CYP3A gene expression in human gut epithelium*

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Received 27 April 1994 accepted 14 June 1994

CYP3A4, a major Phase I xenobiotic metabolizing enzyme present in liver, is also present in human small bowel epithelium where it appears to catalyze significant 'first pass' metabolism of some drugs. To determine whether CYP3A4 or the related enzymes CYP3A3, CYP3A5, and CYP3A7 are present in other regions of the digestive tract, we used CYP3A-specific antibodies to examine histological sections and epithelial microsomes obtained from a human organ donor. CYP3A-related proteins were detected in epithelia throughout the digestive tract and in gastric parietal cells, in pericentral hepatocytes, and in ductular cells of the pancreas. Immunoblot analysis suggested that the major CYP3A protein present in liver, jejunum, colon, and pancreas was CYP3A4 or CYP3A3, whereas CYP3A5 was the major protein present in stomach. Both CYP3A4 and CYP3A5 mRNA were detectable in all regions of the digestive tract using the polymerase chain reaction (PCR); however, only CYP3A4 could be detected by Northern blot analysis. CYP3A7 mRNA was consistently detected only in the liver by PCR and CYP3A3 mRNA was not detected in any of the tissues. We conclude that CYP3A4 and CYP3A5 are present throughout the human digestive tract and that differences in the expression of these enzymes may account for inter-organ differences in the metabolism of CYP3A substrates.

Introduction

Enzymes within the CYP3A subfamily§ are prominent hepatic enzymes that play a major role in the metabolism of a growing list of medications such as cyclosporine A (Kronbach *et al.*, 1988; Combalbert *et al.*, 1989), calcium channel blockers (Guengerich *et al.*, 1986), macrolide antibiotics (Watkins *et al.*, 1985), ethinyl estradiol (Guengerich *et al.*, 1986a), quinidine (Kitada *et al.*, 1987; Guengerich *et al.*, 1986b), midazolam, triazolam (Kronbach *et al.*, 1989), lovastatin (Regina *et al.*, 1991), tamoxifen (Jacolot *et al.*, 1991), and lignocaine (Bargetzi *et al.*, 1989). These enzymes

also appear to be important in the metabolism of potentially harmful dietary contaminants including pyrrolizidine alkaloids (Miranda *et al.*, 1991), aryl hydrocarbons (Shimada *et al.*, 1989), and mycotoxins such as aflatoxin B1 (Shimada & Guengerich, 1989; Aoyama *et al.*, 1990).

Four CYP3A cDNAs have been cloned from a human liver library and these have been termed CYP3A3, CYP3A4, CYP3A5, and CYP3A7 (Nelson *et al.*, 1993). It is unlikely that many additional CYP3A genes exist because a single CYP3A gene is ~30 kb in length (Schuetz, J., unpublished observation) and only 90 kb of human genomic DNA hybridize with a CYP3A cDNA under low stringency conditions (Molowa *et al.*, 1986). The liver enzymes corresponding to each of these CYP3A cDNA's share over 85% amino acid sequence homology. However, in at least some cases, these enzymes have been shown to differ in their catalytic properties and in their regulation (Watkins, 1990; Guengerich, 1989; Wrighton & Stevens, 1992). For example, recombinant CYP3A4 and CYP3A5 are both capable of metabolizing cyclosporin A but the pattern of metabolites

*Presented in part at the American Society for Biochemistry and Molecular Biology, New Orleans, LA, 1990

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§The cytochromes P450 are a gene superfamily that has been divided into families that share greater than 40% amino acid sequence homology and subfamilies that share greater than 55% amino acid homology. In this manuscript, we refer to specific P450s according to the recently recommended 'CYP' nomenclature (Nelson *et al.*, 1993). P450s within the CYP3A subfamily have been termed 'P450III A' in our former publications.

produced by each enzyme differs (Aoyama *et al.*, 1989). In addition, CYP3A4 has been shown to be inducible in liver by medications such as rifampin (Pichard *et al.*, 1990; Watkins *et al.*, 1989) whereas CYP3A5 does not appear to be inducible (Wrighton *et al.*, 1990).

CYP3A4 is also expressed in human small bowel enterocytes where it appears to be the major cytochrome P450 present (Watkins *et al.*, 1987; Kolars *et al.*, 1992b). CYP3A4 expressed in the enterocyte appears to account for significant 'first pass' metabolism of orally administered cyclosporine A and probably other medications (Kolars *et al.*, 1991; Hebert *et al.*, 1992). We have shown that the CYP3A4 substrate aflatoxin B1 (AFB1) is also metabolized within human small bowel enterocytes resulting in the formation of intracellular AFB1-macromolecular adducts (Kolars *et al.*, 1994). As enterocytes normally slough from the villus tip within 5–6 days (Lipkin, 1987), AFB1 adducts formed within the enterocytes will be released to the gut lumen where they may pass harmlessly in the stool. Thus, enterocyte CYP3A4 may contribute to a protective barrier that limits the systemic absorption of potentially toxic dietary compounds (Kolars *et al.*, 1994).

