Pergamon

Biochemical Pharmacology, Vol. 49, No. 5, pp. 717–726, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(94)00493-5

EXPRESSION OF FUNCTIONAL CYTOCHROME P4501A1 IN HUMAN EMBRYONIC HEPATIC TISSUES DURING ORGANOGENESIS

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(*Received* 27 *June* 1994; *accepted* 23 *September* 1994)

Abstract—Investigations with chemical inhibitors and with inhibitory antibodies specific for cytochrome P4501A-catalyzed ethoxyresorufin (ethoxyphenoxazone) O-deethylation and 2-acetylaminofluorene (*N*-2-fluorenylacetamide) ring hydroxylation indicated that cytochrome(s) P450 of the 1A subfamily was functionally expressed in human embryonic hepatic tissues at very early stages (days 50–60) of gestation. Lack of detectable capacity of hepatic microsomal enzymes to catalyze either N-hydroxylation of 2-acetylaminofluorene or O-demethylation of methoxyresorufin indicated that functional cytochrome P4501A2 is expressed minimally or negligibly in human embryonic hepatic tissues. By contrast, profound inhibition of the ring hydroxylation of 2-acetylaminofluorene and of the O-deethylation of ethoxyresorufin by 7,8-benzoflavone as well as by anti-cytochrome P4501A1 antibodies indicated the presence of significant levels of functional cytochrome P4501A1 in hepatic microsomes of human embryos. Using the reverse transcriptase-linked polymerase chain reaction with specific oligonucleotide primers, we also detected significant expression of cytochrome P4501A1 mRNA in human embryonic livers. Polymerase chain reaction amplification, cloning and sequencing of the corresponding cDNA provided evidence that the cytochrome P4501A1 mRNA expressed in human embryonic tissues was identical to that expressed in adult human tissues. The results of the study have important implications in terms of the embryotoxic effects of chemicals that are known to be substrates, inhibitors or inducers of cytochrome P4501A1 and to which pregnant women are exposed.

Key words: cytochrome P4501A1; CYP1A1; human embryos; sequence analysis; teratogenesis; embryotoxicity

Investigations of the prenatal expression of CYP† isoforms have demonstrated that human prenatal hepatic tissues express functional, xenobiotic-biotransforming CYPs whose orthologous forms are not detectable in the prenatal livers of common experimental animals, such as rats, mice and rabbits [1, 2]. Particularly striking in this regard is the expression in human fetal livers of relatively high levels of functional CYP3A7, an isoform that reportedly is expressed at minimal or negligible levels in normal livers of human adults [3, 4]. In contrast, CYP3A4 is expressed at high levels in hepatic tissues of human adults but not during prenatal life. No members of the CYP3A subfamily are known to be expressed constitutively in the hepatic tissues of rodents or rabbits during prenatal life although steroidal induction of CYP3A has been demonstrated in rodents at later stages (fetal period) of gestation [5, 6].

In relatively recent years, reports suggesting the expression of CYP(s) of the 1A subfamily in human hepatic tissues during the fetal period have appeared in the literature. The fetal period extends from approximately day 60 of gestation until term; prior to this, the conceptus is referred to as an embryo rather than as a fetus. Immunodetection of P4501A in human fetal liver at 16–20 weeks of gestation was reported by Murray *et al.* [7] and at 17–32 weeks of gestation (P450HFLb) by Kitada *et al.* [8]. In contrast, Maenpaa *et al.* [9] recently reported a lack of immunodetectability of P4501A in human liver microsomes studied during the fetal period. Omiecinski *et al.* [10] reported detection of P4501A1 mRNA in human embryonic livers with the PCR but did not provide evidence for expression of an enzymically functional isoform. Earlier studies from our laboratory [11] indicated the capacity of human embryonic hepatic preparations to catalyze the carbon-monoxide inhibited O-deethylation of EROD, a reaction often regarded as diagnostic for CYPs of the 1A subfamily. However, we were unable to observe significant inhibition of the reaction with relatively high concentrations of the CYP1A-selective inhibitor ANF or with an inhibitory polyclonal antibody raised against CYP1A1, and tentatively concluded that enzymically *functional* CYP1A was expressed in human embryonic liver

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† Abbreviations: CYP, cytochrome P450 protein; EROD, 7-ethoxyresorufin *O*-deethylase; MROD, 7-methoxyresorufin *O*-demethylase; PROD, 7-pentoxyresorufin *O*-dependentylase; BZROD, 7-benzyloxyresorufin *O*-debenzylase; 3MC, 3-methylcholanthrene; AAF, 2-acetylaminofluorene; RT-PCR, reverse transcriptase-polymerase chain reaction; ANF, 7,8-benzoflavone; *CYP*, cytochrome P450 gene or cDNA; and G6P, glucose-6-phosphate.

during organogenesis at negligible levels. More recently, we found that enzymes present in cell-free preparations of human embryonic hepatic tissues catalyzed the ring-hydroxylation of AAF, a CYP1A substrate, and that the reaction appeared to be inhibitable by ANF [12]. These observations, in apparent conflict with the earlier results obtained with resorufin analogs, suggested that a CYP1A or functionally related isoform(s) appeared to be expressed in human livers during the period of organogenesis (which extends roughly through days 18–60 of gestation) at clearly detectable levels.

The primary purpose of these investigations, therefore, was to examine the question as to whether readily measurable levels of *functional* CYP1A are expressed in human hepatic *embryonic* tissues during organogenesis, the period of gestation widely held to be most sensitive to the teratogenic and dysmorphogenic effects of environmental chemicals. In addition, we wished to determine more precisely the identity(ies) of any enzymically functional CYP1A or CYP1A-similar isoforms detected in human embryonic tissues and, finally, to find reasons for the apparently conflicting literature data. The practical importance of these questions is underlined by previous studies indicating that only very low levels of CYP1A1 in conceptal tissues of rats exposed to inducers of CYP1A1/2 were sufficient to effect conversion of proteratogenic chemicals to reactive intermediates capable of causing profound morphologic abnormalities in rat embryos [13, 14]. Thus, the implications for adverse effects of environmental chemicals on human prenatal development are considerable.

