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Biochemical Pharmacology 68 (2004) 73–83
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Cell-, tissue-, sex- and developmental stage-specific expression of mouse flavin-containing monooxygenases (*Fmo*s)

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Received 13 January 2004; accepted 19 February 2004

Abstract

The cell-, tissue-, sex- and developmental stage-specific expression profiles of five members of the flavin-containing monooxygenase (FMO) family, FMO1, 2, 3, 4 and 5, were investigated in 129/SV mice, using isoform-specific antisense RNA probes. In situ hybridization localized FMO1 and 5 mRNAs to the perivenous, and FMO 2, 3 and 4 mRNAs to the periportal, regions of the liver. In kidney, each FMO mRNA is localized to the distal and proximal tubules and collecting ducts; FMO1 mRNA is present also in the glomerulus. In lung, FMO1 and 3 mRNAs are expressed in the terminal bronchiole, and FMO1 mRNA also in the alveoli. FMO1 mRNA is present in neurons of the cerebrum and in the choroid plexus. RNase protection assays showed that the most abundant isoform in newborn liver, lung, kidney and brain, and in adult lung and kidney is FMO1, but in adult liver FMO5 is present in greatest amounts. In liver, lung and kidney, expression of *Fmo1*, *3* and *5* peaks at 3 or 5 weeks of age, but in the brain, *Fmo1* expression is greatest in newborns. In the kidney, FMO5 mRNA abundance is fourfold greater in males than in females, at all stages of development. Our results demonstrate that *Fmo1*, *2*, *3*, *4* and *5* exhibit distinct cell-, tissue-, sex- and developmental stage-specific patterns of expression.

Keywords: Flavin-containing monooxygenase; Mouse; Tissue; Development; Gene regulation; In situ hybridization

1. Introduction

The flavin-containing monooxygenases (FMOs) [EC 1.14.13.8] constitute a family of proteins that play an important role in the detoxification of many foreign chemicals, including psychoactive drugs, pesticides and dietary-derived compounds [1]. Humans have five genes, named *Fmo1–5* [2], and six pseudogenes, *Fmo6P–11P* [3]. In mice, there are nine *Fmo* genes, which are named *Fmo1–6* and *9*, *12* and *13* [3].

FMO1 and 3 are considered to be the most important members of the FMO family with respect to the metabolism of foreign chemicals [4]. In humans, FMO1 is expressed in fetal kidney and liver and in the adult kidney, but not in adult liver [5]. This is in contrast with other mammals, such as pig [6], rabbit [7], rat [8] and mouse [9], which do express FMO1 in adult liver. The expression of FMO3 is switched on in the liver after birth in humans [5,10] and in mice [9]. In most humans, functional FMO2 is not expressed because of nonsense and/or frameshift mutations in the *FMO2* gene [11–13]. An allele encoding a full-length functional FMO2 is present at a frequency of 13% in individuals of African descent [14]. In rabbit [15], mouse [16] and guinea pig [17], a full-length functional FMO2 is expressed in the lung. But the *FMO2* gene of *Rattus norvegicus* encodes a non-functional protein [18]. Current evidence indicates that FMO6, in humans, also does not code for a functional protein [13,19]. FMO5 is expressed in the liver of humans [20], rabbit [21], guinea pig [20] and mouse [22] and FMO4 mRNA has been detected in low amounts in several human tissues [23,24]. Analysis of mouse sequence databases revealed no cDNAs for FMO12 and 13 and just one, isolated from a 0-day neonate mouse head library, for FMO9 [3]. Thus, none of these three genes appears to be significantly expressed in the adult mouse.

Abbreviations: CYP, cytochrome P450; FMOs, flavin-containing monooxygenases; RT-PCR, reverse transcription-PCR; DEPC-PBS, diethylpyrocarbonate-phosphate buffered saline

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doi:10.1016/j.bcp.2004.02.036

Members of the family exhibit marked developmental stage- and tissue-specific patterns of expression, which differ among species [3,5]. Differences in the patterns of expression of FMOs have implications for the ability of an organism to respond to substrates of these enzymes that are present in its environment and diet, or that are used as therapeutic drugs in clinical or veterinary medicine.

Hormonal regulation appears to play a key role in some of the species- and gender-specific differences observed in FMO expression. For example, in mice *Fmo3* is down-regulated in adult male liver by testosterone [25], whereas male rats have a higher hepatic FMO-related enzyme activity than do females [26]. In vitro studies, using co-cultures of male rat hepatocytes, showed that 17 beta-estradiol [27] and thyroid hormones [28] down-regulate the expression of FMO. In humans the symptoms of a fish-like body odour, associated with the inherited disorder trimethylaminuria (fish-odour syndrome), are exacerbated in some females during menstruation [29], indicating a hormonally mediated decrease in the abundance of FMO3, whose gene, when mutated, gives rise to the disorder [30].

The mouse is increasingly being used as a model organism as researchers seek to understand the function of mammalian genes and the role of specific proteins in health and disease. We have investigated the expression profiles, in the mouse, of five members of the mammalian FMO family, FMO1, 2, 3, 4 and 5. To define these profiles precisely, we have used both in situ hybridization analysis and quantitative RNase protection assays with mouse FMO isoform-specific antisense RNA probes. This allows a comparison of the abundance and cellular location of each isoform within a single tissue, among tissues, and at different stages of development. The quantitative nature of RNase protection assays means that direct comparisons can be made between data derived from the mouse and published data obtained from human tissues.

2. Materials and methods

2.1. Animals

Animals were purchased from Harlan and maintained on the Harlan Teklad TRM Rat/Mouse diet. They had free access to food and water and were sacrificed by cervical dislocation.

2.2. Sexing of newborn

The sex of newborn 129/SV mice was determined by PCR-amplification of a Y chromosome-specific sequence, from tail DNA, using the primer pair: forward, YMTFP1, 5′-ctggagctctacagtgatga-3′; reverse, YMTRP1, 5′-cagttaccaatcaacacatcac-3′ [31]. Tail DNA was isolated as described [32]. As a positive control, a sequence of the myogenin gene was amplified using the primer pair: forward, Omla, 5′-ttacgtccatcgtggacagcat-3′, reverse, Omlb, 5′-tgggctgggtgttagtcttat-3′ [33]. Primers were synthesized by Eurogentech.