CYP3A enzymes also appear to be expressed in epithelia in other regions of the human digestive tract. Microsomes prepared from human esophagus, stomach, and colonic mucosa have been reported to weakly react on immunoblots with a CYP3A specific monoclonal antibody (de Waziers *et al.*, 1989). Tissue sections from human esophagus, stomach, and colon have also been reported to react with a polyclonal antibody raised against a CYP3A protein (Murray *et al.*, 1988). Furthermore, human stomach and colonic mucosa have been shown to be capable of metabolizing cyclosporine A (Tjia *et al.*, 1993; Vickers *et al.*, 1993; Weber *et al.*, 1992). However, the relative rates of formation of specific cyclosporine A metabolites in stomach (Weber *et al.*, 1992) and colon (Vickers *et al.*, 1993) differ from those characteristic of CYP3A4 (Aoyama *et al.*, 1989) suggesting that other members of the CYP3A gene family may be prominent in these organs. We therefore examined the entire alimentary tract from one individual to determine the identity and location of the CYP3A enzymes present.

Methods

Human tissue

Tissue specimens were obtained from the digestive tract of a 31 year-old female whose heart and liver were being procured for orthotopic transplantation. Medications received prior to procurement

included dopamine, vasopressin, furosemide, cimetidine, levothyroxine, and diazepam. The patient had also received dexamethasone (16 mg per day) and phenytoin (400 mg per day) which are known inducers of CYP3A in liver (Pichard *et al.*, 1990; Watkins *et al.*, 1987) for 7 days prior to tissue procurement. Full thickness biopsies from the esophagus, stomach fundus, stomach antrum, duodenum, jejunum, ileum, and colon as well as biopsies from the liver and pancreas were obtained in the operating room immediately following procurement of the heart and liver. Portions of each tissue were placed in buffered formalin for analysis and the remaining tissue was immediately processed in the operating room. Portions of the stomach, small intestine, and colon were rinsed vigorously with cold saline and opened with a longitudinal cut. The mucosa was removed by scraping with a microscope slide. Approximately 2 g of mucosal scraping from each location was placed in 20 ml of cold solution 'C' (5 mM histidine pH 7.0, 0.25 M sucrose, 0.5 mM EDTA, 40 $\mu\text{g ml}^{-1}$ phenylmethylsulfonyl fluoride, Bonkovsky *et al.*, 1985) and immediately frozen in liquid nitrogen for future preparation of microsomes. Approximately 1 g of scraped mucosa from each location was added to 10 ml of 10 mM Tris-HCl buffer (pH 7.4) containing 4 M guanidine isothiocyanate and 7% 2-mercaptoethanol and immediately frozen for future preparation of RNA. Portions of the liver, pancreas, and rinsed esophagus were immediately frozen in liquid nitrogen. Reagents used were obtained from Sigma except where otherwise noted. All studies were approved by the Scientific Committee of the Organ Procurement Agency of Michigan.

Immunohistochemistry

Formalin fixed full thickness specimens obtained from each region of the digestive tract were subjected to immunoperoxidase staining using the Zymed Streptavidin-Biotin System Histostain Kit (Zymed Laboratories, South San Francisco, CA, 94080). In brief, formalin fixed paraffin embedded tissues were sectioned and deparaffinized. The slides were incubated with 10% non-immune serum for 10 min followed by incubation with the primary antibody (see Antibodies below). One hundred μl of primary antibody in dilutions that optimized specific staining was added to the slide for 20–60 min followed by careful washing. The section was then sequentially incubated with a biotinylated second antibody, streptavidin-peroxidase conjugate, and finally the chromogen aminoethyl carbazole (AEC) with 0.3% hydrogen peroxide. Thorough washing with phosphate buffered saline (PBS) was performed after each incubation and the

tissue was counterstained with Hematoxylin. Immunoreactive protein was identified by red staining.

Preparation of microsomes

Microsomes were prepared from the epithelial cells obtained from all gut tissues by calcium precipitation (Bonkovsky *et al.*, 1985). Microsomes were prepared from the liver and pancreas by differential centrifugation as previously described (van der Hoeven & Coon, 1974). Microsomal pellets from all tissues were resuspended in 0.5–1.0 ml of solution 'D' (glycerol 20% wt/vol, 100 mM Tris-HCl pH 7.4, Bonkovsky *et al.*, 1985) to obtain a final protein content of 4–16 mg ml⁻¹ as measured by a modification of the technique of Schacterle (Schacterle & Pollack, 1973). Microsomal protein yield from esophagus was insufficient for immunoblotting.