MATERIALS AND METHODS

Chemicals and reagents. Resorufin, ANF and dicoumarol were purchased from the Aldrich Chemical Co., Milwaukee, WI. $NADP^+$, NADPH, G6P, G6P dehydrogenase and 3MC were purchased from the Sigma Chemical Co., St. Louis, MO. [9-^{14}C]AAF (52.3 Ci/mol) was purchased from the Amersham Corp., Arlington Heights, IL, and was further purified (>99.5% purity) with HPLC using a reverse phase, Whatman Partisil ODS-2 Magnum (M9 10/25) column and eluting isocratically with a methanol–water gradient (80–100%, 30 min). Reference standard hydroxylated AAF metabolites (7-OH-AAF, 5-OH-AAF, 3-OH-AAF, 1-OH-AAF, 9-OH-AAF and N-OH-AAF) were obtained from the Chemical Repository of the National Cancer Institute, Midwest Research Institute, Kansas City, KS, and were used without further purification. 9-oxo-AAF was synthesized and purified (>99% purity) in our laboratory in accordance with previously described methods [15, 16]. Resorufin ethers (also often referred to as phenoxazone ethers) were synthesized according to methods described by Mayer *et al.* [17]; resorufin ethers and resorufin were purified (>99.5%) by the methods described by Klotz *et al.* [18]. Spectrophotochemical grade DMSO was purchased from the J. T. Baker Chemical Co., Phillipsburg, NJ. All other chemicals used were of the highest purity commercially available.

Human embryonic tissues. Hepatic tissues from human embryos were procured through the Central Embryology Laboratory of the University of Washington (Department of Pediatrics), Seattle, WA. All procedures involving handling and assay of these tissues were in accordance with the guidelines of the Human Subjects Review Committee at the same institution. Tissues were obtained immediately following surgical procedures (dilatation and curettage), placed on ice and delivered to the laboratory within 3–4 hr. Gestational ages of the liver specimens obtained were 50–60 days as estimated from measurements of foot lengths. Upon arrival in the laboratory, tissues were frozen immediately by placing in storage at −70°. For experiments designed to draw comparisons, rat conceptal tissues at day 11 of gestation were prepared from control and 3MC-treated pregnant rats as described previously [11].

Enzymic analyses. Because of the very small quantities of tissue obtainable at this stage of gestation, and because of the nature of the experimental design (i.e. comparisons of cytosolic with microsomal fractions), it was necessary to pool tissues from 8–10 embryos. Pooled tissues were thawed and homogenized by hand in a glass homogenizing vessel (Potter) with a ground glass pestle in 3 vol. of ice-cold, 0.1 M potassium phosphate buffer (pH 7.4). The resultant homogenate was centrifuged at 600 *g* for 5 min, and an aliquot of the supernatant fraction was saved for subsequent analyses. The remainder of the 600 *g* × 5 min supernatant fraction was centrifuged at 104,000 *g* for 1 hr. The 104,000 *g* × 1 hr supernatant fraction (designated as cytosolic fraction) was likewise saved for subsequent analyses. The 104,000 *g* × 1 hr sediment was washed by resuspending in 5–6 mL of 0.1 M potassium phosphate buffer (pH 7.4) and recentrifuging at 104,000 *g* for 1 hr. The resultant sediment was resuspended in a volume of 0.1 M (pH 7.4) potassium phosphate buffer equivalent to the original volume of the cytosolic fraction and centrifuged at 8000 *g* for 20 min to eliminate large fragments of particulate material. The resultant 8000 *g* × 20 min supernatant fraction was designated as the microsomal fraction (microsomes) and also was utilized in subsequent analyses.

Rates of O-dealkylation and O-debenzylation of resorufin ethers were assessed fluorimetrically according to slightly modified methods originally described by Burke *et al.* [19]. Unless otherwise specified, the incubation mixtures contained 0.04 to 0.3 mg of embryonic hepatic protein (0.1 mL) from one of the above-described fractions, resorufin ether substrate (10 μM) dissolved in 10 μL of DMSO, 1.0 mM NADPH, an NADPH-regenerating system consisting of 5.0 mM G6P and 1 unit of G6P dehydrogenase, 0.01 mM dicoumarol and sufficient Tris–HCl buffer (0.1 M, pH 7.8) to yield a total volume of 1.0 mL. After obtaining a baseline, reactions were initiated by additions of NADPH, and temperatures of the reaction mixtures were maintained at 37° during measurements of resorufin generation. Reactions were monitored continuously as a function of time with a Farrand recording fluorimeter with excitation and emission wavelengths of 530 and 585 nm, respectively. Generation of resorufin was measured for at least 10 min, and the

Table 1. HPLC separation of 2-acetylaminofluorene (AAF) and its metabolic oxidation products*

Reference compound	Retention time (min)	
	System 1	System 2
AAF	34.2	33.4
N-OH-AAF	29.6	28.3
1-OH-AAF	27.7	27.4
3-OH-AAF	24.1	26.7
9-oxo-AAF	19.8	25.3
5-OH-AAF	12.3	19.8
9-OH-AAF	11.0	16.2
7-OH-AAF	8.8	11.5
Solvent front	3.4	1.3

* Details of the two separation procedures, designated as Systems 1 and 2, are provided in Materials and Methods.

slopes of the lines for the first 3 min were used to calculate the rates of the reactions. The extent of fluorescence increase was calibrated against a known concentration of purified resorufin standard in each assay. The limit of detection was 0.1 pmol/min for each ether. Reactions assessed with methoxyresorufin, ethoxyresorufin, pentoxyresorufin and benzyloxyresorufin as substrates are referred to as MROD, EROD, PROD and BZROD activities, respectively.

