EXPRESSION PROFILES OF DRUG-METABOLIZING ENZYME CYP3A AND DRUG EFFLUX TRANSPORTER MULTIDRUG RESISTANCE 1 SUBFAMILY mRNAs IN RAT SMALL INTESTINE

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ABSTRACT:
The purpose of this study is to examine the expression profiles of CYP3A1, CYP3A2, CYP3A9, and CYP3A18 mRNAs as well as multidrug resistance (mdr1a and mdr1b) mRNAs in the liver and small intestine of normal male Wistar rats using a reverse transcription-polymerase chain reaction (PCR). In the rat liver, the PCR products for CYP3A1, CYP3A2, and CYP3A18 were readily detectable, whereas CYP3A9 was slightly and mdr1a and mdr1b barely detected. Surprisingly, no PCR products for CYP3A1 and CYP3A2 were detected in the small intestine, but those for CYP3A9, CYP3A18, and mdr1a were readily detectable, and a faint band for mdr1b was also observed. Both CYP3A9 and CYP3A18 levels were found to be high in the duodenum and decreased from the top to bottom of the gut, indicating regional differences in both CYP3A9 and CYP3A18 expression in the small intestine. In contrast, mdr1a expression increased gradually from the upper to lower intestine. Consequently, it was suggested that drug metabolism in the small intestine of normal rats was mediated by CYP3A9 and CYP3A18 rather than CYP3A1 and CYP3A2. Also, regional differences of CYP3A9, CYP3A18, and mdr1a expression were found in the small intestine. The distributions of CYP3A9 and CYP3A18 were different from the distribution of mdr1a, suggesting the cooperative action of drug clearance pathways. This information is important to drug metabolism research based on ex vivo and in vivo studies using rats.

The cytochrome P450 (P450)-dependent microsomal mono-oxygenase system consists of a flavoprotein, cytochrome P450 reductase, and a multigene superfamily of hemeproteins (the cytochromes P450). Cytochromes P450 belonging to the CYP3A subfamily are among the most abundant, being predominantly expressed in the liver, but are also present in the gut, leukocytes, and brain (Miksys and Tyndale, 2002; Ding and Kaminsky, 2003). In addition, these CYP3A enzymes catalyze the 6β-hydroxylation of steroids such as cortisol, testosterone, and estradiol and are responsible for the metabolism of numerous drugs, including psychotropic, cardiac, analgesic, hormonal, immunosuppressant, antibiotic, and anticancer drugs. Thus, the CYP3A subfamily plays an important role in the detoxification of endogenous substances and xenobiotics.

In the rat, four CYP3A cDNAs have been recently characterized: CYP3A1 [also referred to as CYP3A23 (Kirita and Matsubara, 1993; Komori and Oda, 1994)], CYP3A2, CYP3A9, and CYP3A18. CYP3A1 had been identified in the liver after the treatment of male rats with a synthetic steroid, pregnenolone 16α-carbonitrile (Gonzalez et al., 1986). CYP3A9 is the most recently cloned member of the family, and its mRNA was detected only in adult rats, with a higher level of expression in females (Wang et al., 1996; Mahnke et al., 1997). CYP3A18 is expressed predominantly in males (Strotkamp et al., 1995; Nagata et al., 1996). However, their existence in rat small intestine has been reported using various types of experimental methods, whereas some have reported the CYP3A subfamily was not expressed in rat small intestine (Watkins et al., 1987; de Waziers et al., 1990; Kolars et al., 1992; Zhang et al., 1996; Chiba et al., 1997). Since various types of reports exist, an understanding of their expression profiles is very important to drug metabolism research based on ex vivo and in vivo studies using rats.

It has been demonstrated that the substrate specificity of CYP3A4 in humans overlapped with that of the MDR1 gene (mdr1a and mdr1b in rat) product, the multidrug efflux transporter P-glycoprotein. It is now well accepted that P-glycoprotein is expressed in normal tissues with excretory function, i.e., intestine, liver, kidney, etc., and P-glycoprotein expressed throughout the body plays an important role in the pharmacokinetics of drugs that are substrates for P-glycoprotein (Fromm, 2002; Lin, 2003). Therefore, the impact of P-glycoprotein on pharmacokinetics as well as CYP3A4 has increased, and its expression profiles are also important.

