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ONTOGENIC EXPRESSION OF HUMAN CARBOXYLESTERASE-2 AND CYTOCHROME P450 3A4 IN LIVER AND DUODENUM: POSTNATAL SURGE AND ORGAN-DEPENDENT REGULATION[1]

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Abstract

Human carboxylesterase-2 (CES2) and cytochrome P450 3A4 (CYP3A4) are two major drug metabolizing enzymes that play critical roles in hydrolytic and oxidative biotransformation, respectively. They share substrates but may have opposite effect on therapeutic potential such as the metabolism of the anticancer prodrug irinotecan. Both CES2 and CYP3A4 are expressed in the liver and the gastrointestinal tract. This study was conducted to determine whether CES2 and CYP3A4 are expressed under developmental regulation and whether the regulation occurs differentially between the liver and duodenum. A large number of tissues (112) were collected with majority of them from donors at 1-198 days of age. In addition, multi-sampling (liver, duodenum and jejunum) was performed in some donors. The expression was determined at mRNA and protein levels. In the liver, CES2 and CYP3A4 mRNA exhibited a postnatal surge (1 versus 2 months of age) by 2.7 and 29 fold, respectively. CYP3A4 but not CES2 mRNA in certain pediatric groups reached or even exceeded the adult level. The duodenal samples, on the other hand, showed a gene-specific expression pattern at mRNA level. CES2 mRNA increased with age but the opposite was true with CYP3A4 mRNA. The levels of CES2 and CYP3A4 protein, on the other hand, increased with age in both liver and duodenum. The multi-sampling study demonstrated significant correlation of CES2 expression between the duodenum and jejunum. However, neither duodenal nor jejunal expression correlated with hepatic expression of CES2. These findings establish that developmental regulation occurs in a gene and organ-dependent manner.

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[2]The authors indicate no potential conflict of interest.

1. INTRODUCTION

Personalized medicine is an ultimate goal of health professionals and inter-individual variability presents the major challenge to achieve this goal (Patel et al., 2013; Preissner et al., 2013; Schleidgen et al., 2013; Stoll et al., 2013). While many factors are contributing to inter-individual variability, biotransformation is recognized as one of the major contributing factors (Preissner et al., 2013; Stoll et al., 2013). There are three types of biotransformation, commonly referred to as phase I (Lewis, 2003), phase II (Deenen et al., 2006) and phase III reactions (You, 2004). Phase I and II reactions are accomplished by drug-metabolizing enzymes. Phase III reactions, without chemical modifications, are accomplished by drug transporters. The human genome contains ~150 biotransformation genes with known pharmacological and toxicological significance (Lewis, 2003; You, 2004; Deenen et al., 2006; Preissner et al., 2013; Stoll et al., 2013). Many biotransformation genes are expressed in a wide range of organs and tissues. However, the highest expression of many biotransformation genes occurs in the liver and the gastrointestinal (GI) tract (Xie et al., 2002; Zancanella et al., 2012). The expression of these genes, on the other hand, exhibits large inter-individual variability, up to 100-fold in some cases (Pearce et al., 2001). Genetic and environmental factors as well as disease status are known to regulate the expression of these genes (Song et al., 2005; Liu et al., 2008; Zanger et al., 2014).

We and other investigators have shown that the expression of biotransformation genes is developmentally regulated in rodents and humans (Morgan et al., 1994; Yang et al., 2009; Hines, 2013; Lu, 2013; Peng et al., 2013). Based on immunoblotting analysis (Morgan et al., 1994), one to two week old rats express no hydrolase A or B, two major rat carboxylesterases. Consistent with little expression of carboxylesterases, the intrinsic hydrolytic clearance of the pyrethroid deltamethrin in 10-day old rats is only ~3% of adult rats (Anand et al., 2006). Even in 4-week old rats, the intrinsic clearance is less than half of that of adults (Anand et al., 2006). In addition, young animals are generally much more sensitive to pesticides such as organophosphates and pyrethroids (Sheets et al., 1994; Liu et al., 1999; Anand et al., 2006). Carboxylesterases are known to protect against these chemicals by hydrolysis in the case of pyrethroids or scavenging mechanism in the case of organophosphates. Human carboxylesterase-1 (CES1) and 2 (CES2) in the liver are developmentally regulated (Yang et al., 2009; Zhu et al., 2009; Shi et al., 2011).

