Cynomolgus Macaque CYP4 Isoforms Are Functional, Metabolizing Arachidonic Acid

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(Received 8 August 2010/Accepted 6 November 2010/Published online in J-STAGE 19 November 2010)

ABSTRACT. Cytochrome P450 (CYP) is important for metabolism of not only xenobiotics such as drugs, but also endogenous compounds including arachidonic acids. CYP4A11, CYP4F3v2, CYP4F11, and CYP4F45 have been identified in cynomolgus macaque, an animal species widely used for investigation of drug metabolism due to its evolutionary closeness to human. However, their metabolic functions have not been investigated. In this study, proteins were heterologously expressed in *Escherichia coli* and characterized by metabolic assays using arachidonic acids as substrates that are metabolized by CYP4 isoforms in human. The results showed that all four CYPs metabolized arachidonic acids. Therefore, cynomolgus macaque CYP4A11, CYP4F3v2, CYP4F11, and CYP4F45 are functional enzymes.

KEY WORDS: arachidonic acids, cynomolgus macaque, cytochrome P450, liver, macaque.

J. Vet. Med. Sci. 73(4): 487–490, 2011

Cynomolgus macaque is a species widely used in pharmacological and toxicological studies due to its evolutionary closeness to human. Due to the importance of cynomolgus macaque, cytochrome P450 (CYP) cDNAs have been identified and characterized in this species. Cynomolgus macaque CYPs have high sequence identities and similar metabolic function to human CYPs in the same subfamily [4], except for CYP2C76, which does not correspond to any human CYP [8]. Cynomolgus macaque CYP4A11, CYP4F3v2, CYP4F11, and CYP4F45 have been identified [10], but their functions remain to be investigated.

The CYP4F subfamily, and to some extent CYP4A, is responsible for the metabolism of arachidonic acid and its metabolites such as leukotrienes, prostaglandins, epoxyeicosatrienoic acids, and hydroperoxyeicosatetraenoic acids, and hydroxyeicosatetraenoic acids (HETEs) [7]. These metabolites have roles in many biological processes such as edema, allergic response, inhibition of platelet aggregation, fever generation, and pain response [7]. Considering that cynomolgus macaque CYPs are highly homologous to human CYPs, cynomolgus CYP4A and CYP4F isoforms might also metabolize arachidonic acids. A previous study indicated the involvement of CYP4F-like protein in metabolism of ebastine, an H₁-antihistamine prodrug, in the small intestine of cynomolgus macaque [2]; however, no CYP4Fs have been identified in cynomolgus macaques. In this study, therefore, cynomolgus macaque CYP4A11, CYP4F3v2, CYP4F11, and CYP4F45 were analyzed in metabolic assays using arachidonic acids (as substrates) and recombinant proteins.

To prepare the cynomolgus macaque CYP4A11, CYP4F3v2, CYP4F11, and CYP4F45 recombinant pro-

teins, expression plasmids were prepared and expression of proteins was carried out as described previously [5, 9]. Briefly, expression plasmids were prepared by polymerase chain reaction (PCR) using plasmids containing cynomolgus macaque CYP4A11 (DQ074797), CYP4F3v2 (DQ074799), CYP4F11 (DQ074800), or CYP4F45 (DQ074798) cDNA template. The PCR primers (Table 1) were designed to modify the N-terminus of the protein to enhance protein expression. The PCR products were subcloned into the pCW vector containing human NADPH-CYP reductase cDNA. Insert sequences were confirmed by sequencing using the ABI PRISM BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) and an ABI PRISM 3730 DNA Analyzer (Applied Biosystems). Expression of the CYP proteins in *E. coli*, preparation of membrane fractions, and measurement of CYP and reductase content were performed as described previously [5, 9].

Arachidonic acid assays were performed in mixtures (0.5 ml) containing CYP protein (50 pmole) or cynomolgus macaque liver microsomes (0.5 mg, XenoTech, LLC, Lenexa, KS, U.S.A.), an NADPH-generating system (1.55 mM NADP⁺, 3.3 mM glucose 6-phosphate, and 0.4 unit/ml glucose 6-phosphate dehydrogenase), and ¹⁴C-arachidonic acids (16 kBq/100 nmol/ml), in 50 mM potassium phosphate buffer (pH 7.4). The reactions were incubated at 37°C for 60 min, and were terminated by addition of 0.25 ml of ice-cold acetonitrile. The resultant mixtures were centrifuged (7,500 × *g*, 4°C, 10 min). The unchanged compound and metabolites in the supernatants were detected by high-performance liquid chromatography (HPLC) using a reverse-phase Inertsil ODS-3 analytical column (4.6 × 250 mm, 5 μm particle size; GL Sciences, Tokyo, Japan). The mobile phase used was a linear gradient system consisted of 50–100% acetonitrile in 0.1% acetic acid in the time range from 0.0 to 40.0 min, by mixing solvent A (0.1% acetic acid