In this study, the expression profiles of CYP3A1, CYP3A2, CYP3A9, and CYP3A18 mRNAs as well as mdr1a and mdr1b mRNAs were examined in both the liver and small intestine of normal male Wistar rats using a reverse transcription-polymerase chain reaction (RT-PCR).
Materials and Methods

Animals. Nine-week-old male Wistar rats (240–250 g) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). After an overnight fast with free access to water, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (Nembutal injection; Dainippon Pharmaceutical Co., Ltd., Osaka, Japan), and sacrificed by exsanguination via the aorta in the abdomen. Animal experiments were carried out in accordance with the Guidelines for Animal Experimentation at Kyoto Pharmaceutical University.

RNA Extraction. The liver was excised quickly after exsanguination, and parts (3-mm squares) were cut out. In the case of the small intestine, it was excised quickly after exsanguination and rinsed in ice-cold 0.9% NaCl solution. The small intestine (total of ca. 90 cm) was divided into three parts: upper (almost corresponding to the duodenum, ~30 cm immediately distal to the pyloric valve), middle (almost corresponding to the jejunum, the 30 cm between the upper and lower sites), and lower (almost corresponding to the ileum, the last 30 cm before the cecum). Each part was re-divided into three (about 10 cm each), and the centers (1 cm distal) were cut out to obtain nine segments. These were designated as S1, S2, S3, S4, S5, S6, S7, S8, and S9, in the direction from the upper to lower intestine of normal rats, and stored at ~80°C until the RNA extraction.

For the extraction of RNA, first the aliquots (3-mm squares) of tissue samples were homogenized in RNA later solution using a pestle, then their total RNA was extracted using a GenElute Mammalian Total RNA kit (Sigma-Aldrich) according to previous reports (Takara et al., 2002, 2003a,b) with modifications.

RT-PCR. RT-PCR analysis was performed according to modifications described in previous reports (Takara et al., 2002, 2003a,b). Aliquots (0.3 μg) of total RNA were used for reverse transcription with an RNA PCR kit (AMV) ver. 2.1 (Takara Shuzo Co., Ltd., Shiga, Japan). PCR amplification was initiated by one cycle of 95°C for 1 min followed by 25 cycles for CYP3A9 and GAPDH, 28 cycles for CYP3A1 and CYP3A2, and 30 cycles for CYP3A18 and mdr1b (for mdr1a and mdr1b). PCR products were separated for amplification of CYP3A and the mdr1 subfamily, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are listed in Table 1 (Vos et al., 1998; Hoen et al., 2000; Yokogawa et al., 2002) and were synthesized by Prolog K. K. Japan (Kyoto, Japan). PCR amplification of cDNA was performed in a total reaction volume of 25 μl including 5 μl of RT product using an RNA PCR kit (AMV) ver. 2.1 (Takara Shuzo Co., Ltd., Shiga, Japan). The RT reaction was conducted in 15 μl of RT reaction mixture, and this was incubated at 30°C for 10 min and subsequently at 42°C for 30 min in a thermal cycler (iCycler Thermal Cycler; Bio-Rad, Hercules, CA). The RT reaction was terminated by heating at 99°C for 5 min, followed by cooling at 5°C for 5 min in a thermal cycler (Bio-Rad), giving the RT product.

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Results

Expression Profiles of CYP3A and mdr1 Subfamily mRNAs in Rat Liver and Small Intestine. The mRNA expression profiles of CYP3A and the mdr1 subfamily were compared in the liver and small intestine (Fig. 1). In normal rat liver, the PCR products for CYP3A1, CYP3A2, and CYP3A18 were readily detectable, whereas little CYP3A9 mRNA was detected (Fig. 1A). In addition, faint bands were obtained for mdr1a and mdr1b mRNAs. Surprisingly, no PCR products for CYP3A1 and CYP3A2 mRNAs in the small intestine of normal rats were detected (Fig. 1B). In contrast, the PCR products for CYP3A9, CYP3A18, and mdr1a mRNAs were readily detectable, and a faint band for mdr1b was also observed.