2.3. RNA isolation

Total RNA was isolated from liver, lung, kidney and brain of 129/SV female and male mice of various ages (newborn, 3, 5 and 8 weeks (adult)), using the ULTRASPEC RNA isolation system (Biotecx). For newborns, samples were pooled and RNA isolated from a total of eight males or four females.

2.4. cDNA cloning and synthesis of antisense RNA probes

cDNA fragments encoding FMO1, 2, 3, 4 and 5 were generated by reverse transcription-PCR (RT-PCR). Total RNA (5 μg), isolated from adult female liver (FMO1, 3, 4 and 5) or lung (FMO2), was reverse transcribed using the First Strand cDNA Synthesis kit for RT-PCR (AMV) (Roche Molecular Biochemicals), according to the manufacturer's recommendations. Double-stranded DNA molecules were generated by PCR using *Taq* polymerase (Qiagen Ltd.). Appropriate primer pairs for the amplification of each FMO sequence were designed by reference to DNA sequences stored under the accession numbers MMU87456 (FMO1), AF184981 (FMO2), U87147 (FMO3), AI390626 (I.M.A.G.E. 692387) (FMO4) and U90535 (FMO5). Oligonucleotides used were: FMO1, forward 5′-tgtctctggacagtgggaagt-3′, reverse 5′-cattccaactacaaggactcg-3′ (amplifies nucleotides 661–877: MMU87456); FMO2, forward 5′-cgaagacgctattgattgcag-3′, reverse 5′-ggcactatcagaggactgga-3′ (amplifies nucleotides 1314–1642: AF184981); FMO3, forward 5′-caccactgaaaagcacggta-3′, reverse 5′-gtttaaaggcacccaaccatag-3′ (amplifies nucleotides 418–862: U87147); FMO4, forward 5′-cgtaagtggcctgtcctctat-3′; reverse 5′-ccactggccagtttcagagaa-3′ (amplifies nucleotides 62–404: AI390626); FMO5, forward 5′-atcacacggatgctcacctg-3′, reverse 5′-gcttgcctacacggttcaag-3′ (amplifies nucleotides 1261–1494: U90535).

Amplification reactions were carried out using a Techne PCR machine (SLS). DNA was denatured at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing for 30 s at 58 °C (FMO1 and 2), 57 °C (FMO3) or 62 °C (FMO4 and 5), and extension at 72 °C for 30 s, followed by a final extension step of 5 min at 72 °C.

PCR products were cloned into the TOPO TA vector (Invitrogen). The inserts were then excised using *Eco*RI and cloned into the *Eco*RI site of pBluescript (Stratagene Europe) to produce the plasmids pBSmFMO1(217), pBSmFMO2(328), pBSmFMO3(445), pBSmFMO4(343) and pBSmFMO5(235). To generate antisense probes plasmids were first linearized with *Bam*HI (pBSmFMO1(217)), *Xba*I (pBSmFMO2(328)), *Hin*dIII (pBSmFMO3(445) and pBSmFMO4(343)) or *Xho*I (pBSmFMO5(235)), then

transcribed using the In Vitro Transcription kit (Stratagene Europe), [α-^{32}P]CTP (800 Ci/mmol; Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) and T3 (FMO1 and 2) or T7 RNA polymerase (FMO3, 4 and 5). Sense probes were generated in the same way except that plasmids were linearized with *Hin*dIII (pBSmFMO1(217) and pBSmFMO2(328)), or *Bam*HI (pBSmFMO3(445), pBSmFMO4(343) and pBSmFMO5(235)) and transcribed using T7 (FMO1 and 2) or T3 (FMO3, 4 and 5) RNA polymerase.

2.5. RNase protection assays

Synthesis in vitro of radiolabeled antisense and sense RNAs and RNase protection assays were performed as described previously [23,34,35]. Gels were exposed to a phosphorimaging plate (BAS-MP2040, Fuji) and signals quantified using Image Guage version 3.01 software (Fuji Photo Film Co. Ltd.). RNA amounts, in terms of molecules/μg of total RNA, were determined by reference to a standard curve of undigested probe. Values were converted to molecules/cell by using the average RNA content of a mammalian cell (5 pg) [36]. Results from RNA samples for different developmental stages are represented as spot images, which indicate the level of expression of a particular FMO RNA. Spot intensities were set relative to molecules of RNA/cell and are indicated in Fig. 5.

2.6. Tissue sections

Adult male and female mice were perfused with freshly prepared 10% (v/v) formalin (Sigma-Aldrich) in diethylpyrocarbonate-treated phosphate-buffered saline (DEPC-PBS) (Sigma-Aldrich). Tissues to be sectioned (liver, lung, kidney, brain) were excised and fixed overnight at 4 °C in 10% formalin. After two 20-min washes in DEPC-PBS, tissues were dehydrated in graded ethanol, cleared in Histoclear (National Diagnostics/Fisher Scientific), infiltrated with wax and embedded. Sections, 10–15 μm thick, were cut, using a Leica RM2155 microtome, fixed onto Superfrost slides (BDH) and dried overnight at 37 °C.

2.7. In situ hybridization probes

Sense and antisense probes for in situ hybridization were produced from the plasmids pBSmFMO2(328), pBSmFMO3(445), pBSmFMO4(343) and pBSmFMO5-(235). Probes derived from pBSmFMO1(217) proved unsuitable for in situ hybridization. Probes for the analysis of FMO1 mRNA were instead produced from pBSmFMO1(350). The insert of this plasmid was generated using the primers forward, 5′-agctgagagagcctgtgctaa-3′, and reverse, 5′-gggttgttcaggtcctaaaag-3′, which prime the amplification of nucleotides 1135–1484 of mouse FMO1 mRNA. PCR conditions were as above, with an annealing temperature of 58 °C.