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Table 1. Primers used for preparation of expression plasmids

CYP		Sequence (5'-3')
CYP4A11	F	GGAATTCCATATGGCTCTGTTATTAGCAGTTTTTAGCAGACTCCTGGGTAGTGTG
	R	GCTCTAGACCCTCAGAGCTGGTCCCTTG
CYP4F3v2	F	GGAATTCCATATGGCTCTGTTATTAGCAGTTTTTCTGGGCCTCGGGCCGGT
	R	GCTCTAGACTCAGCTCAGGGGCTCCAC
CYP4F11	F	GGAATTCCATATGGCTCTGTTATTAGCAGTTTTTCTGGGCCTCGGGCCGGT
	R	GCTCTAGATGGGTGGGTGGGTAGGACAG
CYP4F45	F	GGAATTCCATATGGCTCTGTTATTAGCAGTTTTTCTGGGCCTCGGGCCGGT
	R	GCTCTAGACTCAGCTCAGGGGCTCCAC

F, forward primer; R, reverse primer.

Table 2. Metabolism of arachidonic acids by cynomolgus macaque CYP4 isoforms

CYP	Rate of arachidonic acid metabolism	
	(nmol/min/nmolCYP)	
CYP4A11	0.63	
CYP4F3v2	2.98	
CYP4F11	4.59	
CYP4F45	0.81	
Liver	0.82	

Metabolic assays were carried out using arachidonic acids (100 nmol/ml) as substrates, and cynomolgus macaque liver microsomes (1.03 nmol CYP/mg protein/ml) or protein (100 pmol CYP/ml; CYP4A11, CYP4F3v2, CYP4F11, and CYP4F45) heterologously expressed in *E. coli*. Results are presented as means of duplicate determinations.

solution) with solvent B (0.1% acetic acid in acetonitrile solution), at the flow rate of 1.0 ml/min. Re-equilibration was performed from 40.1 to 50.0 min at 50% solvent B. The HPLC eluent was introduced into a Packard 515TR radiodetector (Perkin Elmer Life and Analytical Sciences, Boston, MA, U.S.A.). Metabolism of arachidonic acids was assessed by measuring the disappearance of arachidonic acid(s) in the reaction.

The results showed substantial arachidonic acid metabolism by cynomolgus macaque CYP4A11, CYP4F3v2, CYP4F11, and CYP4F45 (Table 2), indicating that these CYP4 enzymes are functional. Human CYPs highly homologous to these macaque CYPs, CYP4A11, CYP4F2, CYP4F3, and CYP4F11, are involved in metabolism of

arachidonic acids. Human CYP4A11 is involved in ω -hydroxylation of arachidonic acid, and the subsequent formation of 20-HETE, although it metabolizes lauric acid to a greater extent, another saturated fatty acid [3]. Similarly, human CYP4F2/3v2 and CYP4F12 are also involved in the formation of 20-HETE and 18-HETE, respectively [3]. In contrast, human CYP4F11 metabolizes arachidonic acids much less efficiently in comparison to other CYP4F enzymes [6]. Involvement of cynomolgus macaque CYP4A and CYP4F enzymes in metabolism of arachidonic acid suggests that CYP4A and CYP4F enzymes in cynomolgus macaque and human share similar metabolic properties.

The functional similarities of CYP4A and CYP4F isoforms might be, at least partly, explained by sequence identity, phylogeny, and genomic organization. BLAST (National Center for Biotechnology Information) homology search results showed high sequence identities of CYP4A and CYP4F amino acids (91–95%) between cynomolgus macaque and human (Table 3). In a phylogenetic tree created based on the CYP4A and CYP4F amino acids of cynomolgus macaque and human using DNASIS Pro (Hitachi Software, Tokyo, Japan), cynomolgus macaque CYP4A11, CYP4F3v2, CYP4F11, and CYP4F45 were closely clustered with the corresponding human CYPs (Fig. 1). These results indicate evolutionary closeness of CYP4A and CYP4F isoforms between cynomolgus macaque and human.