For analyses of biotransformation of AAF, reaction vessels contained 1.9 mM NADPH, 1.01 μCi of [9-^{14}C]AAF (42.4 μM, final concentration), 3.4 mM G6P, 2 units of G6P dehydrogenase, 3–4 mg of 600 $g \times 5$ min supernatant protein and sufficient potassium phosphate buffer solution (0.1 M, pH 7.4, containing 0.1 M potassium fluoride for inhibition of deacetylation) to provide a total volume of 0.5 mL. Reactions were initiated by additions of labeled AAF, carried with shaking in an atmosphere of air for 120 min at 37° and terminated by the rapid addition of 1.5 mL of ice-cold, 1.0 M sodium acetate (pH 6.0). The mixture was then extracted four times with 10 vol. of peroxide-free diethyl ether. The ether extracts were pooled and evaporated to dryness; the residue was redissolved in 20 μL of a 95% ethanol solution containing the metabolite standards. Separation of the generated metabolites by HPLC was accomplished using two separate systems designated as systems 1 and 2 (Table 1). For system 1 we used slight modifications of a method previously described [20] as follows: A 10-μL aliquot of the ethanol solution was injected onto a Zorbax C-8 column, utilizing a Beckman HPLC system. Initial composition of the mobile phase was 26:74 isopropyl alcohol:0.01 M acetic acid (v/v, pH 3.3). Each solvent contained 0.01% (w/v) deferoxamine mesylate (Desferal, Ciba). A constant flow rate of 1.2 mL/min was maintained throughout. The solvent ratio was changed linearly to 29:71 over the first 10-min period and then held at that composition for a period of 18 min. Over the next 10 min, the ratio was changed linearly to 54:46 and then held at that ratio for 7 min. Finally, a 100% concentration of isopropanol was achieved with a linear gradient over a 1-min period. Fractions were collected for 30 sec each, and 90 fractions were obtained. System 2 was utilized to provide a better separation of the 5-, 7- and 9-hydroxy metabolites as follows: a 10-μL aliquot of the ethanol solution was injected onto a Rainin Microsorb Short-one C-8 column (3 mm, 100 × 4.6 mm), utilizing the same Beckman HPLC system. Initial composition of the mobile phase was 16:84 isopropanol:0.01 M acetic acid (v/v, pH 3.3). Each solvent contained 0.01% deferoxamine mesylate. Isocratic elution was continued for 17 min and then changed linearly to 40:60 isopropanol:acetic acid over a period of 3 min. After reaching the 40:60 ratio, elution was continued for 15 min. A constant flow rate of 1.0 mL/min was maintained throughout. Fractions were collected for 30 sec each, and 50 fractions were collected. For both systems 1 and 2, elution of standards (Table 1) was monitored by UV absorbance at 253 nm for each sample injected onto the HPLC column. Both systems were used in each assay of AAF biotransformation. System 1 was used to quantitate N-OH-AAF, 1-OH-AAF, 3-OH-AAF and 9-oxo-AAF. System 2 was used for quantitation of 5-OH-AAF, 9-OH-AAF and 7-OH-AAF. 7-OH-AAF and 9-oxo-AAF were quantifiable in both systems and were used as cross checks on metabolite quantifications. For assays, one aliquot of the 95% ethanol extract was subjected to separation with system 1 and a second aliquot was subjected to separation with system 2. Fractions were collected, and 3 mL of Aquasol (New England Nuclear) were added to each collection vial. Samples were counted for a time period sufficient to achieve less than a 2.5% error with 95% confidence intervals. Counting efficiency was 85%. Radioactivity remaining in the aqueous phase of the original incubation mixture (after diethyl ether extractions) was also quantified by liquid scintillation spectrometry. For all enzyme assays, results were expressed as pmol/min/mg protein. Protein concentrations were assayed according to the method of Lowry *et al.* [21].

Antibody preparation. Polyclonal antibodies were raised against rat hepatic P4501A1 by intradermally injecting 0.25 mg of the corresponding purified cytochrome in Freund's adjuvant into adult, female New Zealand White rabbits according to slight modifications [22] of methods described by Thomas *et al.* [23]. For antibody inhibition studies, human embryonic hepatic microsomal fractions for EROD or 600 $g \times 5$ min supernatant fractions for AAF hydroxylation were preincubated for 15 min at 25° with IgG fractions (0.5 to 5.0 mg IgG protein per incubation vessel) prepared from the antisera by passage through a Protein A-Sepharose CL-4B column [24].