As a positive control for hepatic lobe regional expression, we constructed the plasmid pBSGS(313), which encodes nucleotides 911–1222 of the mouse glutamine synthetase mRNA [37]. Female liver RNA was reverse transcribed, as above, and amplified using the primer pair forward, 5′-gcgggaggagaatggtctg-3′, and reverse, 5′-cctgtttcgttgaggagacacg-3′, with an annealing temperature of 60 °C. The PCR product was cloned into TOPO TA, released by *Eco*RI digestion and sub-cloned into the *Eco*RI site of pBluescript.

To produce antisense probes from pBSmFMO1(350) and pBSGS(313), plasmids were linearized with *Bam*HI and transcribed from the T3 promoter. Sense probes were transcribed from the T7 promoter, following linearization with *Hin*dIII. Enzymes for the linearization and transcription of pBSmFMO2(328), pBSmFMO3(445), pBSmFMO4(343) and pBSmFMO5(235) were as described above for the production of RNase protection probes. Digoxigenin-labeled antisense and sense probes were generated using the DIG RNA Labelling Kit (SP6/T7) (Roche Molecular Biochemicals), according to the manufacturer's recommendations. Probes were diluted 1:5 in DEPC-treated water and stored at −70 °C.

2.8. In situ hybridization

Tissue sections were de-waxed in xylene (2×, 10 min) and rehydrated in the following ethanol series (made up in DEPC-PBS): 100% (2×), 75, 50, 25% (3 min each), followed by two 5-min washes in DEPC-PBS. They were then fixed in paraformaldehyde (Sigma-Aldrich), 4% (w/v) in DEPC-PBS, for 20 min, and washed 3 × 5 min in PBS. Sections were incubated at 37 °C for 30 min in Proteinase K (Roche Molecular Biochemicals) (20 μg/ml in DEPC-PBS), except for brain sections, which were incubated in Proteinase K (10 μg/ml) for 15 min. Sections were placed immediately in paraformaldehyde, 4% (w/v) in DEPC-PBS, for 20 min, then washed in PBS for 3 × 5 min. Dehydration was for 1 min in each of a series of ethanols in PBS: 25, 50, 75 and 100% (2×). They were then air dried, and hybridized at 55–60 °C overnight. Probes were used at dilutions of 1:500 or 1:1000 in hybridization buffer (0.2 M NaCl, 5 mM EDTA, 10 mM Tris–HCl, pH 7.5, 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 50% (v/v) deionized formamide (Sigma-Aldrich), baker's yeast tRNA (0.1 mg/ml) (Roche Molecular Biochemicals), 10% (w/v) dextran sulfate (Sigma-Aldrich), 1× Denhardt's solution (50× stock, Sigma-Aldrich)).

After hybridization sections were washed at 65 °C, once for 15 min and twice for 30 min each, in 1× SSC (0.15 M NaCl, 0.01 M NaH_2PO_4, 1 mM EDTA), 50% (v/v) formamide, 0.1% (v/v) Tween-20, then at room temperature, twice for 30 min each in 100 mM maleic acid (Sigma-Aldrich), pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween-20 (MABT) and once for 1 h in blocking solution (MABT containing 2% (w/v) blocking reagent (Roche Molecular

Biochemicals) and 20% (v/v) heat-inactivated sheep serum (Chemicon International)). Sections were then incubated overnight at 4 °C with digoxigenin alkaline phosphatase-conjugated antibody (Fab fragments, Roche Molecular Biochemicals), diluted 1:1500 in blocking solution, washed at room temperature, five times for 10 min each, in MABT, and twice for 10 min each in prestaining buffer (100 mM Tris–HCl, pH 9, 100 mM NaCl, 5 mM $MgCl_2$). For color development, sections were incubated at room temperature for up to 24 h in 100 mM Tris–HCl, pH 9, 100 mM NaCl, 5 mM $MgCl_2$, 0.2 mM 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; Roche Molecular Biochemicals), 0.2 mM nitroblue tetrazolium salt (NBT; Roche Molecular Biochemicals) and 5% (w/v) polyvinyl alcohol (average MW 70–100 kDa; Sigma-Aldrich). Color development was stopped by rinsing the slides several times in distilled water. Sections were then dehydrated in a graded series of ethanols, as described above, and mounted in XAM medium (BDH).

3. Results and discussion

We have used in situ hybridization to determine, and compare, the cell type-specific expression of *Fmo1*, *2*, *3*, *4* and *5* in liver, lung, kidney and brain of the adult 129/SV mouse. These experiments show that the regional localization in the liver lobule of FMO1, 2, 3, 4 and 5 mRNAs is not the same. FMO1 and 5 mRNAs are detected across the acinus, with a concentration gradient decreasing from the perivenous to periportal region (Fig. 1a and h). In contrast, expression of FMO2 and 4 is restricted to the periportal region (Fig. 1b and e). In female adult mice the mRNA encoding FMO3 also localizes to a region of cells surrounding the portal triad (Fig. 1c). No hybridization signal was detected with the antisense probe for FMO3 mRNA in sections of adult male liver (Fig. 1d). The periportal localization of mRNAs encoding FMO2, 3 and 4 was confirmed using, as a control, an antisense probe for glutamine synthetase mRNA, whose expression, in mouse, is confined to a 2- to 3-cell layer surrounding the central vein [37]. Antisense probes for glutamine synthetase and FMO3, used separately (Fig. 1f and c) or together (Fig. 1g), show no overlap in their site of expression of the corresponding mRNAs. No hybridization signal was observed in sections hybridized with any of the corresponding five FMO sense RNA probes (data not shown).

The hepatic distribution of mRNAs encoding FMO1 and 5 is similar to that of mRNAs for other phase I enzymes, such as the cytochromes P450 (CYPs), most of which are more highly expressed in hepatocytes of the perivenous region [38]. The localization of expression of FMO3 to the periportal region is unusual for a protein whose role is considered to be predominantly one of xenobiotic metabolism. The location of FMO3 may serve to protect the liver acinus via a first-pass effect, but may also contribute to increased toxicity and, possibly, carcinogenesis, if a potentially harmful chemical is activated by FMO3. For example, thiourea, phenylthiourea and α-naphthylthiourea are toxic to mouse C3H/10T1/2 cells expressing human FMO3, but not to those expressing human FMO1 [39]. Therefore, the differential pattern of FMO expression within the liver lobule should be considered when carrying out both in vivo and in vitro hepatic toxicological studies on substances that are substrates for these enzymes.