We recently showed that the developmental regulation of human CES1 in the liver consists of a postnatal surge followed by an incremental increase throughout the entire adolescence (Yang et al., 2009; Zhu et al., 2009). Based on the level of CES1 mRNA, the postnatal surge of human CES1 is completed at the age of approximately two months (Zhu et al., 2009). The present study was undertaken to determine whether the ontogenic expression pattern of CES1 represents a common phenomenon among biotransformation genes in humans. This study focused on CES2 and cytochrome P450 3A4 (CYP3A4). In humans, CES1 and CES2 together represent as much as 90% of hydrolytic capacity toward drugs and other xenobiotics (Yan, 2012; Sanghani et al., 2009). CYP3A4 is a member of the cytochrome P450 mixed-function oxidase system (Klein and Zanger, 2013). This oxidase is involved in the metabolism of more than 50% drugs and other xenobiotics. CES2 and CYP3A4 in humans are functionally linked in terms of tissue distribution and coupled metabolism. For

example, both CES2 and CYP3A4 are abundantly expressed in the liver and the GI tract (Xie et al., 2002; Fakhoury et al., 2006; Paine et al., 2006). Importantly, some drugs are metabolized by both CES2 and CYP3A4 and their relative activity has profound therapeutic consequences (Yang et al., 2007). The anticancer drug irinotecan, for example, is hydrolyzed to produce SN-38, a metabolite with potent anticancer activity. In contrast, irinotecan undergoes oxidation by CYP3A4 to produce two major oxidative metabolites: NPC (7-ethyl-10-(4-amino-1-piperidino) carbonyloxycamptothecin and APC (7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin. Both NPC and APC are less active than the parent compound (Fakhoury et al., 2009).

This study tested a large number of human liver and duodenal samples for the expression of CES2 and CYP3A4 by Western blotting and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In the liver, the levels of CES2 and CYP3A4 mRNA exhibited a postnatal surge by 2.7 and 29 fold, respectively. The duodenal samples, on the other hand, showed a gene-specific expression pattern at mRNA level. CES2 mRNA increased with age but the opposite was true with CYP3A4 mRNA. Nevertheless, CES2 and CYP3A4 protein increased with age in both liver and duodenum. The multi-sampling study demonstrated significant correlation of CES2 expression between the duodenum and jejunum in humans. However, neither duodenal nor jejunal expression correlated with hepatic expression of CES2.

2. MATERIALS AND METHODS

2.1. Chemicals and supplies

Ponceau S was purchased from Sigma (St. Louis, MO). *TaqMan* probes were from Life Technologies Corporation (Grand Island, NY). The *TaqMan* assay identification numbers were: CES2, Hs00187279_m1 (NM_198061); CYP3A4, Hs00604506_m1 (NM_017460.3); and polymerase (RNA) II, Hs01108291_m1 (NM_000937). Random primers and M-MLV reverse transcriptase were purchased from Promega Corporation (Madison, WI). The RNAzol B reagent was from Tel-Test Inc (Friendswood, TX). Antibody against glyceradehyde-3-phosphate dehydrogenase (GAPDH) was from Abcam (Cambridge, MA). Unless otherwise specified, all other reagents were purchased from Fisher Scientific (Pittsburgh, PA).

2.2. Preparation of RNA and S9 fractions

A total of 112 human tissue samples were used in this study including 59 liver, 43 duodenal and 10 jejunal samples. Multi-sampling was performed in 10 donors, thus, there was a total of 92 donors. Among them, 59 were pediatric (1-332 days of age) and 23 adult donors. Majority of the donors (54%) were Caucasian-American. The tissues were acquired primarily from the University of Maryland Brain and Tissue Bank for Developmental Disorders (Baltimore, MD). Total RNA was isolated with RNAzol B and the integrity was verified by agarose gel electrophoresis described previously (Yang et al., 2007). S9 fractions were prepared by differential centrifugation as described previously (Yang et al., 2007). The use of the human samples was approved by the Institutional Review Board.

2.3. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RNA (0.1 μg) was subjected to the synthesis of the first strand cDNA in a total volume of 25 μL with random primers and M-MLV reverse transcriptase (Yang et al., 2009). The reactions were conducted at 25°C for 10 min, 42°C for 50 min and 70°C for 10 min. The cDNAs were then diluted 6 fold and qPCR was performed with *TaqMan* Gene Expression Assay as described previously (Yang et al., 2007; Yang et al., 2009). The PCR amplification was conducted in a total volume of 20 μL containing universal PCR master mixture (10 μL), gene-specific *TaqMan* assay mixture (1 μL), and cDNA template (3 μL). The cycling profile was 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60 °C, as recommended by the manufacturer. Amplification and quantification were done with the Applied Biosystems 7900HT Real-Time PCR System. All samples were analyzed in triplicate and the signals were normalized to polymerase (RNA) II and then expressed as relative levels of mRNA.

