heart, liver, kidney,