In the kidney, FMO1, 2, 3, 4 and 5 mRNAs are primarily localized to the distal tubules of the cortex (Fig. 2, data not shown). The five mRNAs were also detected in the proximal tubules and the collecting ducts of the medulla (Fig. 2c and d, data not shown). Xenobiotic-metabolizing enzymes, such as CYPs and glutathione *S*-transferases, have been localized to the cells of the proximal tubules, distal tubules and the thick ascending limb, and in the medulla [40], suggesting these as sites of renal detoxification. FMO1 mRNA was also detected in the glomerulus (Fig. 2a and b), the site of initial filtration of blood arriving at the kidney from the afferent arterioles.

FMO1 mRNA is localized to the endothelial lining of the alveoli, which includes types I and II endothelial cells (Fig. 2e), and the cells lining the terminal bronchiole, which include the Clara cells. However, the particular cell type in which FMO1 mRNA is present could not be identified because of the thickness of the sections analyzed (10–15 μm). The intensity of the hybridization signal in the terminal bronchiole lining was greater than that in the lining of the alveoli. FMO2 mRNA is localized to the lining of the terminal bronchiole and the endothelial cells lining the alveoli; however, the hybridization signals were weak (data not shown). In contrast, in rabbits FMO2 has been identified as the major lung isoform [15]. FMO3 mRNA is specifically localized to the lining of the terminal bronchiole (Fig. 2f) and unlike the situation in liver, is present in both male and female adult mice. No signal was observed in the endothelial cells of the alveoli. No discernible signal was observed by in situ hybridization for FMO4 mRNA (data not shown). FMO5 mRNA was localized to the lining of the alveoli and terminal bronchiole (data not shown).

The localization of FMO1 and FMO5 mRNAs in the mouse lung corresponds to the regions in which FMO2 protein is found in rabbit lung [41]. The terminal bronchiole is the first part of the lung to be exposed to exogenous substances, and the presence, in this region, of FMOs provides a protective mechanism to the organism. However, the localization of expression of *Fmo1* and *3* genes to the terminal bronchiole may also predispose the lung to the toxic effects of chemicals that are activated by FMOs [42].

In situ hybridization of mouse brain sections localized FMO1 mRNA to the neurons of the cerebrum (Fig. 3a and b) and to the choroid plexus (Fig. 3c), a region rich in blood vessels. FMO5 mRNA also was detected in the neurons of the cerebrum (data not shown). No expression of FMO mRNAs was detected in astrocytes, in contrast to