2.4 Western analysis

S9 fractions (8-20 μg) were resolved by 7.5% SDS-PAGE in a mini-gel apparatus and transferred electrophoretically to nitrocellulose membranes. In some cases, membranes were rinsed once in TBST buffer (20 mM Tris-HCl, 150 mM NaCl and 0.05% Tween 20) and then blocked in 5 % non-fat milk as described previously (Sanghani et al., 2009; Xiao et al., 2012). In other cases, membranes were washed once in 0.1% acetic acid solution and stained in 0.1% Ponceau S solution for 5 min. The membranes were then washed twice in 5% acetic acid solution and then blocked in 5 % non-fat milk. The blots were incubated with an antibody against CES2, CYP3A4 and GAPDH, respectively. The preparation of the antibodies against CES2 and CYP3A4 was described elsewhere (Zhu et al., 2000). In both cases, the antigens were synthetic peptides conjugated with keyhole limpet hemocyanin. The sequence of CES2 peptide was H_2N-CQELEEPEERHTEL-COOH, and of CYP3A4 was H_2N-CVKRMKESRLEDTQKHRVDFLQ-COOH. The primary antibodies were subsequently localized with goat anti-rabbit IgG conjugated with horseradish peroxidase, and horseradish peroxidase activity was detected with a chemiluminescent kit (SuperSignal West Pico). The chemiluminescent signals signal was captured by Carestream 2200 PRO imager.

2.5. Other analyses

Protein concentrations were determined with BCA assay (Pierce) based on bovine serum albumin standard. Data are presented as mean ± SD. Statistical analyses were performed with SPSS-PASW Statistics 20. Significant differences were tested according to Spearman for correlation or One-way ANOVA followed by a DUNCAN's test for comparison of means. In all cases, significant differences were made when p values were less than 0.05.

3. RESULTS

3.1. Expression of CES2 and CYP3A4 mRNA as a function of age

We have shown that the expression of human CES1 exhibits a two-phase developmental regulation: a fast surge in the early period after birth followed by an incremental phase

toward the end of adolescence (Shi et al., 2011). This study was undertaken to determine whether the two-phase developmental regulation represents a common phenomenon among drug-metabolizing enzymes in humans. This study focused on CES2 and CYP3A4, two major drug-metabolizing enzymes (Yan, 2012). CES2 and CYP3A4 share many substrates and their metabolism may have different clinical consequences (Smith et al., 2005). We initially tested a large number of individual liver tissues collected at birth to 198 days of age. As described for human CES1 (Shi et al., 2011), samples were divided into several groups: I (1-31 days), II (35-70 days), III (89-119 days) and IV (123-198 days). As a control, adult livers (≥18 years) were included and designated group V. Each group had 10-14 individual samples. Among them, 33 were male and 26 female donors (Table I).

The expression of CES2 and CYP3A4 was determined by RT-qPCR with a *Taqman* probe and Western blotting. As shown in Fig. 1, the relative level of CES2 mRNA in group I-V was 26, 70, 88, 80 and 100%, respectively. The level of CYP3A4 mRNA in these groups was 2, 65, 110, 98 and 100%, respectively. Clearly, both genes exhibited a postnatal surge (group I versus II) with a magnitude of 2.7 and 29 fold, respectively. For CES2 mRNA, none of the postnatal groups reached the adult level. In contrast, the level of CYP3A4 mRNA in groups III and IV reached or even exceeded the adult level (Fig. 1). The levels of the corresponding proteins, on the other hand, showed a greater age-dependent increase for both enzymes (Fig. 1). The expression of mRNA for both genes exhibited large inter-individual variations with CYP3A4 mRNA being much greater (Fig. 1).

3.2. Correlation of ontogenic expression between hepatic CES2 and CYP3A4

Both CES2 and CYP3A4 mRNA exhibited a postnatal surge, however, the magnitude differed markedly (Fig. 1). In addition, the level of CYP3A4 but not CES2 mRNA in certain pediatric groups reached or even exceeded the adult level. These observations pointed to potential differences in the molecular mechanisms supporting the ontogenic regulation of these two genes. To shed light on this possibility, correlation studies were performed on age as well as on each other. As shown in Fig. 2A, the level of CES2 mRNA was significantly correlated with age and the correlation was slightly greater during the period of 1-70 days of age ($r = 0.478$) than that of 1-198 days ($r = 0.359$). Similar trend of correlation was detected on the level of CYP3A4 mRNA over age ($r = 0.539$ versus $r = 0.350$). Importantly, the levels of CES2 and CYP3A4 mRNA were correlated well. The correlation coefficient for the period of 0-198 days was 0.318 and the correlation was much improved for the period of 1-70 days ($r = 0.859$), suggesting that the same or similar mechanism support the ontogenic expression of CES2 and CYP3A4, particularly during the first two months after birth.