RNA preparation, first strand cDNA synthesis, PCR amplification of cDNA and Southern blotting. In these experiments, human embryonic hepatic tissues from 6–8 embryos were pooled and homogenized as described previously [25]. Total RNA was extracted from the hepatic homogenates as described by Chomczynski and Sacchi [26], and mRNA was isolated with a Poly(A^+) Quick mRNA purification kit (Stratagene, LaJolla, CA). Reverse transcription of human embryonic hepatic mRNA was performed with oligo dT_{15} (Promega, Madison,

WI) and AMV reverse transcriptase (Life Sciences, Inc., St. Petersburg, FL) as described by Omiecinski *et al.* [10]. The PCR sense primer utilized was 5′-TAGACACTGATCTGGGCTGCAG-3′ and the antisense primer was 5′-GGGAAGGCTCCATC-AGCATC-3′. These primers were designed to target the cDNA within the corresponding genomic exon 7 of human CYP1A1 [27, 28]. PCR reactions were performed with a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). The reaction mixture consisted of 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.0 mM $MgCl_2$, 0.2 mM of each dNTP, 25 pmol each of sense and antisense primers, 5 μL of cDNA and 2.5 units of Taq polymerase (Promega) in a total volume of 50 μL. After the addition of 100 μL of mineral oil, 30 cycles of amplification were performed with denaturation at 93° for 1 min, annealing at 54° for 1.5 min, and extension at 72° for 1 min. A 25-μL aliquot of the PCR-amplified cDNA was electrophoresed on 2.0% agarose gels and stained with ethidium bromide for visualization of the amplified product(s). DNA molecular size markers were co-electrophoresed in each experiment. Electrophoresed cDNA was denatured as described by Sambrook *et al.* [29], transferred to nitrocellulose membranes for Southern blots as described by Schatz [30], and hybridized with ^{32}P-labeled 5′-CAGGCAGGATCCCTTAGGCT-3′ as an internal 20-mer oligonucleotide probe [10]. The nitrocellulose membranes were then washed and exposed to XAR Kodak X-ray film with intensifying screens (Fisher Scientific, Seattle, WA).

Cloning and sequencing of the RT-PCR-amplified cDNA. A second 25-μL aliquot of the PCR reaction mixture was electrophoresed on an ethidium bromide-containing, 2.0%, low-melting-point agarose gel together with DNA molecular size markers. A cDNA band at the expected length was then excised from the gel and purified according to methods described by Qian and Wilkinson [31]. Briefly, the slice of low-melting-point agarose gel containing the cDNA was placed in a microcentrifuge tube and 3 vol. of 1 × TE buffer (10 mM Tris–HCl, 1.0 mM EDTA, pH 8.0) were added. To melt the agarose, the tube was incubated at 70° for 5 min with brief vortexing during the incubation. The sample was then quick-frozen at −70° for at least 5 min, followed by thawing and centrifugation at 16,000 *g* for 1–2 min to sediment the agarose with the cDNA remaining in the supernatant. The agarose-free cDNA was then precipitated with ethanol and treated with large-fragment (Klenow) DNA polymerase I (Promega) to repair the ends of the cDNA [30]. The resultant blunt-ended cDNA was then linked to *Eco*RI adaptor (Promega) in accordance with the manufacturer's instructions. Bluescript vector (SK-; Stratagene Cloning Systems, LaJolla, CA) was used as the vector that was cut with *Eco*RI (GibcoBRL, Grand Island, NY) and treated with calf intestinal alkaline phosphatase prior to utilization for ligation. Sequencing then was performed on both strands with the Sequenase 2 System provided by the U.S. Biochemical Life Science Co. (Cleveland, OH) in accordance with procedures outlined by the manufacturers.

RESULTS

Investigations of homogenate subfractions. Centrifugation of 600 *g* × 5 min supernatant fractions of human embryonic hepatic homogenates at 104,000 *g* for 60 min and subsequent analyses of the 104,000 *g* supernatant (cytosolic fraction) and the further processed sediment (microsomal fraction, see Materials and Methods) revealed that substantial EROD activity was present in the cytosolic fraction, although activity was also readily measurable in the microsomal fraction (Table 2). The cytosolic fractions contained 2.5 to 3.0 mg protein/mL, whereas microsomal fractions, with volumes adjusted to be equal to the original volumes of the cytosolic fractions, contained 0.6 to 0.8 mg protein/mL. Thus the contribution of microsomal protein to the 600 *g* × 5 min supernatant fraction was roughly 4- to 5-fold less than the contribution of cytosolic protein to the 600 *g* × 5 min supernatant fraction. In terms of EROD activities in human embryonic hepatic 600 *g* × 5 min supernatant preparations, the contribution of the cytosolic fraction was more than 5-fold greater than the contribution of the microsomal fraction since specific activities in the cytosolic fractions were somewhat higher than specific activities observed in the microsomal fractions. For comparative purposes, cytosolic and microsomal fractions from day 11 rat conceptuses were also studied. Rat conceptal microsomal fractions exhibited no detectable activity unless embryos were preexposed *in utero* to 3MC, an inducer of P4501A1. Rat conceptal cytosolic fractions, on the other hand, exhibited easily measurable and roughly equal EROD activities in both control and treated conceptuses.

Comparisons among various resorufin ether substrates. Investigations of human embryonic hepatic dealkylation and debenzylation activities in cytosolic versus microsomal fractions were conducted with methoxy-, ethoxy-, pentoxy- and benzyloxyresorufin ethers as substrates (Table 3). With these substrates, easily measurable MROD, EROD, PROD and BZROD activities, respectively, were determined with the cytosolic fraction as enzyme source. Highest activities were observed with the benzyloxy ether as substrate and lowest activities with the methoxy ether. However, the microsomal fraction exhibited detectable activities with only the ethoxy and benzyloxy ethers as substrates. Microsomal activities with the ethoxy ether were roughly 3-fold higher than those observed with the benzyloxy ether. PROD and MROD activities both were below the limits of detectability in repeated experiments with microsomal fractions.

