Expression of Cytochromes P450 in Fetal, Infant, and Juvenile Liver of Cynomolgus Macaques

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Summary: Preclinical data of fetal, infant, and juvenile animals are important for the prediction of drug toxicity in fetuses and children. However, expression of drug-metabolizing enzymes, including cytochromes P450 (CYPs), have not been fully investigated in fetal, infant, or juvenile liver of the cynomolgus macaque, an animal species important for preclinical studies. In this study, hepatic expression of 20 cynomolgus macaque CYPs (mfCYPs) in the CYP1–4 subfamilies that are relevant to drug metabolism was measured in fetuses, infants, and juveniles using DNA microarrays. Expression of most mfCYPs, including those moderately or abundantly expressed in postnatal livers such as mfCYP2A23, mfCYP2A24, mfCYP2B6, mfCYP2C9, mfCYP2C19, mfCYP2C76, mfCYP2D17, mfCYP2E1, mfCYP3A4, and mfCYP3A5 was much less abundant in fetal livers, but increased substantially after birth. In contrast, expression of mfCYP2C8 in fetal livers was not substantially different from postnatal livers. Since human CYP3A7 is expressed more abundantly in fetal livers than in adult livers, mfCYP3A7, an ortholog of human CYP3A7, was analyzed by quantitative polymerase chain reaction. Expression of mfCYP3A7 in fetal livers was much lower than that in postnatal livers, and greatly increased after birth, unlike the expression of human CYP3A7. These results indicate that expression of most mfCYPs examined was low in fetal livers, but increased greatly in postnatal livers, with a few exceptions such as mfCYP2C8.

Keywords: cytochrome P450; expression; cynomolgus macaque; liver; fetus; juvenile

Introduction

It is well known that drug disposition and response are different between pediatric and adult populations, and this can lead to adverse drug reactions, including grey baby syndrome with chloramphenicol, kernicterus with sulfadiazine, neurotoxicity with hexachlorophene, fatality with benzyl alcohol, and cardiotoxicity with anthracyclines. The incidence of these adverse drug reactions has led to legislative changes to encourage clinical trials in pediatric populations in the United States (FDA Modernization Act 1997), Best Pharmaceuticals for Children Act (2002), and in Europe [Regulation EC No. 1901/2006 on Medicinal Products for Pediatric Use (2006)]. Similarly, the acquisition of preclinical juvenile animal data has been encouraged or is sometimes required for prediction of toxicity in children. Variability in drug response is partly caused by pharmacokinetic and pharmacodynamic determinants. Analysis of 45 commonly used drugs indicated that the half-lives of these drugs were 3–9 times longer in neonates than in adults, possibly due to differences of expression of drug-metabolizing enzymes in fetal, neonatal, and adult livers. Cytochrome P450 (CYP) is a family of drug-metabolizing enzymes involved with three-quarters of drugs that are eliminated through metabolism. In humans, approximately 20 CYP isoforms in the CYP1–4 families are largely responsible for drug metabolism. Most of these CYP
isoforms are expressed much less abundantly in fetal livers than in adult livers. In contrast, human CYP3A7 accounts for the majority of total CYP content in fetal and neonatal liver.\(^3\)\(^-\)\(^5\) In fetal liver, the expression of human CYP3A7 is detectable as early as 50 to 60 days of gestation,\(^6\)\(^-\)\(^8\) and expression continues to increase during pregnancy, reaching a maximum at 2 weeks after birth; expression then decreases to a low level during adult stages.\(^5\) On the other hand, human CYP3A4, a dominant CYP3A in adult liver, is expressed at low level in human fetal liver, but expression increases greatly after birth.

The cynomolgus macaque (Macaca fascicularis) is a non-human primate species with great importance in preclinical studies of drug metabolism and toxicity tests due to its evolutionary closeness to humans. In particular, the cynomolgus macaque is a useful animal model for evaluating drug-metabolizing enzymes, as is evident in thalidomide toxicity, which results in teratogenicity in cynomolgus macaques and humans, but not in the rat.\(^8\)

In the cynomolgus macaque, more than 20 CYPs have been identified, and most of those isoforms have sequence identities and metabolic properties similar to those in humans.\(^9\) However, investigation of expression and function of drug-metabolizing enzymes has been limited to adult stages in the cynomolgus macaque. Therefore, expression of cynomolgus macaque CYPs (mfCYPs) in fetal, infant, and juvenile liver needs to be investigated if this species is to be used in preclinical pediatric drug studies. In this paper, we designate mfCYP2C20, mfCYP2C43, mfCYP2C75, mfCYP3A8, and mfCYP4F45 as mfCYP2C8, mfCYP2C9, mfCYP2C19, mfCYP3A4, and mfCYP4F2, respectively, as recommended by the P450 Nomenclature Committee.\(^9\)

In this study, expression of 20 mfCYPs was measured in fetal, infant, and juvenile livers using DNA microarrays. Moreover, expression of mfCYP3A47, an ortholog of human CYP3A7, was analyzed by quantitative polymerase chain reaction (qPCR), along with mfCYP3A4 and mfCYP3A5, during the same developmental stages.