Immunoblot analysis

Microsomal protein 2.5–100 µg, from each tissue (except esophagus) was subjected to immunoblot analysis as previously described (Watkins *et al.*, 1987). In brief, proteins were separated by electrophoresis in 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) (32 mA per gel, 4 h) and then electrophoretically transferred to nitrocellulose filters. The filters were then blocked overnight in PBS containing 10% calf serum 3% bovine serum albumin or 0.3% Tween 20. Filters were then incubated sequentially with a murine monoclonal antibody (see Antibodies below) or a rabbit polyclonal antibody followed by peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG, with extensive washing in cold PBS after each incubation. Immunoreactive proteins were visualized by incubating the filters in a solution containing 3,3'-diaminobenzidine tetrahydrochloride (0.83 mM) and hydrogen peroxide (0.005%).

To enhance the sensitivity of our immunoblotting technique, a chemiluminescence detection system (Amersham, Arlington Heights, IL) was also used. The proteins were separated by electrophoresis and transferred to nitrocellulose filters as described above. The filters were blocked for 1 h with TBS (0.15 M NaCl, 0.3% Tween 20) containing 5% non-fat dry milk (Carnation). The filters were then incubated sequentially with the primary antibody followed by the appropriate conjugated secondary antibody as described above. Extensive washings were done with TBS containing 0.3% Tween 20 and 0.25% non-fat dry milk after each incubation. After a final washing with TBS containing only 0.3% Tween 20, the filters were placed in seal-a-meal bags with 0.0625 ml cm⁻² each of reagent 1 and reagent 2 (Amersham ECL Western blotting reagent kit). These reagents were

mixed with the filters for 1 min and then discarded. The filters were then exposed to Hyperfilm-ECL (Amersham) for 1 s to 15 min depending on the band intensity. All experiments were performed in duplicate.

Antibodies

Three different antibodies were used in these studies. The polyclonal antibody used in our immunohistochemistry studies was raised in a rabbit against a purified human liver CYP3A protein termed 'HLp' (Watkins *et al.*, 1985) which is believed to represent CYP3A4 and possibly CYP3A3 proteins. This antibody has been well characterized, appears to recognize all known CYP3A proteins on immunoblots (Kolars *et al.*, 1992a), and does not react with purified proteins within the *CYP1* and *CYP2* gene families (data not shown). The monoclonal antibody used in our immunoblot analysis, termed 13-7-10, is a murine monoclonal antibody that appears to also recognize all human CYP3A proteins (Beaune *et al.*, 1985; Watkins *et al.*, 1987; Wrighton *et al.*, 1990) but does not react with formalin fixed tissue sections (Kolars *et al.*, 1992a). The CYP3A5 specific antibody used on immunoblots is a polyclonal antibody raised in rabbits to purified CYP3A5. Antibodies to epitopes common to other CYP3A proteins were removed from the anti-CYP3A5 IgG fraction by immunoabsorption against HLp (Wrighton *et al.*, 1990).

Preparation of RNA

The method of Chomczynski and Sacchi (Chomczynski & Sacchi, 1987) was used to isolate RNA with the following modifications. Approximately 1 g of tissue was added to 10 ml of 10 mM Tris-HCl buffer (pH 7.4) containing 4 M guanidine isothiocyanate and 7% 2-mercaptoethanol. Cells were lysed with a hand dounce homogenizer. RNA was quantified by determining the UV absorption at 260 nm after suspension in water. Yields were adequate from all tissues except esophagus where sufficient total RNA was available for the polymerase chain reaction but not hybridization studies.

RNA hybridization studies

Thirty µg of total RNA isolated from liver or gut epithelia was subjected to electrophoresis in 1.0% agarose gels (prepared with 5X MOPS buffer containing 2.2 M formaldehyde) and a 1X MOPS buffer for 4 h at 80 V. The ethidium staining of the resolved ribosomal RNA bands confirmed that little degradation had occurred in all tissues. The RNA was then transferred by capillary action onto nylon and cross-linked with 1200 J of UV radiation. Hybridization was initially

performed using a human CYP3A cDNA (55b) as previously described (Molowa *et al.*, 1986). Hybridization studies were also performed using an anti-sense 30-mer oligonucleotide corresponding to a hypervariable region of human CYP3A4 (see Table 1).