Investigations of inhibition of human embryonic hepatic EROD activities. Studies of the inhibitory effects of a polyclonal antibody raised against adult rat hepatic P4501A1 (anti-P4501A1) are presented in Table 4. In preliminary experiments, this antibody preparation was shown to be highly effective in inhibiting the EROD activity of microsomes prepared from the livers of 3MC-induced, adult male rats (95–98% inhibition). The antibody exhibited no statistically significant inhibitory effects in reaction vessels containing the 600 *g* × 5 min supernatant or

Table 2. Ethoxyresorufin *O*-deethylase (EROD) activities in subfractions of human embryonic hepatic (days 50–60 of gestation) and rat whole conceptal (day 11 of gestation) homogenates*

Tissue	Homogenate fraction	EROD activity (pmol/mg/min)
Human liver	600 *g* × 5 min supernatant	2.73 ± 0.62
Human liver	Cytosolic fraction†	6.46 ± 1.84
Human liver	Microsomal fraction‡	4.63 ± 1.91
Rat conceptus	600 *g* × 5 min supernatant	1.29 ± 0.37
Rat conceptus	Cytosolic fraction†	2.48 ± 0.75
Rat conceptus	Microsomal fraction‡	ND§
Rat conceptus, 3MC‖	Cytosolic fraction†	2.58 ± 0.83
Rat conceptus, 3MC‖	Microsomal fraction‡	7.97 ± 2.26

* Values in the table represent the means ± SD of specific activities (pmol/mg protein/min) of 3–4 experiments. In each experiment, tissues were pooled from 8–10 human embryos or from 15–20 rat conceptuses. Generation of resorufin was assayed as detailed in Materials and Methods.

† 104,000 *g* × 60 min supernatant fraction.

‡ 104,000 *g* × 60 min sediment after previous centrifugation at 8000 *g* × 20 min.

§ ND indicates that activities were below the level of detectability (<0.1 pmol/min).

‖ 3MC indicates that conceptuses were exposed *in utero* to 3-methylcholanthrene. Pregnant dams were given a single intraperitoneal injection of 3MC in corn oil at 40 mg/kg 48 hr prior to removal of the conceptuses for assays.

Table 3. Oxidative O-dealkylation and O-debenzylation of resorufin ethers catalyzed by human embryonic hepatic microsomal and cytosolic fractions*

Homogenate fraction	Substrate	Specific activity (pmol/mg/min)
Cytosolic fraction†	Methoxyresorufin	4.23 ± 1.12
Microsomal fraction‡	Methoxyresorufin	ND§
Cytosolic fraction	Ethoxyresorufin	6.46 ± 1.51
Microsomal fraction	Ethoxyresorufin	4.63 ± 2.27
Cytosolic fraction	Pentoxyresorufin	4.94 ± 0.86
Microsomal fraction	Pentoxyresorufin	ND
Cytosolic fraction	Benzyloxyresorufin	8.28 ± 2.69
Microsomal fraction	Benzyloxyresorufin	1.59 ± 0.48

* Results are from three or four separate experiments in which livers were pooled from 8–10 embryos; values are means ± SD. Other conditions were as detailed in Materials and Methods.

† 104,000 *g* × 60 min supernatant fraction.

‡ 104,000 *g* × 60 min sediment after previous centrifugation at 8000 *g* × 20 min.

§ ND indicates that activities were below the level of detectability (<0.1 pmol/min).

cytosolic fractions of human embryonic hepatic homogenates as enzyme sources. But, in experiments with microsomal fractions from the same embryonic livers, inclusion of anti-P4501A1 produced approximately 72% inhibition of EROD activity. Likewise, ANF produced no significant inhibition when the human embryonic hepatic 600 *g* × 5 min supernatant or cytosolic fractions were utilized as enzyme sources but effected approximately 77% inhibition in the corresponding microsomal fractions.

Inhibition of human embryonic hepatic AAF oxidation. In previous investigations, we demonstrated the capacity of several human embryonic tissue preparations (hepatic, adrenal, pulmonary, renal and cardiac) to catalyze the biotransformation of AAF to several oxidized metabolites [12]. Results of the current investigations (Table 5) show that anti-P4501A1 antibody and ANF each profoundly inhibited conversion of AAF to its 7-, 5- and 3-hydroxylated metabolites when catalyzed by human embryonic hepatic enzymes. Catalyses of the conversions of AAF to its 9-OH and 9-oxo metabolites by human embryonic preparations were shown not to be P450-mediated [12] and conversion to the 1-hydroxy and N-hydroxy-AAF metabolites in these investigations were too low to permit

Table 4. Inhibition of human embryonic hepatic ethoxyresorufin *O*-deethylase (EROD) activities by 7,8-benzoflavone (ANF) and by rabbit anti-rat P4501A1 antibody (anti-P4501A1)*

Homogenate fraction	Inhibitor	EROD activity (pmol/mg/min)
600 *g* × 5 min supernatant	None	2.92 ± 0.62
600 *g* × 5 min supernatant	Preimm. IgG†	2.79 ± 1.23
600 *g* × 5 min supernatant	Anti-P4501A1†	3.07 ± 0.87
600 *g* × 5 min supernatant	ANF	2.74 ± 0.55
Cytosolic fraction‡	None	4.68 ± 1.72
Cytosolic fraction	Preimm. IgG§	5.12 ± 2.03
Cytosolic fraction	Anti-P4501A1§	5.26 ± 1.95
Cytosolic fraction	ANF	4.46 ± 0.84
Microsomal fraction‖	None	6.16 ± 1.23
Microsomal fraction	Preimm. IgG¶	6.23 ± 2.28
Microsomal fraction	Anti-P4501A1¶	1.75 ± 0.47
Microsomal fraction	ANF	1.40 ± 0.28

* Values are means ± SD of specific activities (pmol/mg/min) from 3–4 experiments with pooled livers (8–10/pool). ANF was added to reaction vessels at 0.01 mM concentrations and anti-P4501A1 antibody in the quantities indicated.

† IgG protein (2.5 mg) preincubated with 600 *g* × 5 min supernatant (see Materials and Methods) was added to reaction vessels.

‡ 104,000 *g* × 60 min supernatant fraction.