### Materials and Methods

**Animals, tissues, and RNA isolation:** Liver samples were collected from 22 cynomolgus macaques (purchased in China or Indonesia) at 11, 15, or 19 weeks of gestation (the second to third trimester); at 1, 6, 12, and 18 months postnatal; and at 2–3 years of age (Table 1), following the standard protocols established at Shin Nippon Biomedical Laboratories, Ltd. (Kagoshima, Japan). Although the relationship of age between cynomolgus macaques and humans is not precisely known, 1 year for the cynomolgus macaque would be roughly 3 years for a human. This study was reviewed and approved by the Institutional Animal Care and Use Committee of Shin Nippon Biomedical Laboratories, Ltd. Liver samples were collected and flash-frozen in liquid nitrogen. Total RNA was isolated from these liver samples using TRIzol (Invitrogen, Carlsbad, CA) or RNaseasy Mini Kit (Qiagen, Valencia, CA), according to the manufacturer’s protocols. The quality of each RNA sample was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), according to the manufacturer’s protocols.

**DNA microarray analysis:** Gene expression analysis was performed using a low-density DNA microarray containing the probes of 20 mfCYPs, namely, mfCYP1A, mfCYP2A23, mfCYP2A24, mfCYP2B6, mfCYP2C18, mfCYP2C8, mfCYP2C9, mfCYP2C19, mfCYP2C76, mfCYP2D17, mfCYP2E1, mfCYP2J2, mfCYP3A4, mfCYP3A5, mfCYP4A43, mfCYP4A11, mfCYP4F2, mfCYP4F3, mfCYP4F11, and mfCYP4F12, as described previously.\(^10\) Expression data were filtered to remove background signals and were normalized globally per DNA microarray. Mean expression of each gene was calculated based on signals detected by the DNA microarray probe spotted in triplicate. The expression levels in fetal and postnatal animals (≤18 months) were compared with that of the reference group (animals of 2–3 years), and the ratios were log\(_2\) transformed.

**Quantitative PCR:** To measure expression of mfCYP3A4, mfCYP3A5, and mfCYP3A7 in fetal and postnatal liver samples, real-time reverse-transcription PCR was performed using gene-specific primers and probes as described previously.\(^11\) For mfCYP3A4, gene-specific primers and probe were designed based on the cDNA sequence of mfCYP3A4 (NM_001195758), while those of mfCYP3A5 were the same as described previously.\(^12\) To design primers and probes for mfCYP3A7, the rhesus CYP3A7 cDNA (NM_001195758) sequence was used, since information on the mfCYP3A7 cDNA sequence was not available (see [http://drnelson.uthsc.edu/cytochromeP450.html](http://drnelson.uthsc.edu/cytochromeP450.html)). The CYP cDNAs of the rhesus macaque (a closely related species of the cynomolgus macaque) are generally highly identical (>99%) to orthologous CYP cDNAs of the cynomolgus macaque.\(^9\) The primers used were 5′-CACC-CTGGTGCTCCTCTATT-3′ and 5′-GGCTGTCGACCATAAACAG-3′ for mfCYP3A4, 5′-CAGCTGGTGCTCCTCTATC-3′ and 5′-TGTCGGGATCTGTGATGGT-3′ for

| Table 1. Characteristics of the 22 cynomolgus macaques |
|----------------|----------------|
| Stage          | N   | Sex         |
| Fetal          |     |             |
| 11 weeks of gestation | 1  | Female 1   |
| 15 weeks of gestation | 2  | Male 1, Female 1 |
| 19 weeks of gestation | 2  | Male 1, Female 1 |
| Postnatal      |     |             |
| 1 months       | 2   | Male 1, Female 1 |
| 6 months       | 2   | Male 2     |
| 12 months      | 5   | Male 4, Female 1 |
| 18 months      | 3   | Male 2, Female 1 |
| 2–3 years      | 5   | Male 5     |
Results and Discussion

Using a low-density DNA microarray containing probes for 20 mfCYPs, age-dependent mfCYP expression in liver was analyzed using 22 cynomolgus macaques, including fetuses (11, 15, and 19 weeks of gestation), infants (1 and 6 months postnatal), and juveniles (12 and 18 months postnatal and at 2–3 years of age) (Table 1). Each mfCYP expression level in fetal, infant, and juvenile animals (≤18 months postnatal) was compared to and described relative to mean expression levels in the animals aged 2–3 years that were used as the reference group for comparison. The heat map for mfCYPs showed a distinguishable expression pattern between prenatal and postnatal stages: expression of most mfCYPs was low in fetal livers, but increased greatly in postnatal livers (Fig. 1), especially those expressed moderately or abundantly in postnatal liver (mfcYP2A23, mfcYP2A24, mfcYP2B6, mfcYP2C9, mfcYP2C19, mfcYP2C76, mfcYP2D17, mfcYP2E1, mfcYP3A4, and mfcYP3A5).

mfcYP1A1 hepatic expression was very low during the fetal stages, greatly increased during the early postnatal stage, reached the maximum at 12 months old, and decreased slightly thereafter (Fig. 2). mfcYP1A1 is expressed much more abundantly than mfcYP1A2 in adult livers.13,14 In contrast, mfcYP1A2 is a predominant CYP1A in adult human liver. Human CYP1A2 is not expressed, or is barely expressed, during fetal and early neonatal stages, but it is clearly expressed in infants aged 1–3 months.15 These results indicate that the developmental expression pattern in liver from fetal to postnatal stages is similar for mfcYP1A1 and human CYP1A2.