Polymerase chain reaction

RNA from tissue samples was reverse transcribed to cDNA by incubating 1 µg of RNA with 750 pmol oligo-dT (Pharmacia, Piscataway, NJ), 1 µl 25 mM dNTP, 5 µl RT buffer (500 mM Tris pH 8.3, 500 mM KCl, 80 mM MgCl₂, 100 mM DTT) and 1 µl reverse transcriptase (Seikagaku America Inc, Rockville, MD) in a reaction volume of 50 µl for 1 h at 41° C. 10 µl of this reaction mixture was then added to 5 µl 1 mM dNTP, 5 µl Taq buffer (400 mM KCl, 0.1% gelatin), 1 U Taq polymerase (Perkin-Elmer, Norwalk, CT), 1 µg of an antisense synthetic 30-mer primer relatively specific for either CYP3A3, CYP3A4, CYP3A5, or CYP3A7 (see Table 1) and 1 µg of a sense 30-mer oligonucleotide primer in a final reaction volume of 50 µl. The oligonucleotide primers selected span a region of the CYP3A7 gene that contains an intron (J. Schuetz, personal communication). Due to the high homology of the CYP3A genes, it is likely that the primers selected also span an intron in the other three genes. Samples were amplified with a Perkin-Elmer DNA thermal cycler for 32 cycles using a 45 s denaturation step (at 94° C), extension for 75 s (at 72° C) and no annealing step. These stringent conditions were chosen to maximize the specificity of the amplification. A final 72° C extension step was performed for 10 min at the end of the 32 cycles. Twenty µl of each sample was electrophoresed at 65 V on a 1% agarose 2% NuSieve (FMC Bioproducts, Rockland, ME) 1X TAE gel containing 0.004% ethidium bromide. Amplification of the appropriate segment was confirmed by visualizing a band of appropriate length under UV light. All CYP3A primers are referred to by the sequence number of the first base pair (Table 1).

Cloning and nucleotide sequence analysis

DNA fragments amplified by the polymerase chain reaction were purified by electrophoresis in 1% low melting temperature agarose and ligated directly into the plasmid pBluescript (Stratagene, La Jolla, CA) which had been modified as previously described (Marchuk *et al.*, 1991). Briefly, pBluescript was cut with *Eco* RV to create blunt ends. A single 5' T overhang was added by incubating the cut plasmid with Taq Polymerase in the presence of only dTTP. The pBluescript and PCR product were then ligated utilizing the TA overlap of the T from the plasmid and the 5' A overhang characteristically left on Taq poly-

Table 1. CYP3A human oligonucleotides

Gene	Sense primers	Anti-sense primers
CYP3A3	5'GGC TAT CAC AGA TCC TGA CAT GAT CAA ACT3' 306* (16)	5' ACT CTA CAC AGA CAA TGA GAG AGC TCC GGA 1700* (16)
	5' TGG ACC CAG AAA CTG CAT TGG CAT GAG GTT 3' 1374* (20)	5' TGT GGG ACT CAG TTT CTT TTG AAT TCT TAT 3' 895* (16)
CYP3A4	5' CCT TAC ACA TAC ACA CCC TTT GGA AGT 3' 1375* (41)	5' CAT TGG ATG AAG CCA TCT CAT TTC AGA GTC 3' 1658* (16)
CYP3A5	5' CCC AGT TGC TAT TAG ACT TGA 3' 1188* (20)	5' AGC TCA ATG CAT GTA CAG AAT CCC CGG TTA 3' 1727* (41)
CYP3A7	5' AGT ATA GAA AAG TCT GGG GTA TTT ATG ACT 3' 203* (42)	5' GGG GCA CAG CTT TCT TGA AGA CCA 3' 1619* (20)
		5' TAT TGA GAG AAC GAA TGG ATC TAA TGG 3' 649* (42)

*number refers to 5' base pair location in referenced sequence.

merase generated PCR product. The ligated DNA was transfected into DH5 alpha *E. coli* and the successful recombinants identified as white colonies. Plasmid containing the PCR insert was purified using the Magic Miniprep kit (Promega, Madison, WI) and sequenced by the Sanger method (Sanger *et al.*, 1977) using Sequenase (USB, Cleveland, OH). Results were confirmed by sequencing each DNA segment at least three times. Comparisons of the determined sequence with the published sequences for CYP3A3, CYP3A4, CYP3A5, and CYP3A7 were made using the Genetics Computer Group CAP Program.

Results

To determine the distribution of CYP3A gene products along the gastrointestinal tract, portions of esophagus, stomach, small intestine, colon, liver, and pancreas were obtained from the digestive tract of a single human organ donor who had received the CYP3A inducers dexamethasone and phenytoin prior to organ procurement surgery.

Immunohistochemistry

The monoclonal antibody (13-7-10) did not react with formalin fixed tissue and could not be used

for immunohistochemistry. We therefore used a rabbit polyclonal antibody that has been shown to be highly specific for CYP3A proteins and which we have previously used in immunohistochemistry studies in rat and human (Kolars *et al.*, 1992a, b). CYP3A immunoreactive protein was detected in mature epithelial cells in each of the tissues studied (Figs 1 and 2) at dilutions up to 1:1280. Preimmune serum from the same rabbit did not react with these tissues, even at dilutions of 1:150 (not shown). Moreover, when the rabbit serum containing this antibody was preincubated with purified antigen (i.e. HLp), staining of each tissue was completely inhibited (not shown). No detectable reaction occurred between the CYP3A5 specific antibody and sections obtained from the stomach or small intestine (the only tissues examined with this antibody).