§ IgG protein (2.0 mg) preincubated with cytosolic fraction (see Materials and Methods) was added to reaction vessels.

‖ 104,000 *g* × 60 min sediment after previous centrifugation at 8000 *g* × 20 min.

¶ IgG protein (0.5 mg) preincubated with microsomal fraction (see Materials and Methods) was added to reaction vessels. This concentration strongly inhibited (95–98%) EROD activities in microsomal preparations from MC-induced, adult rats.

accurate assessments of inhibitory activity. No evidence of significant N-hydroxylating activity was observed in any experiment. In the experiments with ANF, inhibitions of human embryonic AAF hydroxylating activities at the 3-, 5-, and 7-carbon positions were 69–78% and with anti-P4501A1, inhibition was 57–64%.

Detection of CYP1A1 gene (CYP1A1) transcripts and sequencing. Results of the enzymic analyses obtained in the above-described experiments strongly suggested the expression of functionally active CYP1A1 in human embryonic hepatic tissues. Attempts to detect *CYP*1A1 transcripts with RT-PCR yielded positive signals (Fig. 1A) on ethidium bromide stained agarose gels. No signal was detected when reverse transcriptase was omitted from the reverse transcription reaction. Experiments with Southern blotting yielded results consistent with the idea that the amplified cDNA generated in the experiments with RT-PCR was complementary to adult human *CYP*1A1 mRNA. The RT-PCR-amplified cDNA exhibited a size of approximately 146 bp and hybridized with a 20-mer internal nucleotide probe selective for *CYP*1A1 cDNA (Fig. 1B). For further characterization, cloning and sequencing of the RT-PCR-amplified cDNA, in accordance with procedures described in Materials and Methods, indicated an exact identity [27, 28] with the sequence for human adult mammary cell *CYP*1A1 cDNA for a 146 bp sequence (from genomic position 7076 to 7221; accession No. X02612) within the seventh exon [27, 28]. The partial sequence is depicted in Fig. 2.

DISCUSSION

The detection of easily measurable levels of *functional* CYP1A1 as well as *CYP*1A1 mRNA in tissues of the human embryo during organogenesis is of importance primarily because of the capacity of CYP1A1 to catalyze the bioactivation of a large number of foreign organic chemicals including many to which human embryos are often exposed. This discovery raises questions pertaining to the possible detrimental effects of environmentally ubiquitous CYP1A1 substrates on developing human embryos during organogenesis and of the influence of varying levels of embryonic CYP1A1 on the magnitude and/or incidence of such potential effects. It is clear from the results presented that the quantities of functional CYP1A1 detected in human embryonic livers were low, particularly in comparison with those measurable in livers of adult rats previously exposed to inducing doses of 3MC or similar inducing agents. Nevertheless, a series of previous investigations (reviewed in Refs. 13 and 14) has demonstrated that even extremely low levels of rat conceptal CYP1A1 are sufficient to bioactivate AAF (a CYP1A1 substrate) to the extent that profound morphologic abnormalities were elicited. Clearly, absolute levels

Table 5. Inhibition by 7,8-benzoflavone (ANF) and by rabbit anti-rat P4501A1 antibody (anti-P4501A1) of the hydroxylation of 2-acetylaminofluorene (AAF) catalyzed by 600 g × 5 min supernatant fractions of human embryonic hepatic homogenates*

AAF metabolite	Inhibitor	% Inhibition
7-OH-AAF	ANF†	78
9-OH-AAF	ANF	0
5-OH-AAF	ANF	69
9-oxo-AAF	ANF	ND‡
3-OH-AAF	ANF	73
1-OH-AAF	ANF	ND
N-OH-AAF	ANF	ND
7-OH-AAF	Anti-P4501A1§	62
9-OH-AAF	Anti-P4501A1	0
5-OH-AAF	Anti-P4501A1	64
9-oxo-AAF	Anti-P4501A1	ND
3-OH-AAF	Anti-P4501A1	57
1-OH-AAF	Anti-P4501A1	ND
N-OH-AAF	Anti-P4501A1	ND

* Percentages of inhibition in the table are the mean values from replicate determinations of a single experiment run with a pooled supernatant from 10 embryos. The experiments were repeated with very similar results. Assay procedures are detailed in Materials and Methods. Ranges of specific activities for each AAF metabolite were very similar to those reported in a previous publication [12].

† ANF was added to reaction vessels at a final concentration of 0.01 mM.

‡ ND indicates that quantities of metabolite were below the limits of detectability and could not be evaluated for inhibition.

§ IgG protein (5.0 mg) preincubated with 600 g × 5 min supernatant fraction (see Materials and Methods) was added to reaction vessels. These concentrations strongly inhibited (85–90%) the 7-hydroxylation of AAF in microsomal preparations from MC-treated, adult rats. An equal quantity of preimmune IgG protein was added to the corresponding controls.

are of far lesser importance than the ratio of bioactivation to bioinactivation or other defenses. The latter have been only minimally investigated in human embryonic tissues.