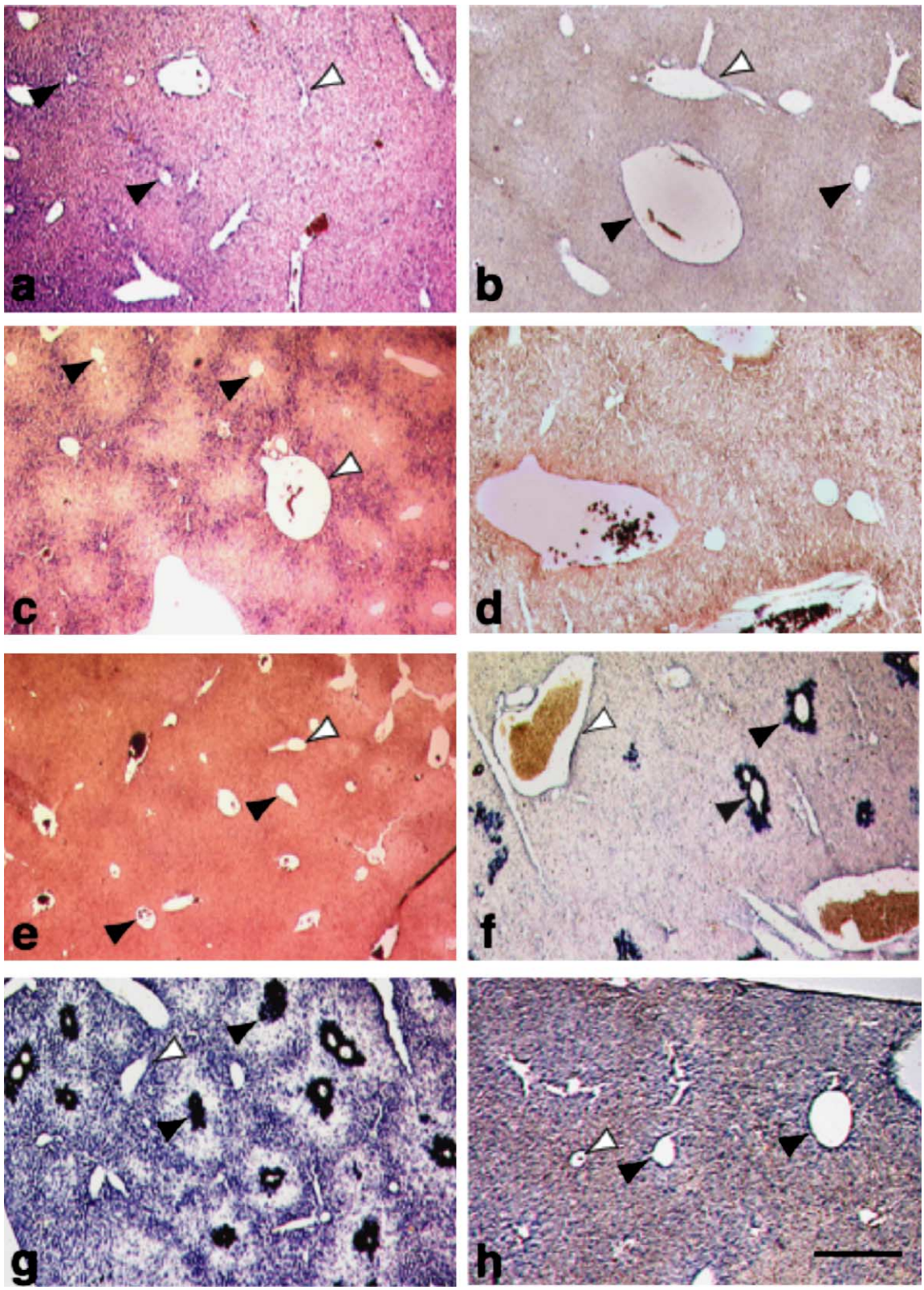

Fig. 1. Localization of FMO mRNAs in mouse liver. Antisense probes for mRNAs encoding FMO1 (a), FMO2 (b), FMO3 (c, d), FMO4 (e) and FMO5 (h) were hybridized to sections from female (a–c, e, h) and male (d) liver. Sections shown in male (f) and female (g) were hybridized with antisense probes for glutamine synthetase and FMO3. Female sections were from the same animal. Black arrowheads indicate the central veins and white arrowheads the portal vein. Scale bar represents 200 μm.

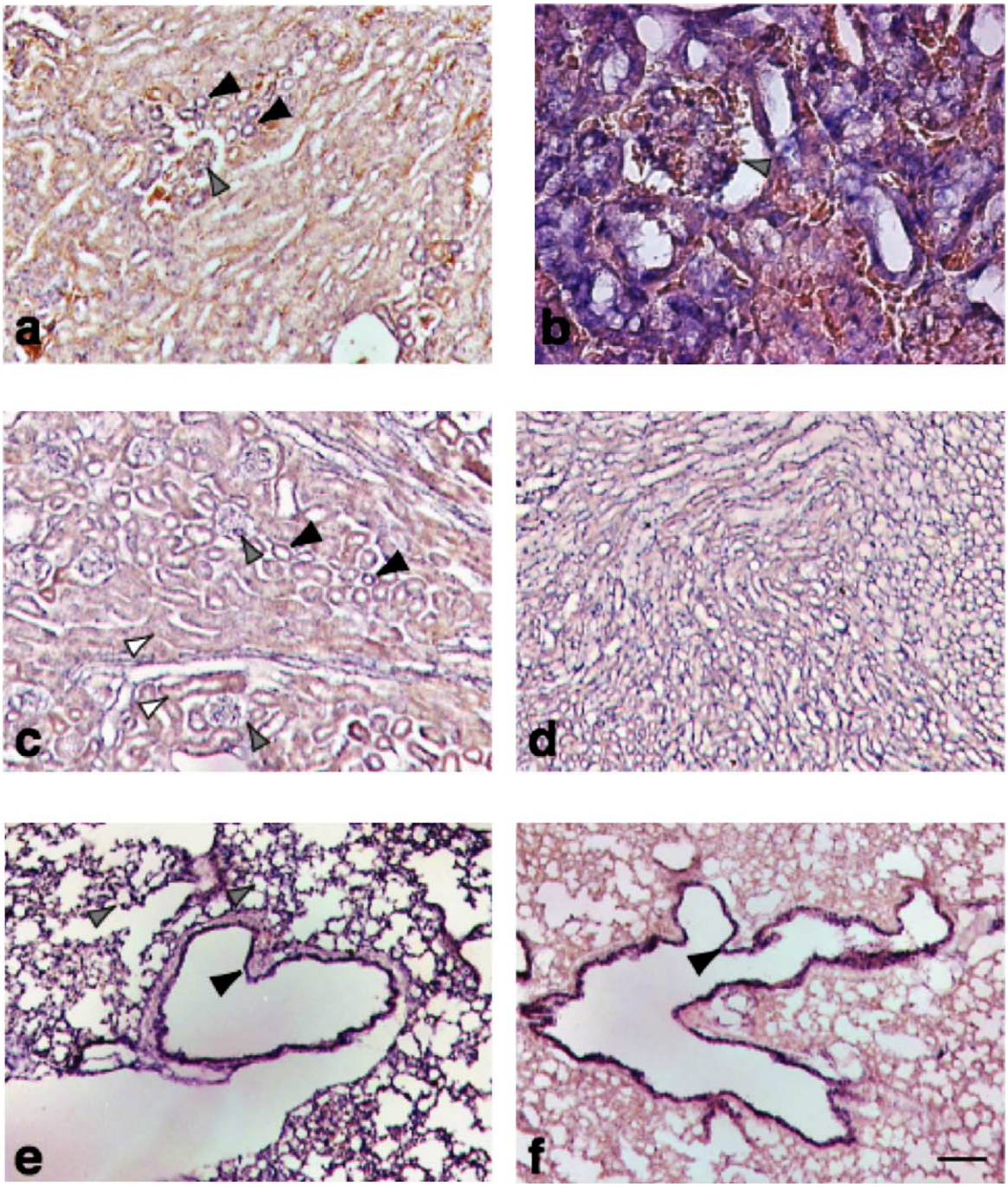

Fig. 2. Localization of FMO mRNAs in mouse kidney and lung . In situ hybridization of male kidney using antisense probes for mRNAs encoding FMO1 (a, b) and FMO5 (c, d). Sections from a single animal are shown. Arrowheads indicate distal convoluted tubules (black), proximal convoluted tubules (white) and glomerulus (grey). Sections from the same female lung were hybridized with antisense probes for mRNAs encoding FMO1 (e) or FMO3 (f). Arrowheads indicate terminal bronchiole (black) and epithelial cells lining alveoli (grey). Scale bar represents 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the study of Di Monte [43], who detected FMO activity in primary cultures of rat astrocytes.

3.1. Global profiling of Fmo1, 2, 3, 4 and 5 gene expression

Using quantitative RNase protection assays and probes specific for each of the FMO isoforms, we have directly compared the abundance of mRNAs for the five FMO family members in a single tissue. Such analysis also makes it possible to compare the amounts of a single FMO mRNA in different tissues. We analyzed the expression of *Fmo1*, *2*, *3*, *4* and *5* in male and female 129/SV and C57BL/6J mice. As the results obtained in both strains were the same we show only the results obtained with 129/SV mice (Fig. 4).