3.3. Duodenal expression of CES2 and CYP3A4

In addition to the liver, the GI tract expresses high levels of drug metabolizing enzymes. We next tested whether the duodenum shares with the liver in the ontogenic expression of CES2 and CYP3A4. A total of 43 duodenal samples were collected and divided into several groups: dI (1-70 days), dII (76-141 days), dIII (163-332 days) and dIV (adult, ≥ 18 years) with each group having 9-13 individual samples (Table II). It should be noted that grouping of duodenal samples was different from that of liver samples. Two considerations were made for the grouping of duodenal samples. The total number of duodenal samples was

fewer than that of liver samples. Importantly, the liver samples exhibited similar patterns of age-dependent mRNA expression of CES1 and CYP3A4 (Fig. 1). In contrast, these two genes exhibited different patterns of mRNA expression (Fig. 3).

The levels of CES2 mRNA and protein exhibited age-related increases in groups dI, dII and dIII (Fig. 3), although the increase in CES2 mRNA between group dIII and dIV was minimal. The adult group (dIV), nevertheless, exhibited much greater individual variation in CES2 mRNA (Left of Fig. 3A). Ponceau S staining was used for the normalization of loading as the levels of several commonly used house-keeping proteins (e.g., GAPDH) showed large group-group variations. The correlation of CES2 mRNA with age among the duodenal samples (0-332 days) was statistically significant ($p < 0.017$) and the correlation coefficient was 0.412 (Fig. 3A). Interestingly, the correlation among the 0-96 day-samples had a smaller coefficient (r = 0.283) and did not reach statistical significance. Overall, the correlation study demonstrated that CES2 mRNA exhibited a similar pattern of ontogenic expression in the liver and duodenum (Figs. 1 and 3).

In contrast to the liver, CYP3A4 mRNA in the duodenum exhibited a major difference in the age-related expression. The level of liver CYP3A4 mRNA was correlated positively with age (Fig. 2B). However, the level of duodenal CYP3A4 mRNA decreased with age (Fig. 3B). In the duodenal samples, a 25% decrease of CYP3A4 mRNA was detected from group dI (1-70 days) to group dII (76-141 days) or dIII (163- 332 days). Overall, the level of CYP3A4 mRNA in the duodenal samples among pediatric donors exhibited a trend of inverse correlation with age, although the inversed correlation did not reach the level of statistical significance ($r = -0.145$, $p = 0.421$). Interestingly, during the first three months after birth (1-96 days), the level of CYP3A4 mRNA showed a trend of positive correlation with age ($r = 0.184$, $p = 0.546$) (Fig. 3B). Again, the correlation coefficient did not reach statistical significance. Among all duodenal samples, large individual variations were detected with coefficients of variation ranging from 1.78 to 2.46. The adult duodenal samples, compared with group dI samples, showed a 67% decrease in CYP3A4 mRNA (Left of Fig. 3B). Interestingly, the level of CYP3A4 protein in this group was much greater than that of all pediatric duodenal groups.

3.4. Organ-specific ontogenic expression of CES2

The studies with groups-based samples demonstrated large individual variations, although the variations tended to be smaller with the liver than duodenal samples (Figs. 1 and 3). To ascertain the contribution of potential individual variations in the expression, we next determined the expression of CES2 in the liver and intestinal samples from the same donors. This was of significance as this study would specify whether individuals expressing relatively high levels of CES2 in the liver also express high levels of this enzyme in his/her GI tract. We collected liver, duodenum and jejunum from 10 individuals. With an exception of a single adult donor, all donors were pediatric from 1 to 196 days of age. Once again, both RT-qPCR and Western blotting were used for the expression determination. CES2 mRNA but not CES2 protein was detected in all samples. While there were exceptions, the relative abundance of CES2 protein occurred in an order of the liver, the duodenum and the jejunum (Fig. 4A). Donor 10, on the other hand, expressed the highest level of CES2 protein

in the duodenum. Donor 3 expressed comparable levels of CES2 protein among all three organs and donor 4 did not express detectable CES2 protein in either organ. Based on the level of CES2 mRNA, duodenal and jejunal expression was significantly correlated with a coefficient of 0.359 ($p = 0.023$). However, neither duodenal nor jejunal expression was correlated with liver expression (Fig. 4B).

4. DISCUSSION

Personalized medicine is an ultimate goal of health professionals and inter-individual variability presents the major challenge (Patel et al., 2013; Preissner et al., 2013; Schleidgen et al., 2013; Stoll et al., 2013). Biotransformation is the major contributor to individual variability (Preissner et al., 2013; Stoll et al., 2013). The expression of drug-metabolizing enzymes is regulated largely by environmental factors and disease status, whereas in children, these factors are compounded by developmental regulation (Hines, 2013). We have recently made a concerted effort and laid groundwork on the ontogenic expression of biotransformation genes (Yang et al., 2009; Shi et al., 2011). In this study, we tested a large number of liver and small intestinal samples from pediatric donors for the expression of CES2 and CYP3A4, two major drug-metabolizing enzymes (Sanghani et al., 2009; Klein and Zanger, 2013). While both genes were developmentally regulated, the overall outcomes varied depending on a gene and an organ. In the case of CES2, age-dependent increases were detected in the liver and the GI tract at both mRNA and protein levels (Figs. 1 and 2). However, the age-related increases in CYP3A4 were detected at protein in both organs but not mRNA level (Figs. 1 and 3).