An earlier investigation from this laboratory [11] suggested that functional CYPs of the 1A subfamily were not detectable in liver, lung, kidney, adrenal gland or heart of human embryos at days 50–60 of gestation. This tentative conclusion was based on the observation that, although EROD activity was readily detectable in such tissues, it was not inhibited significantly by antibodies inhibitory to CYP1A1 isoforms or by ANF, a potent and selective CYP1A inhibitor. The results of the current investigation show that the reason for lack of detection of inhibitory effect was due to a somewhat unexpected and surprisingly high activity in the cytosolic fraction that is not inhibited by either ANF or anti-P4501A1 antibody. Because the earlier reported investigations dealt with measurements of *O*-deethylase activities in 600 g × 5 min supernatant fractions, which contain components of both the cytosolic and microsomal fractions, lack of detectability of inhibition is now understandable. Since more than 80% of the EROD activity measured in the 600 g × 5 min supernatant fractions of human embryonic hepatic homogenates can be accounted for by the cytosolic fraction (an unexpected finding) and inhibitions of microsomal EROD activity by antibody and ANF were in the approximate range of 60–70%, it is now apparent from the above considerations why significant inhibition was not detected in the human embryonic hepatic 600 g × 5 min supernatant fractions utilized in previous investigations [11]. Discovery of significant EROD activity in cytosolic fractions in these studies thus represents a key element in the resolution of past questions pertaining to catalytically functional CYP1A1 expression in both rodent and human embryonic tissues. The nature of cytosolic fraction components responsible for EROD activity remains an important question and presents a major challenge for future investigations. We are currently investigating these issues. It is clear, however, that the EROD activity detected in cytosolic fractions is not unique to human embryonic liver because such activity was also found in cytosolic fractions of rat conceptal homogenates (Table 2) as well as in cytosolic fractions of several tissues of adult rats (liver, lung, kidney—data not presented). Nor was the activity of the cytosolic fraction unique to the ethoxyresorufin substrate. Similar activity also was found in cytosolic fractions with the methoxy, pentoxy and benzyloxy ether derivatives as substrates (Table 3). In contrast, O-dealkylation of neither methoxyresorufin nor pentoxyresorufin was detectable in microsomal fractions even though readily measurable O-deethylation of ethoxyresorufin and O-debenzylation of benzyloxyresorufin proceeded with the same tissue preparations. Rigorous characterizations of the cytosolic fraction activities, *O*-debenzylase activity of the microsomal fractions, and their responsible respective catalysts all remain for future investigations.

Evidence that the catalyst primarily responsible for EROD and AAF ring hydroxylating activities in the microsomal fractions of human embryonic livers is, in fact, CYP1A1 is provided by the following observations. (1) The microsomal activities each were inhibited markedly by both ANF and anti-CYP1A1, each of which is an established inhibitor of CYP1A1. (2) Significant participation of a catalytically functional CYP1A2 seemed very unlikely in view of the lack of detectable O-demethylation of methoxyresorufin, a reaction that is very effectively catalyzed by both rodent and human CYP1A2 isoforms [11, 32, 33], as well as the lack of detectable N-hydroxylation of AAF, a reaction likewise known to be very effectively catalyzed by both human and rodent CYP1A2 [12, 32]. Because the data obtained convinced us that *functional* CYP1A2 was either absent from or present at extremely low levels in the tissues investigated, further consideration of CYP1A2 was not pursued. (3) Expression of *CYP*1A1 mRNA in the same tissue was in agreement with results reported by Omiecinski *et al.* [10] with PCR. (4) Sequence data obtained in these experiments provided evidence that the embryonic cytochrome P4501A1 is identical to adult human CYP1A1. We were unable to detect CYP1A1 protein in human embryonic tissues with immunoblots (data not