In the liver, CYP3A immunoreactive protein was most prominent in centrilobular hepatocytes (i.e. zone 3) and was not detected within biliary epithelium or other cell types of the portal tract (Fig. 1A). In the small intestine, CYP3A protein was not detected in the crypt cells but was readily apparent in villus enterocytes (Fig. 1B). CYP3A protein did not appear to be uniformly distributed within the enterocyte, but

Fig. 1. Immunoperoxidase staining of liver (a) and jejunum (b) following incubation with an antibody (anti-HLp) that reacts with all CYP3A proteins (original magnification $\times 300$). The areas of dark staining indicate the location of CYP3A proteins.

(a)

(b)

(c)

(d)

Fig. 2. Immunoperoxidase staining of full thickness specimens following incubation with anti-HLp (original magnification $\times 300$). The red staining indicates the location of CYP3A proteins in (a) esophagus; (b) stomach; (c) colon; and (d) pancreas.

appeared to be largely located at the apex of the cell, adjacent to the microvillus border (Fig. 1B).

In esophagus (Fig. 2A), faint staining was observed in the functional layer of the squamous epithelium. Some staining of the superficial desquamating cells was also seen but this may represent non-specific staining ('edge artifact'). In the stomach (Fig. 2B), staining was localized primarily to parietal cell cyto-

plasm with faint staining noted in the mature epithelial cells of the upper crypt. In the colon (Fig. 2C), staining was also observed in the mature epithelial cells of the upper crypt. As in the small intestine, no staining was observed in the submucosa. In the pancreas (Fig. 2D), staining was observed predominantly in the ductular epithelium and to a lesser degree in the acinar cells.

Immunoblot analysis

Microsomes were prepared from each of the tissues as described in Methods. The microsomes were then subjected to immunoblot analysis.

When immunoblots were developed using the immunoperoxidase technique, only microsomes from liver and jejunum appeared to react with the monoclonal antibody 13-7-10, producing a single protein band which comigrated with purified CYP3A4 (not shown). However, development of the immunoblots using the more sensitive chemiluminescence technique revealed immunoreactive protein in all of the tissues examined. In addition to liver and jejunum, 13-7-10 produced single reactive bands in microsomes prepared from stomach, ileum (not shown), colon and pancreas (Fig. 3). The reactive protein in stomach microsomes appeared to migrate slightly slower on the SDS polyacrylamide gels than did the reactive proteins in the other tissues (Fig. 3 and data not shown). Several bands of lower molecular weight were also identified in microsomes prepared from pancreas which may represent digested fragments of CYP3A enzymes.

Duplicate immunoblots were next developed with a CYP3A5 specific antibody (Fig. 3). This antibody

did not appear to react with the liver and jejunal microsomes (2.5 and 5 μ g protein, respectively) but produced distinct bands in stomach and colon microsomes (75 μ g protein). CYP3A5 immunoreactive protein was clearly most abundant in the stomach (Fig. 3). CYP3A5 was not clearly identified in pancreas microsomes. When 50 μ g of liver and jejunal microsomes were analysed on a separate immunoblot, CYP3A5 protein was detected in both tissues. The CYP3A5 specific antibody did not appear to be cross reacting with CYP3A4 in liver and jejunum because the relative intensity of the bands produced by the two antibodies was not similar (Fig. 3).

RNA studies

First, CYP3A cDNA was hybridized to RNA prepared from each tissue under low stringency. Hybridization was detected only in jejunum and liver (Fig. 6), indicating that the abundance of CYP3A mRNA's was low in other regions of the digestive tract. An identical pattern of hybridization was obtained when the blot was stripped and reprobed with a CYP3A4 specific oligonucleotide under stringent conditions (not shown) which is consistent with our prior

Fig. 3. Immunoblot of microsomes prepared from digestive tract tissues developed with a monoclonal antibody (13-7-10) that recognizes all CYP3A proteins or an antibody (anti-CYP3A5) that selectively recognizes CYP3A5 (see methods).

observations that CYP3A4 is the major CYP3A mRNA present in human enterocytes (Kolars *et al.*, 1992b).

We next prepared cDNA from the total RNA obtained from each organ as described in Methods. Each cDNA sample was subjected to 32 cycles of PCR using as primers the CYP3A3, CYP3A4, CYP3A5, and CYP3A7 specific oligonucleotides shown in Table 1. To verify that the amplified segment corresponded to the specific cDNA, we excised each of the amplified fragments from the gel and performed sequence analysis as described in Methods.