The results described in this study, nevertheless, point to an important conclusion about ontogenic expression in human liver. Namely, the postnatal surge, although exceptions may exist, is a general phenomenon among biotransformation genes. The magnitude of the surge, on the other hand, varied depending on a gene. Based on RT-qPCR analysis, the expression of CYP3A4 mRNA showed a surge by 29-fold, but the expression of CES2 mRNA by 2.7 fold only (Fig. 1). We previously reported a 7.1-fold postnatal surge of CES1 mRNA (Shi et al., 2013). The surge of CES2 and CYP3A4 mRNA represented 65-70% of the level in adult liver, whereas in the case of CES1, it represented 50% only (Shi et al., 2013). Following the surge was gradual increases in mRNA expression during the period of 6 months after birth. However, such increases varied markedly from a gene to another. In the case of CES2, 10-20% increases were detected from 70 to 198 days of age, but the level of CES1 mRNA was increased by only 5% during this period (Shi et al., 2013). The level of CYP3A4 mRNA, on the other hand, was increased by as much as 45%. As a matter of fact, the level of CYP3A4 mRNA in groups III (89-119 days) and IV (123-198 days) reached or even exceeded the adult level (Fig. 1). In contrast, the surge of CES2 mRNA was followed by an incremental increase toward adulthood, which was similar to that of CES1 mRNA (Fig. 1) (Yang et al., 2009; Shi et al., 2011). However, the incremental phase of CES2 mRNA represented less percentage than that of CES1 (15 versus 50%).

The level of CYP3A4 but not CES2 mRNA displayed organ-specific ontogenic expression patterns. The hepatic expression of CYP3A4 mRNA, like CES2 mRNA, was correlated significantly during the first 6 months of life ($r = 0.350$, $p = 0.027$) (Left of Fig. 2B).

Similarly to CES2 mRNA, CYP3A4 mRNA was correlated much better with age ($r = 0.539$, $p < 0.014$) when the period of the first 70 days of age was considered (Right of Figs. 2A and B). Importantly, levels of CES2 and CYP3A4 mRNA were correlated significantly with each other (Fig. 2C). These findings suggested that CES2 and CYP3A4 share mechanisms in ontogenic expression, at least in light of hepatic mRNA expression. In contrast, the duodenum exhibited different expression patterns between CES2 and CYP3A4 mRNA. A positive correlation during the first 70 days was detected for both genes (Right of Fig. 3). However, the overall correlation during the first 198 days was opposite. The abundance of CYP3A4 mRNA exhibited a trend of negative correlation with age, while the level of CES2 mRNA positively with age in the duodenum (Fig. 3).

The multi-organ sampling study provided important information on inter-organ differences in terms of gene expression. The level of CES2 mRNA increased with age in both liver and duodenum. However, hepatic and duodenal levels from the same donors showed insignificant correlation (Fig. 4B). On the other hand, duodenal and jejunal levels of CES2 mRNA were significantly correlated (Fig. 4B). The insignificant correlation between the liver and small intestine suggests that the developmental regulation is mediated by different triggers in these two organs. The precise mechanisms remain to be determined, and many changes take place immediately after birth and in the early days/weeks of life, notably on hormones and food intake. On the other hand, many biotransformation genes are expressed in a rapid increasing manner such as CYP3A4 (Fig. 1 and Table I) and CES1 (Yang et al., 2009; Shi et al., 2011). For example, fibroblast growth factor-21 (FGF-21) is induced rapidly (Hondares et al., 2010), and importantly the induction of FGF-21 was diminished in mice lacking functional peroxisome proliferator-activated receptor-α (PPARα) (Hondares et al., 2010).