Hepatic expression levels of mfcYP2A23, mfcYP2A24, mfcYP2B6, mfcYP2C9, mfcYP2C19, mfcYP2C76, mfcYP2D17, mfcYP2E1, mfcYP3A4, and mfcYP3A5 greatly increased from fetal to early postnatal stages (Fig. 2). Similarly, in humans, expression levels of CYP2C9, CYP2C19, and CYP2D6 proteins were low in fetal livers, but drastically increased in postnatal livers.16,17 Expression of human CYP2C9 and CYP2C19 is regulated by the pregnane X receptor (PXR), the constitutively active receptor (CAR), and the glucocorticoid receptor, but only CAR-binding sites at upstream regulatory regions are functional.18–20 CAR expression is substantially lower in fetal and neonatal liver than in adult liver,21 indicating that the role of CAR is minimal in fetal liver. In contrast, expression and function of these transcription factors have not been investigated in the cynomolgus macaque fetus or neonate. To understand the regulatory mechanism for expression of mfcYP2C9/19 in fetal and neonatal liver, it is of great importance to analyze the roles of PXR, CAR, and glucocorticoid receptor for mfcYP2C9/19 expression.

mfcYP2C18, mfcYP2J2, mfcYP4A11, mfcYP4F2, mfcYP4F3, and mfcYP4F11 were also expressed at low levels in fetal liver and expression levels increased in postnatal liver, but the change in gene expression levels from fetal to postnatal stages was relatively small (Fig. 2). In contrast, expression levels of mfcYP2C8, mfcYP3A43, and mfcYP4F12 were not substantially different during the fetal and postnatal stages (Fig. 2). mfcYP3A43 encodes a functional enzyme that metabolizes midazolam, but its expression in adult liver is much lower than those of mfcYP3A4 and mfcYP3A5.22 mfcYP4F12 is predominantly expressed in the adult liver and jejunum,22 but the function of the protein remains to be characterized. mfcYP2C8, an ortholog of human CYP2C8, is relatively abundantly expressed in adult liver and encodes a functional enzyme that metabolizes drugs such as paclitaxel,11 indicating that mfcYP2C8 is also expected to
be expressed abundantly in fetal liver. In humans, CYP2C8 is not expressed, or is expressed at an undetectable level, in fetal liver, indicating that regulation of CYP2C8 expression in liver during development might be different between macaques and humans, although a final conclusion cannot be made without further investigation using a larger number of fetal liver samples collected at more time points.

CYP3A7 is a dominant CYP3A expressed in human fetal livers. Our DNA microarray did not contain a probe for mCYP3A7, an ortholog of human CYP3A7. Therefore,
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The expression patterns of CYP3A7 in liver during development were found to be different between cynomolgus macaques and humans, indicating that different molecular mechanisms are involved in regulation of CYP3A7 transcriptional switches from fetal to postnatal stages. Recent studies have shown that regulation of gene expression during development is associated with dynamic epigenetic modifications, including DNA methylation and histone modifications. In mice, a transcriptional switch during liver maturation from fetal CYP3A (Cyp3a16) to adult CYP3A (Cyp3a11) is partly regulated by histone methylation. Previous studies in humans indicated that differential regulation of CYP3A7 and CYP3A4 is partly accounted for by interactions of transcription factors with the proximal promoters of these genes, although they are regulated by the same transcription factors in vitro. For example, nuclear factor-I and HNF3\(\beta\) play roles in regulation at the proximal promoter of CYP3A7, but not of CYP3A4. Furthermore, variant isoforms of nuclear factor-I found in human prenatal and postnatal liver regulate the expression of CYP3A4 and CYP3A7 differently in in vitro assays. CYP3A developmental expression is most likely regulated by epigenetic modifications as well as by cis- and trans-acting factors.

In conclusion, we obtained expression profiles of 20 mCYPs in livers from fetal and postnatal cynomolgus macaques (up to 2–3 years of age) using a DNA microarray. As is also the case in humans, expression of most mCYPs increased drastically from fetal to postnatal stages, including mCYP2C9, mCYP2C19, mCYP2C76, mCYP2D17, mCYP3A4, and mCYP3A5, which are expressed either modestly or abundantly in adult livers. Furthermore, qPCR analysis showed that mCYP3A7 was also expressed at a low level in fetal liver and that its expression level increased greatly in postnatal livers; similar profiles were found for mCYP3A4 and mCYP3A5. This study is expected to increase the understanding of drug metabolism in fetal, infant, and juvenile macaques and to provide useful information for drug metabolism and toxicity assessments using fetal and juvenile macaques.

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