The genomic organization of *CYP4As* and *CYP4Fs* in the genome was analyzed using BLAT (UCSC Genome Bioinformatics). The analysis showed that *CYP4Fs* form a gene cluster in the macaque and human genome, and that the

Table 3. Sequence identities of cynomolgus macaque and human CYP4 amino acids

Human	Cynomolgus macaque				
	CYP4A11	CYP4F3v2	CYP4F11	CYP4F12	CYP4F45
CYP4A11	95	46	46	45	47
CYP4A22	92	45	45	45	46
CYP4F2	45	92	84	81	94
CYP4F3v2	46	95	84	80	92
CYP4F8	44	80	77	77	80
CYP4F11	46	85	91	82	87
CYP4F12	46	81	80	92	81
CYP4F22	45	67	65	64	67

The BLAST program was used to compare CYP4A and CYP4F amino acid sequences in cynomolgus macaque and human. The highest sequence identity is indicated by bold numbers for each cynomolgus macaque CYP.

Fig. 1. Phylogenetic tree of CYP4A and CYP4F isoforms in cynomolgus macaque and human. The phylogenetic tree was created using DNASIS Pro with cynomolgus macaque and human CYP4A and CYP4F amino acid sequences found in GenBank. The scale bar indicates 0.1 amino acid substitutions per site for distance measurement.

position and direction of the functional genes correspond well between macaque and human (Fig. 2), similar to *CYP4A* (data not shown), suggesting a conserved genomic structure of *CYP4As* and *CYP4Fs* in these species. Furthermore, their tissue expression patterns are similar in cynomolgus macaque and human. In cynomolgus macaque brain, lung, heart, liver, kidney, adrenal gland, small intestine, testis, ovary, and uterus, CYP4A11 and CYP4F3v2 mRNAs are preferentially expressed in liver and kidney, whereas CYP4F11 and CYP4F45 mRNAs are preferentially expressed in liver, kidney, and small intestine [10]. Similarly, human CYP4A11, CYP4F2, and CYP4F3v2 mRNAs are expressed in liver and kidney [3]. The resemblance of *CYP4A* and *CYP4F* genomic organization and tissue expression patterns indicates the molecular similarity of these genes between cynomolgus macaque and human.

This study revealed the involvement of CYP4A and CYP4F in metabolism of arachidonic acids. In human, arachidonic acids are metabolized by numerous CYPs in the CYP1-4 family [7]. Hence, the involvement of other CYPs in the CYP1-3 family need to be analyzed for arachidonic

acid metabolism, to assess the contribution of CYP4A11, CYP4F3v2, CYP4F11, and CYP4F45 to arachidonic acid metabolism in cynomolgus monkey liver. To this end, cynomolgus monkey CYP2J2 and CYP4F12, which were not analyzed in metabolic assays due to the unavailability of their recombinant proteins, also need to be investigated, since their human orthologs (CYP2J2 and CYP4F12) metabolize arachidonic acids.

Considering that some cynomolgus macaque CYP4s (with metabolic capabilities) are expressed in small intestine at the mRNA level, they might play the roles as metabolic enzymes in this tissue. Indeed, a previous study showed that a CYP4F enzyme is responsible for ebastine metabolism in the small intestine of cynomolgus macaque [2]. Similarly, ebastine is metabolized by a CYP4F enzyme (CYP4F12) in human small intestine [1]. The effect of first-pass metabolism on orally administered ebastine takes place largely in the intestine [2], suggesting the important role of CYP4Fs in the intestine.

In conclusion, cynomolgus macaque CYP4A11, CYP4F3v2, CYP4F11, and CYP4F45 are functional drug-metabolizing enzymes, metabolizing arachidonic acids. Human CYP4A and CYP4F enzymes also metabolize arachidonic acids, indicating that cynomolgus macaque and human share CYP4A and CYP4F metabolic properties. Moreover, the high sequence identities and the 1-to-1 relationships in the phylogenetic tree between cynomolgus macaque and human, indicated the evolutionary closeness of CYP4A and CYP4F isoforms between the two species, which was further supported by conserved genomic structures between macaque and human. The results presented in this study suggest the evolutionary closeness and functional resemblance of CYP4A and CYP4F enzymes between cynomolgus macaque and human. The recombinant proteins prepared in this study could be useful for further investigation into their metabolic properties, which will help to increase our understanding of the CYP enzymatic properties in cynomolgus macaque.

ACKNOWLEDGMENTS. We greatly thank Dr. Ryoichi Nagata and Dr. Koichiro Fukuzaki for their support of this work, and Mr. Patrick Gray for reviewing the manuscript.

Macaque chromosome 19

Human chromosome 19

Fig. 2. Genomic structure of the macaque *CYP4Fs*. Analysis of the genome data, using the BLAT program, showed that *CYP4Fs* form a gene cluster in the macaque genome similar to that in human. Macaque *CYP4F3*, *CYP4F11*, *CYP4F12*, and *CYP4F45*, are located in positions and directions corresponding to human *CYP4F3*, *CYP4F11*, *CYP4F12*, and *CYP4F2*, respectively.

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