The CYP3A4 specific primers readily amplified a DNA fragment of appropriate molecular weight in cDNA from the liver, esophagus, stomach (body >> antrum), small intestine (duodenum, jejunum, ileum), colon, and pancreas (Fig. 4). In each cDNA sample, the identity of the amplified DNA was confirmed by sequence analysis to be CYP3A4. Similarly, CYP3A5 specific primers readily amplified a DNA fragment of appropriate molecular weight from each cDNA sample (Fig. 4). In each sample, sequence analysis confirmed that CYP3A5 had been amplified. CYP3A7 specific primers consistently amplified cDNA

Fig. 4. Polymerase chain reaction products obtained from cDNA samples prepared from the indicated organs. Synthetic oligonucleotide primers complimentary to hypervariable regions of the CYP3A cDNAs (see Table 1) were used to amplify fragments of CYP3A5 or CYP3A4 cDNA (see Methods). The identity of each fragment was confirmed by subcloning and nucleotide sequencing.

Fig. 5. Polymerase chain reaction products obtained from tissue cDNA using synthetic oligonucleotide primers that are complimentary to characteristic regions of the CYP3A7 cDNA (Table 1; see Methods). The fragment amplified from liver cDNA was confirmed to be CYP3A7 by subcloning and nucleotide sequencing.

only from liver (Fig. 5) and the identity of the amplified fragment was confirmed by cloning and sequencing. A faint band of appropriate molecular weight was intermittently observed in cDNA prepared from the small intestine (not shown), however, the amplification was insufficient to allow cloning for sequence analysis.

CYP3A3 was not detected in cDNA prepared from the liver or from any of the digestive tract tissues. The selection of CYP3A3 specific primers was complicated by the fact that the base pair homology between CYP3A3 and CYP3A4 exceeds 98%. Primers were chosen from regions where base mis-matches with CYP3A4 could be maximized. The first set of CYP3A3 'specific' primers consisted of a sense primer that shared complete homology with CYP3A4 (CYP3A3 1374 – Table 1) and an anti-sense primer that shared only 24 of 30 bases in common with CYP3A4 (CYP3A3 1658–Table 1). PCR resulted in amplification of a DNA fragment of appropriate molecular weight from cDNA prepared from liver and from each of the digestive tract tissues. However, sequencing of these amplified segments revealed 100% homology with CYP3A4 and 13 base pair mismatches with CYP3A3. Next, the anti-sense primer was replaced by a CYP3A3 primer (CYP3A3 1700 – Table 1) that differed by 4 base pairs at the 3' end from the corresponding CYP3A4 sequence. PCR analysis again resulted in weak amplification of DNA of appropriate molecular weight in some tissues; however, when the amplified fragments were cloned and sequenced they were found to be 100% homologous with CYP3A4. Finally, a third attempt to amplify CYP3A3 was made using primers that differed from CYP3A4 by two base

Fig. 6. Northern blot hybridization of total RNA (30 μ g) prepared from the indicated organs with a 32 P labelled CYP3A cDNA under low stringency conditions (see Methods). Insufficient material prevented hybridization studies with esophagus or pancreas.

pairs at the 3' end of the sense primer (CYP3A3 306 – Table 1) and by three base pairs at the 3' end of the anti-sense primer (CYP3A3 895–Table 1). Using these primers, which should each be relatively specific for CYP3A3, no amplified DNA was detected. Thus, we were not able to identify CYP3A3 mRNA in any of the digestive tract tissues from this patient.

Discussion

In these studies, we have characterized *CYP3A* gene expression throughout the entire human digestive tract. One important feature of our studies was the opportunity to simultaneously compare *CYP3A* gene expression in digestive tract epithelia and liver in a single individual.

In liver, immunohistochemical studies demonstrated that CYP3A immunoreactive protein was most abundant in pericentral hepatocytes (Fig. 1A) which is consistent with previous reports (Murray *et al.*, 1988; Ratanasavanh *et al.*, 1991). Of note, staining was not observed in periportal hepatocytes despite the fact that the patient received prolonged treatment with known inducers of hepatic CYP3A (i.e. dexamethasone and phenytoin) prior to organ procurement surgery. This suggests that zone 1 hepatocytes are not responsive to these inducers.

Hepatocyte microsomes readily reacted with the monoclonal antibody 13-7-10 on Western blots producing a single band which comigrated with purified CYP3A4. CYP3A4, CYP3A5, and CYP3A7 mRNA's were detected in the liver by PCR analysis; however, only CYP3A4 mRNA was detectable by Northern Blot analysis. CYP3A4 therefore was the most abundant CYP3A enzyme in our patient's liver which is consistent with previous reports (Bork *et al.*, 1989).