PPARα has been implicated in the regulation of the expression of carboxylesterases in rodents (Hosokawa et al., 1994; Morgan et al., 1994; Poole et al., 2001), however, its involvement in the regulated expression of human carboxylesterases remains to be established. On the other hand, the CES2 promoter region contains two PPARα putative elements located −907 and −256, respectively (Wu et al., 2002). It has been reported that the CES2 gene has three promoters, designated promoter-1, promoter-2 and promoter-3, respectively (Wu et al., 2002). These promoters use different transcription start sites, thus producing distinct transcripts. It appears that promoter-3 has a broad tissue activity and supports constitutive but low level of expression. In contrast, promoter 1 and promoter 2 show tissue-dependent activity. For example, promoter 1 is more active in the liver than promoter 2, and the opposite is true in the small intestine (Wu et al., 2002). Interestingly, one of the PPARα putative elements is present in promoter 1 and the other in promoter 2. Given the observation that the liver but not small intestine expresses high levels of PPARα (Auboeuf et al., 1997; Zoete et al., 2007), the element in promoter 1 is likely involved in the developmental regulation of CES2 in the liver. On the other hand, PPARδ and PPARγ are expressed much higher in the GI tract. Therefore, these two receptors likely play a role in the developmental regulation of CES2 in the GI tract (Auboeuf et al., 1997). While all PPARs are functionally related, they exhibit different ligand specificity (Zoete et al., 2007). An involvement of different PPARs in the developmental regulation of CES2 between the GI tract and liver provides an explanation to the insignificant correlation on CES2

expression between these two organs, although both organs exhibited significant age-dependent expression (Figs. 1, 2 and 3).

In contrast to RT-qPCR, Western blotting consistently detected age-related increases regardless of genes (CES2 or CYP3A4) or organs (liver and duodenum) (Figs. 1 and 3). The precise mechanisms remain to be determined on the disproportions between mRNA and protein expression of CES2 and CYP3A4. In particular, duodenal samples in the adult group had the lowest level of CYP3A4 mRNA, but the same group expressed the highest level of CYP3A4 protein (Left of Fig. 3B). It is likely that the translational efficiency of intestinal CYP3A4 mRNA increases with age. Alternatively, the expression of certain microRNAs (miRs) that target CYP3A4 transcript increases and thus negatively affects the production of CYP3A4 proteins. In support of this possibility, several miRs have been reported to regulate the expression of CYP3A4 (Pan et al., 2009) or the pregnane X receptor, a major regulator of CYP3A4 expression in response to xenobiotic stimuli (Takagi et al., 2008). Nevertheless, it remains to be determined whether these miRs are expressed in an age-dependent manner.

The precise pharmacological significance of the organ-specific expression remains to be established. In the case of CES2, the contribution to the overall hydrolysis between the liver and the GI tract is likely closer than the difference in the expression. In this study, the entire wall of the small intestine was used. It is not clear whether CES2 is present in the whole section of the wall or primarily in the mucosal layer. Based on the study in puppies (Stander et al., 2010), the mucosal layer takes 60-70% of the thickness of the entire wall. Therefore, the expression levels were probably underestimated if CES2 is exclusively present in the mucosal layer. Likewise, the presence of CES2 in the liver may not be uniform. We previously showed that several rat carboxylesterases were primarily located in the centrilobular regions (Yan et al., 1994; Yan et al., 1995). Finally, the initial concentrations of drugs and other xenobiotics in the mucosal layer of the small intestine are higher than those in the liver after oral administration. Therefore, it is likely that CES2 in the GI tract contributes comparably to the hydrolysis by liver CES2, although CES2 is generally more abundant in the liver (Fig. 4A). In support of this possibility, hydrolysis of the antiplatelet agent prasugrel was comparable between the small intestine and liver in dogs (Hagihara et al., 2011).

In summary, our work points to several important conclusions. First, the postnatal surge of mRNA expression in the liver, although exceptions may exist, is a general phenomenon among biotransformation genes. Second, high-levels of mRNA do not necessarily result in high-levels of protein, and such disproportions likely occur in an organ-specific manner. And third, individuals may have disproportional drug-metabolizing capacities between the liver and the GI tract, two major biotransformation organs. These findings establish that developmental regulation occurs in a gene and organ-dependent manner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviation

CES2	carboxylesterase-2
CYP3A4	cytochrome P450 3A4
GAPDH	glyceradehyde-3-phosphate dehydrogenase
GI	gastrointestinal
PBS	phosphate buffered saline
PPARα	peroxisome proliferator-activated receptor-α
RT-qPCR	reverse transcription-quantitative polymerase chain reaction

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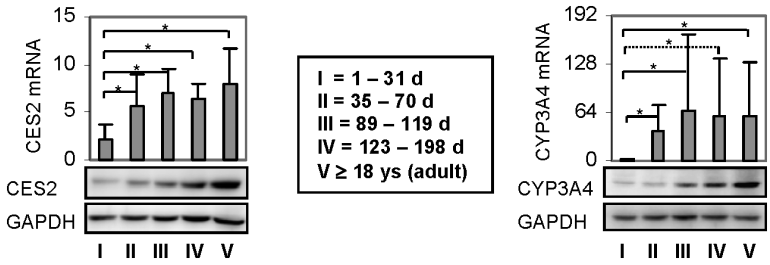