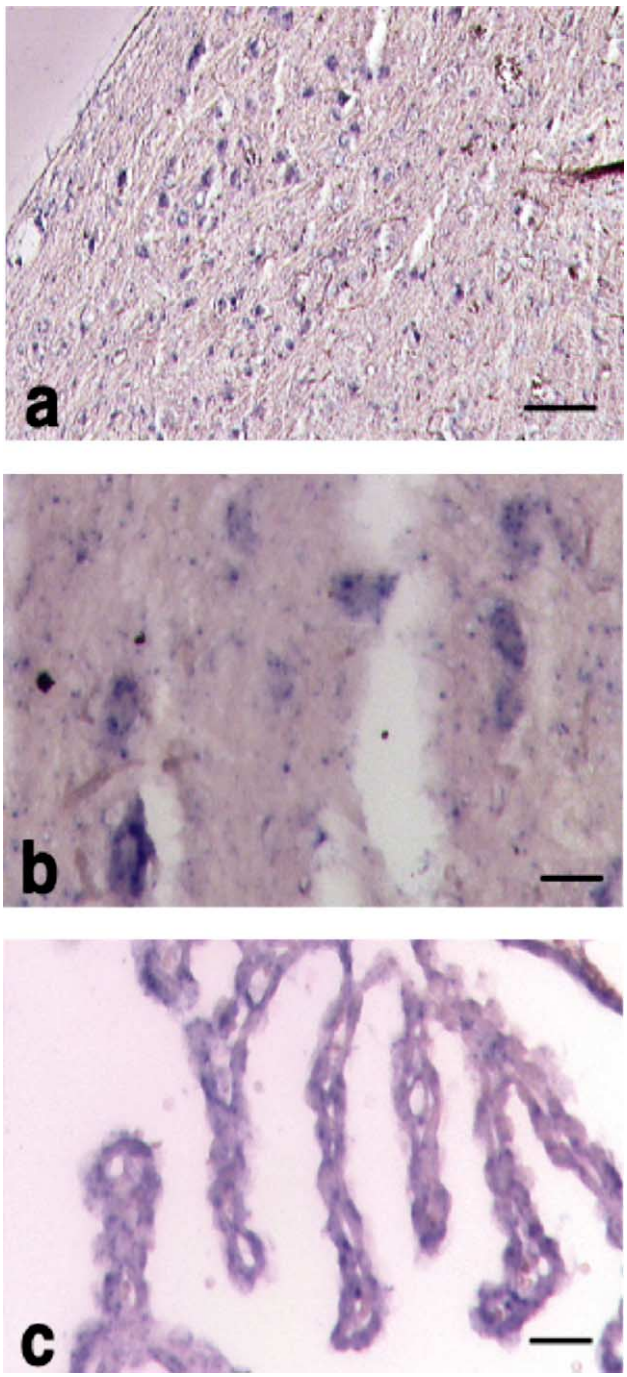

Fig. 3. Localization of FMO1 mRNA in brain. In situ hybridization of an antisense probe for FMO1 RNA to sections of the cerebrum (a, b) and choroid plexus (c) of female mouse brain. Scale bars represent 100 μm (a) and 200 μm (b, c).

The most abundant FMO mRNA isoform in the liver of both sexes is FMO5 (Fig. 4). This mRNA is present in amounts 5-fold greater than FMO1 mRNA and, in female mice, 2.5-fold greater than FMO3 mRNA. FMO2 and 4 mRNAs are expressed only at a low level. No significant sex differences were observed for FMO2, 4 and 5. In females FMO1 mRNA was 1.4-fold more abundant. A similar sex difference was observed in FMO1 protein amounts between female and male CD-1 mice [44]. As noted previously [9], *Fmo3* is down-regulated in the liver of adult male mice. Female liver contained about 80 times as much FMO3 mRNA than did male liver samples. Interestingly, despite the essential lack of hepatic *Fmo3* expression in the adult male, the total FMO mRNA content in the liver of male and female 129/SV mice is similar (87 and 95 molecules/cell, respectively), indicating a possible compensatory effect. The abundance of FMO mRNAs in the female adult liver is FMO5 > FMO3 > FMO1 > FMO2 ≈ FMO4, but in males is FMO5 > FMO1 > FMO2 ≈ FMO4 > FMO3.

In agreement with the study of Cherrington et al. [9], we find the expression of FMO3 in adult mouse liver to be gender-specific to females. However, in contrast to these authors, we found that FMO5, not FMO1, is the most highly expressed FMO isoform in the liver of adult mice. The difference between published data [9] and the results of our present study may be due mouse strain differences or to the nature of the experimental analyses used. For instance, the RNase protection probes we used are specific for individual mouse FMO isoforms, whereas previous studies used rabbit FMO cDNAs and antibodies raised against rabbit FMOs and are therefore not as quantitative or stringent. It is not known whether the high amounts of FMO5 mRNA detected in mouse liver are due to storage of the RNA in the cytosol prior to translation or if these amounts reflect the abundance of FMO5 protein. The lack of purified mouse FMO5 and an isoform-specific antibody prevents the quantification of the protein.

FMO1 mRNA is the most abundant isoform in the mouse lung, with amounts in females being 1.4-fold higher than males (14.37 ± 0.62 molecules/cell and 10.58 ± 0.39 molecules/cell in female and male, respectively). Amounts of FMO1 mRNA are about 3-fold higher than that observed for FMO5 mRNA and about 15-fold higher than for FMO2, 3 and 4 mRNAs. In contrast to rabbits in which FMO2 has been identified as the major lung isoform [15], in the mouse this isoform is expressed in very low amounts (<1 molecule/cell). Thus, the abundance of FMO mRNAs in the female and male adult lung is FMO1 > FMO5 > FMO3 ≈ FMO2 ≈ FMO4.