CYP3A5 protein has a slightly slower mobility on SDS-PAGE than does CYP3A4 giving rise to a 'doublet' when immunoblots of liver microsomes are reacted with the 13-7-10 monoclonal antibody (Aoyama *et al.*, 1989; Wrighton *et al.*, 1989). CYP3A5 has been detected in only 25–30% of adult human livers as judged by standard immunoperoxidase development of immunoblots (Aoyama *et al.*, 1989; Wrighton *et al.*, 1989, 1990). It has therefore been suggested that CYP3A5 is 'polymorphically' expressed (Wrighton *et al.*, 1989). A band corresponding to CYP3A5 was not detected in immunoblots of liver microsomes using the standard immunoperoxidase technique which indicates that our patient would have been classified as a CYP3A5 'non-expressor'. However, immunoblots developed with the CYP3A5 specific antibody using the more sensitive chemiluminescent technique demonstrated that CYP3A5 protein was present in low

concentration in the liver (Fig. 3), which is consistent with our detection of CYP3A5 mRNA by PCR (Fig. 4). These observations suggest that the hepatic CYP3A5 'polymorphism' is not due to a complete lack of expression of the CYP3A5 protein.

We also detected CYP3A7 mRNA in adult liver by PCR. This enzyme, which is the major P450 present in human fetal liver, has also been reported to be expressed in some, but not all, adult livers (Schuetz *et al.*, 1993). The lack of a CYP3A7 specific antibody precluded attempts to detect CYP3A7 protein in this patient's liver.

In the small bowel, CYP3A immunoreactive protein was identified predominantly in mature enterocytes and not in cells lining the villus crypt or in other cell types within the intestinal wall. This is consistent with previous reports (Watkins *et al.*, 1987; Kolars *et al.*, 1992b). CYP3A protein appeared to be largely located at the apex of the enterocytes which is consistent with the location of CYP3A protein in rat enterocytes (Kolars *et al.*, 1992b). CYP3A4 immunoreactive protein appeared to be more abundant in enterocytes than was CYP3A5 as the latter could only be appreciated using the chemiluminescent immunoblot technique. CYP3A4 mRNA was identified both by Northern Blot hybridization and by PCR amplification in contrast to CYP3A5 mRNA which could only be identified using PCR. This confirms our earlier observation that CYP3A4 is the major CYP3A gene expressed in enterocytes (Kolars *et al.*, 1992b) but also indicates that CYP3A5 is expressed in enterocytes at relatively low levels. This pattern of CYP3A4 and CYP3A5 expression is similar to that observed in the liver of this patient. Although CYP3A7 mRNA was readily detected in the liver of this patient by PCR, CYP3A7 mRNA was not consistently demonstrated in the mucosa of the small intestine, suggesting that it is at most a minor enzyme in this organ.

In the stomach, CYP3A immunoreactive protein was identified both in parietal cells as well as in the surface epithelium. Unlike the case in liver and small intestine, the major enzyme expressed in stomach appeared to be CYP3A5. The protein in stomach reacting with the monoclonal antibody 13-7-10 migrated more slowly on the SDS-PAGE gels than did the immunoreactive proteins from the other organs (i.e. CYP3A4) and stomach microsomes readily reacted with the CYP3A5 specific antibody on immunoblots (Fig. 3). As with liver and small intestine, both CYP3A4 and CYP3A5 mRNAs were detected by PCR analysis in the stomach mucosa. We had hoped to use the CYP3A5 specific antibody on the tissue sections to determine whether this enzyme was present in the parietal cells. However, this antibody did not

detectably react with the tissue sections. There may have been relatively few epitopes available to bind to this antibody which had been purified with immunoaffinity adsorption to other CYP3A protein(s) (Wrighton *et al.*, 1990).

In the colon, CYP3A immunoreactive protein was identified by immunohistochemistry in the mature epithelial cells but not in the crypt. Immunoblot analysis identified CYP3A4 and, to a lesser extent, CYP3A5 immunoreactive protein in microsomes prepared from the colonic mucosa. PCR analysis confirmed the presence of both CYP3A4 and CYP3A5 mRNAs in the colon.

In the esophagus, CYP3A immunoreactive protein was detected in the squamous epithelium. Unfortunately, sufficient material was not available to prepare microsomes for immunoblot analysis. PCR analysis indicated that both CYP3A4 and CYP3A5 mRNAs were present in esophagus.

In the pancreas, CYP3A immunoreactive protein was seen in the ductular epithelium and, to a lesser extent, in the islet cells which is consistent with a previous report (Murray *et al.*, 1988). CYP3A4 immunoreactive protein was clearly identified on the immunoblots. CYP3A5 immunoreactive protein may also have been present, but multiple other protein bands were identified on the blots of pancreatic microsomes (Fig. 3). Both CYP3A4 and CYP3A5 mRNAs were detected in pancreas by PCR analysis.