Fig. 1. Hepatic expression of CES2 and CYP3A4 as a function of age

Total RNAs from livers were subjected to RT-qPCR analysis for the levels of CES2 or CYP3A4 mRNA by *Taqman* probe as described in the section of Materials and Methods. The signals from each target were normalized based on the signal from Pol II and expressed as relative levels among all samples. The data are presented as mean ± SD. For Western analysis S9 fractions (8 μg for CES2 and 10 μg for CYP3A4) were resolved by 7.5% SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. The samples were pooled with the same amounts of proteins from individual samples in the same group. The blots were incubated with an antibody against CES2, CYP3A4 or GAPDH and developed with chemiluminescent substrate. The signal was captured by Carestream 2200 PRO imager. *Statistical significance at $p < 0.05$.

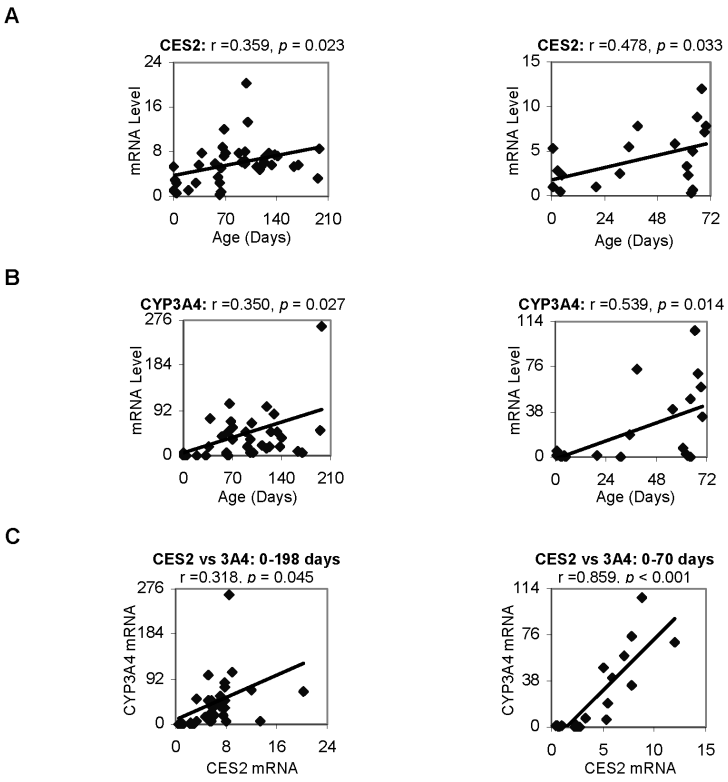

Fig. 2. Correlation analyses between age and levels of CES2 or CYP3A4 mRNA

The correlation was performed with SPSS Statistics 20. (A) Correlation of CES2 mRNA as a function of age:1-198 days (Left) or 1-70 days (Right). (B) Correlation of CYP3A4 mRNA as a function of age:1-198 days (Left) or 1-70 days (Right). (C) Correlation of CES2 over CYP3A4 mRNA as a function of age:1-198 days (Left) or 1-70 days (Right). Correlation coefficients and corresponding *p* values are shown.

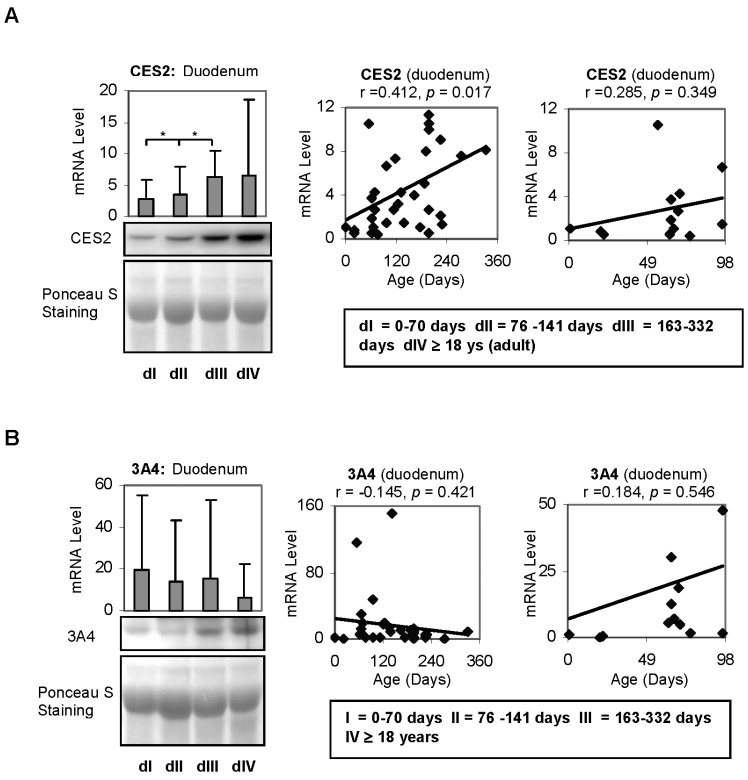

Fig. 3. Duodenal expression of CES2 and CYP3A4 as a function of age and correlation analyses