Regional Differences in CYP3A9 mRNA Expression in Rat Small Intestine. The CYP3A9 mRNA expression increased in the direction from the upper to lower intestine of normal rats, was significantly stronger in the S3 than in the S1 region, and subsequently declined in the S4 region (Fig. 2). The expression from S5 to S9 was almost stable, and not significantly different from that in the S1 region. These levels were similar to those of CYP3A9 in normal liver, compared on a per 0.3-μg total RNA basis.

Regional Differences in CYP3A18 mRNA Expression in Rat Small Intestine. The CYP3A18 mRNA expression increased in the direction from the upper to lower intestine of normal rats (Fig. 3). The expression in S3 was maximal and significantly higher than that in S1, and the expression in the region from S4 to S9 was almost stable. In addition, CYP3A18 mRNA expression throughout the small intestine was remarkably weaker than that in liver, compared on a per 0.3-μg total RNA basis.

Regional Differences in mdr1a mRNA Expression in Rat Small Intestine. From the upper to lower intestine, the mdr1a mRNA expression in normal rats gradually increased (Fig. 4), with the expression in the lower region markedly stronger than that in the upper or middle region. Moreover, the mdr1a mRNA expression level was higher in the small intestine, especially the lower region, than liver, compared on a per 0.3-μg total RNA basis.

Discussion

Consistent with previous reports (Mahnke et al., 1997; Rowlands et al., 2000), the expression of CYP3A1, CYP3A2, CYP3A9, and CYP3A18 mRNAs was observed in the liver of normal male rats (Fig. 1A). Unexpectedly, no signals for CYP3A1 and CYP3A2 were detected in the small intestine of normal rats, but CYP3A9 and CYP3A18 mRNA were observed (Fig. 1B). To rule out inhibition of the PCR caused by the primer interaction, other primer pairs, reported
however, their signals were also unable to be detected, even using the samples derived from different sites of the normal small intestine (data not shown). Is CYP3A1 or CYP3A2 really present in the small intestine of normal Wistar rats? The presence of the CYP3A subfamily in rat small intestine has been reported (Watkins et al., 1987; de Waziers et al., 1990; Chiba et al., 1997), although some authors were unable to detect message for CYP3A1 or CYP3A2 (Zhang et al., 1996; Gushchin et al., 1999). Also, it was reported that a protein in enterocytes of male Wistar rats reacted with a commercially available antibody to CYP3A2, but RT-PCR analysis resulted in the amplification of a product, which corresponded to CYP3A1 and not to

Rats were sacrificed, and their tissues were quickly collected into RNA later solution (Sigma-Aldrich). Total RNA was extracted from the tissue using a GenElute Mammalian Total RNA kit (Sigma-Aldrich). PCR products were separated on a 3% agarose gel stained with 100 ng/ml ethidium bromide to visualize the DNA bands. Data were obtained after densitometric analysis of three electrophoreograms derived from three rats. Results are expressed as percentages of the ratio of CYP3A18 to GAPDH mRNA levels in the S1 group. Each bar represents the mean ± S.E. of three rats.

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CYP3A2 (Kolars et al., 1992). Taking these reports into consideration, CYP3A1, but not CYP3A2, is considered to be expressed in rat small intestine. However, Kolars et al. (1992) have reported that the message for CYP3A1 was able to be detected by RT-PCR in the small intestine of dexamethasone-treated rats, but not detected in normal rats. Furthermore, Zhang et al. (1996) obtained data on whether CYP3A1 was expressed or not in the small intestine, but the expression level was not entirely clarified. Therefore, a moot point remains regarding the expression of CYP3A1 in the small intestine of normal rats. Guschnin et al. (1999) reported the presence of a CYP3A isoenzyme, which is clearly distinguishable from CYP3A1 and CYP3A2, in rat intestinal microsomes using immunoblot, RT-PCR, and Northern blot analysis. In the present study, we were unable to detect the message for either CYP3A1 or CYP3A2 in the small intestine of normal rats using RT-PCR (Fig. 1). Accordingly, CYP3A2 has been confirmed not to be expressed in the small intestine of normal male rats. It was, however, suggested that CYP3A1 expression was absent or extremely weak in the small intestine of normal male rats considering previous reports.