FMO1 and 2 [15,45], FMO4 [23] and FMO5 [46] have been shown to be present in the kidney of various species. Here, we show that, in the mouse kidney, *Fmo1* and *5* genes are subject to gender-specific regulation, with both mRNAs being more highly expressed in the male (23.80 ± 1.97 molecules/cell and 23.5 ± 5.00 molecules/cell, respectively) than in the female (9.40 ± 5.60 molecules/cell and 3.21 ± 0.10 molecules/cell, respectively) (Fig. 4c). The most marked sex difference being exhibited by FMO5 mRNA, which is about sixfold more abundant in male then female kidney. FMO2 and 3 mRNAs are present in low abundance (<1 molecule/cell). The kidney is the tissue in which FMO4 mRNA is expressed in greatest abundance (1.8 molecules/cell). The abundance of FMO mRNAs in the kidney, in female, is FMO1 > FMO5 ≥

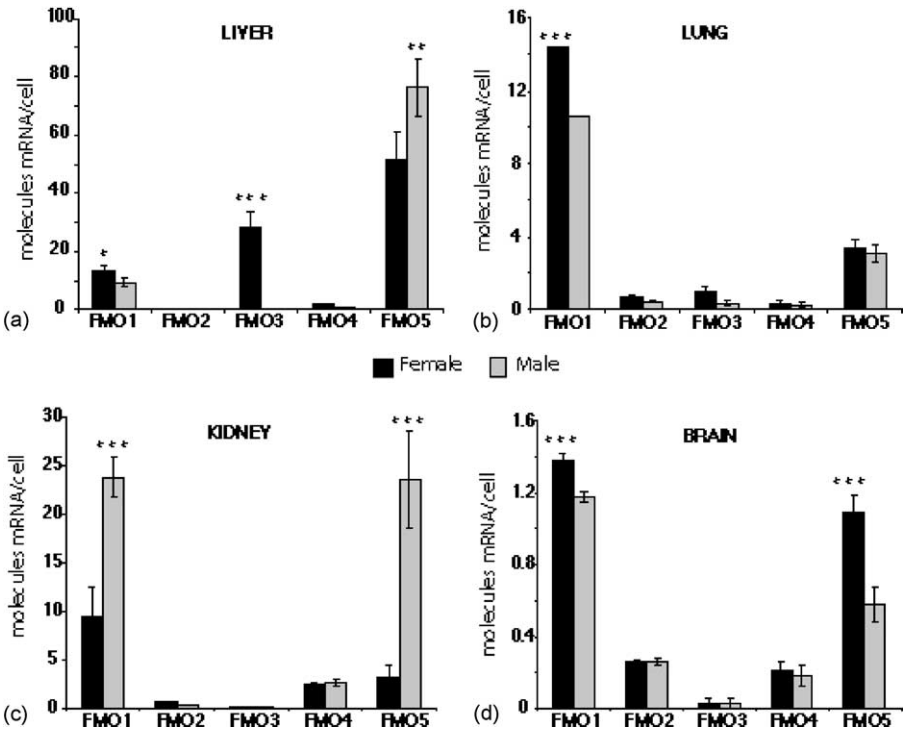

Fig. 4. Abundance of FMO mRNAs. Antisense probes for FMO1, 2, 3, 4 and 5 mRNAs were hybridized to total RNA isolated from the liver ($n = 6$) (a), lung ($n = 7$) (b), kidney ($n = 6$) (c) and brain ($n = 6$) (d) of female and male 129/SV mice. Statistical differences were determined by ANOVA and Student's two-tailed t-test: $^{*}P < 0.005$; $^{**}P < 0.001$, $^{***}P < 0.0001$. n number of animals.

FMO4 > FMO3 > FMO2 and in male FMO1 ≈ FMO5 > FMO4 > FMO2 > FMO3.

FMO activity has been detected in human [47,48] and rat [49,50] brain and FMO4 mRNA in rabbit brain [51]. It is also known that, in mouse and rat, the neurotoxin *N*-methyl-1,2,3,6-tetrahydropyridine (MPTP) undergoes *N*-oxidation by FMOs [52,53]. Our analysis of the expression within the brain of mRNAs encoding FMO1, 2, 3, 4 and 5 shows that FMO1 and 5 mRNAs are the most abundant. FMO5 mRNA is twofold higher in female than in male brain. In the brain, however, the amounts of FMO1 and 5 mRNAs is only 1–10% of those found in the liver (Fig. 4d). FMO2, 3 and 4 mRNAs are present in amounts <1 molecule/cell.

3.2. Developmental stage-specific expression of FMOs

We next investigated how *Fmo* gene expression changes during development and at what age sex differences in the expression of these genes become established. RNA isolated from the liver, lung, kidney and brain of newborn, 3 and 5 weeks post-partum, and adult 129/SV mice was analyzed by RNase protection. Fig. 5 shows a spot image analysis of these data and how the expression patterns of the FMO mRNAs, in each tissue, change with age. In the liver, mRNAs encoding FMO1, 2, 4 and 5 are present in the newborn, but that encoding FMO3 is not. In both sexes, the expression of *Fmo1* and *5* increases in the liver after birth, to reach a peak at 5 weeks post-partum. Subsequently, the abundance of FMO1 mRNA declines in adult females and males, respectively, to amounts similar to those seen in the newborn. *Fmo5* also exhibits an age-related decline in expression. The expression of *Fmo3* peaks in the female at 5 weeks post-partum, but the decline in the abundance of