(A) Duodenal expression of CES2 Total RNAs from duodena were subjected to RT-qPCR analysis for the levels of CES2 mRNA by *Taqman* probe and the signals from each target were normalized based on the signal from Pol II and expressed as relative levels among all samples. The data are presented as mean ± SD. For Western analysis S9 fractions (10 μg) were resolved by 7.5% SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. The blot was stained by 0.1% Ponceau S as described in the section of Materials and Methods. Thereafter the blot was incubated with an antibody against CES2 or GAPDH and developed with chemiluminescent substrate. The signal was captured by Carestream 2200 PRO imager. Once again, the correlation of CES2 mRNA as a function of age was performed with SPSS Statistics 20. *(B) Duodenal expression of CYP3A4* All procedures for the expression and correlation analysis for CYP3A4 were the same as described above. However, the Western blotting used 20 μg of S9 fractions. *Statistical significance at $p < 0.05$.

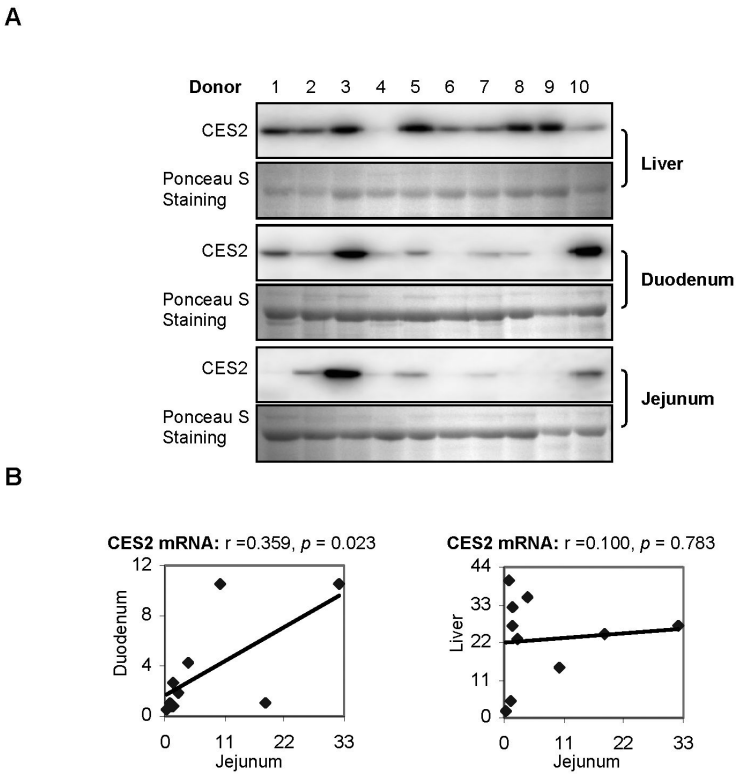

Fig. 4.

Multi-sampling study on the expression of CES2 Liver, duodenal and jejunal S9 fractions (8 μg) were resolved by 7.5% SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. The blots were stained by 0.1% Ponceau S and then incubated with an antibody against CES2 or GAPDH and developed with chemiluminescent substrate. The signal was captured by Carestream 2200 PRO imager. Once again, the correlation of CES2 mRNA between duodenum and jejunum (Left) or liver jejunum was performed (Right) with SPSS Statistics 20. Correlation coefficients and corresponding *p* values are shown.

Table I

Demographic distribution of liver donors

Group	n	Male/Female	CA	AA	H
I (1-31 days)	12	6/6	6	4	2
II (35-70 days)	13	5/8	5	8	
III (89-119 days)	10	7/3	4	6	
IV (123-198 days)	10	8/2	4	5	1
V (adult: ≥18 years)	14	7/7	10	4	

Abbreviation: CA, Caucasian-American; AA, African American; H, Hispanic

Table II

Demographic distribution of duodenal donors

Group	n	Male/Female	CA	AA	H
dI (1-70 days)	11	3/8	6	5	
dII (76-141 days)	9	7/2	3	6	
dIII (163-332 days)	13	9/4	7	6	
dIV (adult: ≥ 18 years)	10	5/5	8	2	

Abbreviation: CA, Caucasian-American; AA, African American; H, Hispanic