Of note, CYP3A9 was observed in the small intestine of normal male rats, and its level was higher in the small intestine than in the liver, although not significantly (Figs. 1 and 2), confirming the results of Robertson et al. (1998) and Guschnin et al. (1999). In addition, CYP3A18 was remarkably expressed in both liver and small intestine of normal male rats (Figs. 1 and 3). The previous reports also indicated that CYP3A18 was expressed predominantly in the liver rather than in the small intestine in normal male Wistar rats, but the hepatic expression of CYP3A18 was of much higher levels in male than in female rats (Robertson et al., 1998). Hence, it was found that CYP3A9 and CYP3A18, but not CYP3A1 and CYP3A2, were predominantly expressed in the small intestine of normal male rats. The expression of both CYP3A9 and CYP3A18 was, however, reported to be remarkably dependent on sex (Robertson et al., 1998). Accordingly, it was suggested that CYP3A9 and CYP3A18 play a major role in drug metabolism mediated by CYP3A9 and CYP3A18 rather than CYP3A1 and CYP3A2 in the small intestine of normal male rats.

Next, we examined whether there were regional differences in CYP3A9 or CYP3A18 expression in the small intestine of normal Wistar rats. Both CYP3A9 and CYP3A18 were found to be highly expressed in the upper site (almost corresponding to the duodenum) of the small intestine (Figs. 2 and 3). It is generally accepted that the level of P450s decreases in the direction from top to bottom of the gut (Kaminsky and Fasco, 1991), supporting the present findings. Therefore, it was found that there were regional differences in both CYP3A9 and CYP3A18 expression in the small intestine of normal rats, indicating the need for performing experiments carefully.

Expression profiles of mdr1a and mdr1b as well as the CYP3A subfamily were also examined. Signals for mdr1a and mdr1b were detected in both liver and small intestine of normal male rats, and the expression of mdr1a in small intestine was remarkable (Fig. 1) and consistent with a previous report (Borst et al., 1993). In addition, the level of mdr1a mRNA in rat small intestine increased significantly from the top to bottom of the gut (Fig. 4). Stephens et al. (2002) also found that the level of P-glycoprotein was higher in ileum than in jejunum by immunoblotting, although in normal mice. Furthermore, the level of mdr1a was higher in small intestine than in liver (Figs. 1 and 4), indicating the importance of P-glycoprotein in the intestine, as others have claimed. Accordingly, it was found that regional differences in the expression of mdr1a mRNA as well as CYP3A9 and CYP3A18 existed in the small intestine of normal male rats. Also, the trends for distribution were markedly different between CYP3A9 and mdr1a, implying cooperative action of the CYP3A family and P-glycoprotein in the drug clearance pathway (as stated below).

Regrettably, the physiological rationale of our findings remains unclear, but we speculate as follows. The jejenum has a relatively short transit time (~40 min in the rat) (Kayne et al., 1993); in other words, the high membrane permeability in the jejenum is essential for the absorption of lipophilic xenobiotics. In the case of such substances, P-glycoprotein-mediated efflux might be offset by passive influx and consequently lead to a net absorption. Thus, P450s are considered essential for the detoxification of xenobiotics, which have penetrated the intestinal epithelial cells. In contrast, the transit time in the ileum was reported to be considerably longer (~140 min in the rat) (Kayne et al., 1993), and thus absorption was able to be performed even at a lower membrane permeability. Given that the membrane permeability is low, P-glycoprotein-mediated efflux but not P450-mediated metabolism is considered to be very efficient for the detoxification of xenobiotics. Therefore, these points are considered to be an interpretation of the high levels of expression of CYP3A9 and CYP3A18 at the upper site and mdr1a at the lower site in rat small intestine.

In conclusion, we clarified the expression profiles of CYP3A and mdr1 subfamily mRNAs in both liver and small intestine of normal male Wistar rats and also the regional differences of CYP3A9, CYP3A18, and mdr1a in the small intestine. The information is very important to drug metabolism research based on ex vivo and in vivo studies using rats.

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