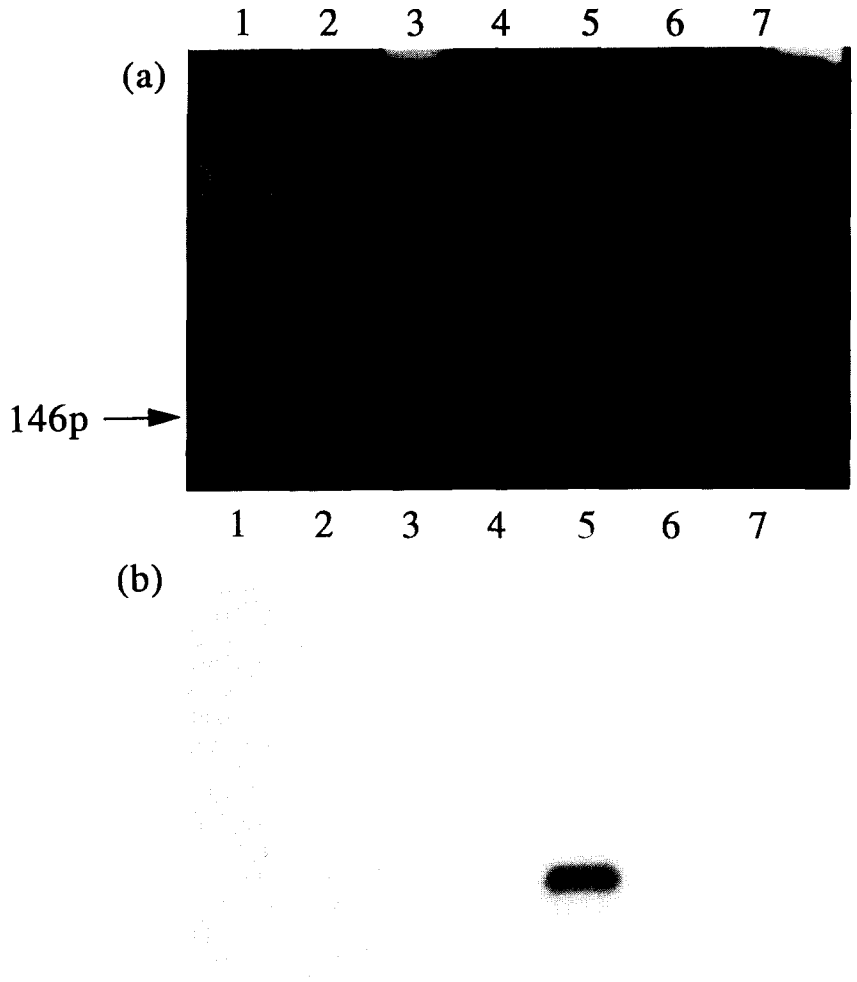

Fig. 1. RT-PCR products generated from human hepatic embryonic tissues at gestational ages 50–60 days, with primers selective for human *CYP*1A1 (see Materials and Methods). (a) Ethidium bromide stained 2.0% agarose gel shows RT-PCR products from the amplification of cDNA synthesized from oligo-dT selected mRNA of embryonic hepatic tissues. Twenty-five microliters of PCR reaction mixture were loaded onto the gel. Lanes 1, 3 and 7, DNA molecular size markers; Lane 2, mRNA from human embryonic livers without reverse transcriptase added to the reverse transcription reaction; Lane 4, first strand cDNA was omitted from the PCR reaction mixture; Lane 5, mRNA from human embryonic livers with reverse transcriptase added to the reverse transcription reaction. Lane 6, sample blank. (b) Southern blotting of the same gel. Hybridization was performed with a ^{32}P-labeled internal probe (see Materials and Methods). The arrow indicates the migration position of the RT-PCR product at a 146-bp size as predicted from usage of the described primer pair. This experiment was repeated twice with virtually identical results.

shown), but those results were consistent with the inability to immunodetect catalytically active CYP1A1 in 3MC-induced rat embryos during organogenesis [22, 25].

Because CYP3A7 is known to be expressed prenatally in human hepatic tissues, the possibility must be considered that a member of the CYP3A subfamily could be responsible for the xenobiotic biotransforming activities observed in these studies. It is known, for example, that members of the CYP3A subfamily (including human CYP3As) can catalyze the ring hydroxylation of AAF [12, 32] and that ANF has been reported to be capable of inhibiting CYP3A-catalyzed reactions [34]. In view of these observations, our previous studies with AAF [12] did *not* rigorously rule out CYP3A participation or definitively demonstrate functional CYP1A1 expression in human embryonic livers. Also, CYP3As are known to catalyze the O-debenzylation of benzyloxyresorufin [5, 6]. However, both human and rodent members of the CYP3A subfamily have been reported to have minimal or no capacity to catalyze O-deethylation of ethoxyresorufin [11, 32] and the catalytic activities

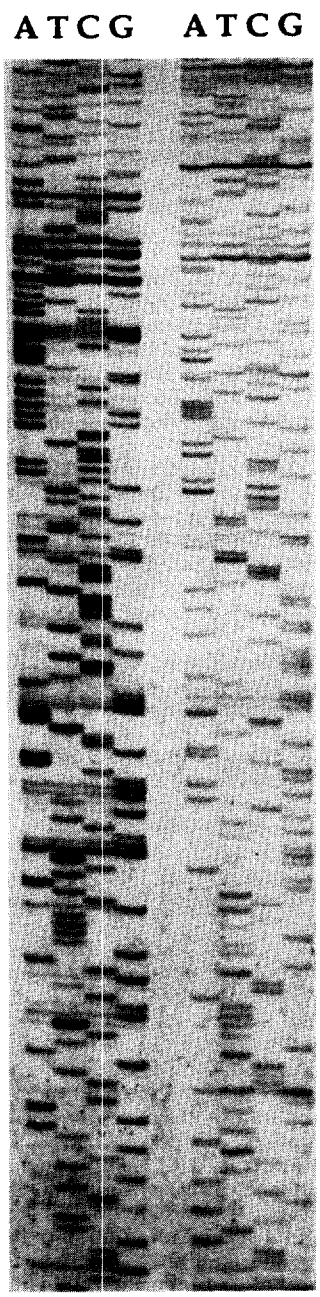

Fig. 2. Partial sequence of the cDNA generated in the RT-PCR reactions (see Materials and Methods). The sequence illustrated is that of the 146 bp cDNA targeted within exon 7, genomic position 7076 to 7221 (accession No. X02612). The 4 left lanes indicate the sequence of the sense strand, and the 4 right lanes indicate the sequence of the antisense strand.

of CYP3A isoforms would not be expected to be inhibited by anti-CYP1A1. Thus, the possibility of significant catalysis of ANF/anti-CYP1A1 inhibitable EROD by human embryonic CYP3A(s) seems quite remote. Also, it has not yet been determined whether CYPs of the 3A subfamily are expressed significantly in human embryonic livers during the early stages of gestation investigated in this study, i.e. during organogenesis. This remains an additional important question for future investigations. Also, future investigations of individual embryos would be desirable because of the possible impact of genetic polymorphisms and environmental influences on the values obtained.

In summary, the data presented here provide convincing evidence that *functionally* active CYP1A1 is expressed in easily measurable quantities in microsomal fractions of human embryonic livers during organogenesis, an early stage of development of especial importance in terms of sensitivity to the embryopathic effects of exogenous chemicals to which embryos may be exposed. The CYP1A1 isoform expressed in embryonic tissues appears to be identical to that expressed in tissues of the human adult. The data also demonstrate for the first time that components of cytosolic fractions (as yet unidentified) of embryonic tissue homogenates can catalyze easily measurable O-dealkylation and O-debenzylation of various resorufin ethers. This observation was an important factor in the resolution of apparently conflicting previous data concerning the expression of CYP1A1 in human embryonic tissues. Finally, the data strongly indicate that enzymically active CYP1A2 is expressed minimally or negligibly in human embryonic liver during organogenesis.

Acknowledgements—The authors wish to acknowledge the expert technical assistance of Julie Pascoe-Mason and Molly Hogan. We wish also to acknowledge the assistance of Drs. Alan G. Fantel and Thomas H. Shepard, Department of Pediatrics, University of Washington, in securing human embryonic tissues for these investigations, supported by NIH grant ES-OHOHI.

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