We were unable to consistently detect CYP3A7 mRNA in any tissue other than liver. We were also unable to detect CYP3A3 mRNA in any tissue including liver despite attempts using three different sets of PCR primers designed to preferentially amplify CYP3A3 cDNA. When DNA amplification did occur, sequence analysis of the generated fragment indicated that CYP3A4 but not CYP3A3 cDNA had been amplified. This suggests that if CYP3A3 mRNA was present in any tissue, it was expressed at a very low level. This was true even though our patient received prolonged treatment with dexamethasone and phenytoin which would be expected to induce CYP3A3 in the liver (Molowa *et al.*, 1986).

Because our study represents the first characterization of CYP3A enzymes in an entire human digestive tract, it is not possible to determine how our findings were influenced by the medications our patient received. The administration of dexamethasone and phenytoin almost certainly resulted in induction of CYP3A4 in the liver and jejunum, as CYP3A4 has been shown to be inducible in both of these organs (Watkins, 1987, 1990; Kolars *et al.*, 1992b). However, it cannot be concluded that CYP3A4 was induced in all tissues examined because there may be

tissue specific differences in responsiveness to inducers. For example, we have shown in rats that dexamethasone treatment resulted in induction of CYP3A1 mRNA in liver, stomach, and small bowel, but not in colon (Kolars *et al.*, 1992a). CYP3A5 appears not to be inducible in liver (Wrighton *et al.*, 1989, 1990) so it seems unlikely that CYP3A5 was induced in any of the tissues that we examined.

It has been demonstrated that there are large interpatient differences in the expression of CYP3A4 as well as CYP3A5 in adult liver (Bork *et al.*, 1989, Wrighton *et al.*, 1989, 1990), and heterogeneity in expression of CYP3A4 in jejunum has also been suggested (Kolars *et al.*, 1992b). Interpatient differences in the expression of CYP3A mRNA in colon has also been observed (McKinnon *et al.*, 1993) and may account for the three–five fold variation in metabolism of cyclosporine A by colon mucosa (Tjia *et al.*, 1993). Future studies will investigate whether similar heterogeneity in expression of these enzymes exists in esophagus and stomach.

Differences in the relative expression of CYP3A4 and CYP3A5 in the epithelium of each digestive tract organ should result in differences in catalytic properties. For example, CYP3A4 metabolizes cyclosporine A to two hydroxylated metabolites (M1 and M17) and one demethylated metabolite (M21) (Aoyama *et al.*, 1989). This is the pattern of metabolites formed when cyclosporine A is placed within the lumen of the human small intestine (Kolars *et al.*, 1991) and this is consistent with CYP3A4 being the major CYP3A enzyme expressed in this organ (Kolars *et al.*, 1992b). Colonic mucosa has also been shown to metabolize cyclosporine A to the M17 and M21 metabolites (Tjia *et al.*, 1993; Vickers *et al.*, 1993) which is consistent with our finding that CYP3A4 predominates in that organ. Human gastric mucosa has been reported to metabolize cyclosporine A but not to the M17 or M21 metabolites (Tjia *et al.*, 1993) which is in keeping with our findings that CYP3A5 is the major CYP3A enzyme present in gastric mucosa. CYP3A5 does not metabolize cyclosporine A to M17 or M21 but produces the M1 metabolite which was not assayed in the stomach metabolism study (Aoyama *et al.*, 1989). Thus, previous studies looking at cyclosporine A metabolism within digestive tract organs are consistent with our findings and indicate that the metabolism of a compound may vary significantly during the course of its absorption.

In summary, of the four known members of the CYP3A gene subfamily, only CYP3A4 and CYP3A5 appear to be expressed in the digestive tract. These enzymes appeared to be present in all organs examined but were most abundant in pericentral

hepatocytes and in mature enterocytes of the small intestine. CYP3A4 appears to be the major CYP3A gene that was expressed in all tissues with the exception of stomach where our data indicates that CYP3A5 was predominant. Differences in the relative expression of CYP3A4 and CYP3A5 may account for organ specific differences in the metabolism of many drugs. Finally, it seems likely that differences in the expression of these genes could also result in differences in the metabolism of potentially harmful dietary contaminants. For example, CYP3A4 has been shown to convert aflatoxin B1 to mutagenic metabolites but CYP3A5 does not appear to have this ability (Aoyama *et al.*, 1990). Our findings may therefore have implications regarding organ specific susceptibility to some environmental diseases, including some gastrointestinal malignancies.

Acknowledgements

This research was supported by the NIH (ES-04238, PBW). Drs Kolars and Lown are recipients of Veterans Administration Career Development Awards. We are grateful to William O. Dobbins III, MD for his helpful discussions regarding this project.

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