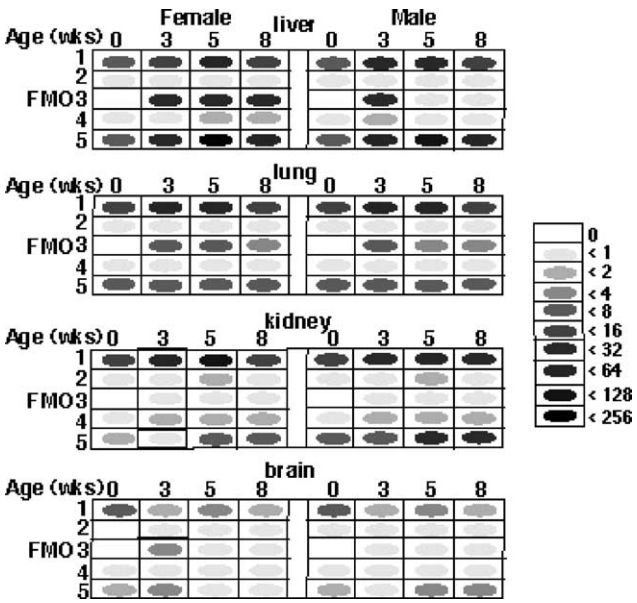

Fig. 5. Expression of FMO mRNAs as a function of tissue, sex and age. Abundance of mRNAs encoding FMO1 (1), FMO2 (2), FMO3 (3), FMO4 (4) and FMO5 (5) in the liver, lung, kidney and brain are shown as a spot image analysis set. The right hand panel shows the spot intensities relative to the number of mRNA molecules/cell.

FMO3 mRNA in the adult is only about 20%. In males, however, the mRNA peaks earlier, at 3 weeks post-partum, and is barely detectable by 5 weeks. FMO2 and 4 mRNAs remain relatively constant at <1–2 molecules/cell and exhibit no significant age-related changes in their expression.

In humans, FMO1 is the predominant fetal hepatic isoform [5] and its expression is suppressed after birth [5,10]. Mice [9], like other mammals [15], maintain hepatic FMO1 expression in adulthood. However, in the 129/SV and C57BL/6J mouse strains, as in man, *Fmo5* is the most highly expressed member of the family in liver [24].

The regulation of *Fmo3* expression by sex steroids in male mouse liver is documented [25]. Our studies show that, in mouse liver, *Fmo1*, *3* and *5* are all up-regulated during puberty, presumably due to hormonal influences, and all are down-regulated in adulthood.

In the kidney, both FMO1 and 5 mRNAs are detectable in the newborn and, as is the case in the liver, the expression of each of the corresponding genes peaks at 5 weeks of age. Although the developmental pattern of expression of *Fmo5* is the same in male and female mice, there is a striking gender difference in the abundance of the mRNA. This is greater, at all stages of development, in the male and the fold-difference between the two genders remains constant throughout development suggesting that the gender difference in *Fmo5* expression is unlikely to be due to hormonal factors.

FMO3 mRNA is undetectable in the kidney of newborns, but its expression increases by 3 weeks. *Fmo4* expression in kidney, although low, was found to be greater at all ages, than in other tissues investigated and the mRNA increased from birth and then remained constant from 3 weeks of age through to adulthood.

In the lung, as is the case for liver and kidney, *Fmo1*, *2*, *4* and *5* are expressed in the newborn and *Fmo3* is not. The abundance of FMO1 mRNA increases about fourfold from birth to 3 and 5 weeks of age, but then declines in the adult to a level that is similar to that in the newborn. In contrast, the abundance of FMO5 mRNA remains relatively constant in both sexes from birth to adulthood. FMO2 and 4 mRNAs are present in low amounts in lung at all developmental stages. From 3 weeks of age to adulthood the amount of FMO3 mRNA remains constant.

In the brain FMO1 mRNA is most abundant in the newborn and drops about 80% by 8 weeks of age. FMO5 mRNA, is only 70% of that of FMO1 mRNA in the newborns. However, FMO1 and 5 mRNAs are present in similar amounts in the brains of 3-, 5- and 8-week-old animals. As in all other tissues examined, FMO3 mRNA was not detected in the brains of newborn mice. But the mRNA was present, in low amounts (<1 molecule/cell), at 3, 5 and 8 weeks of age.

Our results show that FMO1 mRNA is the most abundant FMO mRNA in the liver, lung, kidney and brain of newborn mice. The reasons for the higher expression of *Fmo1* in early development are not yet understood. The predominance of this mRNA does not change with age in the lung and kidney. In the liver, however, FMO5 mRNA is the most highly expressed FMO mRNA from age 3 weeks into adulthood. These two mRNAs were found in all tissues analyzed and showed a number of similarities in their tissue- and cell type-specific expression profiles, suggesting that their genes share common regulatory factors. *Fmo3* is switched on only after birth in liver, lung, kidney and brain. The decline in *Fmo3* expression in the liver of adult male mouse is not observed in the kidney and lung. *Fmo3* is therefore subject to differential, tissue-specific hormonal control. In both sexes mRNAs encoding FMO1, 3 and 5 increase during puberty and decline with age, suggesting a physiological role, during development, for the proteins they encode. *Fmo1*, *3* and *5* are therefore all subject to age-related down-regulation.

A detailed functional analysis of the promoter elements is necessary to define the DNA regulatory elements and transcription factors that control the developmental-, tissue-, cell type- and gender-specific expression of each of the *Fmo* genes. The production of mice, deficient in various FMO activities, will help to define the precise biological roles of the *Fmo* gene family in the metabolism of endogenous and exogenous chemicals in specific cell types and at various developmental stages.

The data presented in this paper and the quantitative nature of the RNase protection assay make it possible to compare directly the amounts of FMO mRNAs produced in mouse and human. For example, in both human and mouse FMO1 mRNA is the most abundant FMO mRNA in the kidney [23]. In both species, the *Fmo3* gene is switched on only after birth, although the mouse, unlike human [5], continues to express *Fmo1* in adult liver. FMO5 mRNA is the most highly expressed FMO mRNA in the liver of both human (about 30 molecules/cell) [24], and in 129/SV mice (51 ± 10 molecules/cell and 76 ± 10 molecules/cell in females and males, respectively). FMO4 mRNA is expressed in low amounts (1–2 molecules/cell) in liver, lung and kidney of both human [23] and mouse.

Determination of the expression profiles of a particular gene or family of genes is a logical next step as we enter the post-genomic phase and decipher the patterns of expression of orthologous genes in different species.

Acknowledgments

We thank Dr. Anoop Kumar, Department of Biochemistry and Molecular Biology, University College London, for help with tissue preparation and photography. The work was supported by a grant from the Wellcome Trust (No. 053590).

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