Dichotomous development of the organic anion transport protein in liver and choroid plexus

RUTH HOUGE ANGELETTI,1 ARI J. BERGWERK,2,3 PHYLLIS M. NOVIKOFF,4 AND ALLAN W. WOLKOFF2,3
1Departments of Developmental and Molecular Biology, 2Medicine, and 4Pathology, and the 3Marion Bessin Liver Research Center, Albert Einstein College of Medicine, Bronx, New York 10461

An important function of the liver is the removal of organic anions from the circulation (5, 34, 40). This is an efficient process in which >50% of bile acids and other organic anions are transported into the liver in a single pass (12, 13, 37). This uptake process is saturable and temperature dependent and requires cellular ATP and extracellular Cl− (25, 41). Similar to the liver, the choroid plexus also transports organic anions as a mechanism for clearance from, and possibly secretion into, the cerebrospinal fluid (25, 27). Previous physiological studies suggest that this organic anion transport process in the choroid plexus is similar to that in the liver (1, 2, 7).

A specific organic anion transport protein (OATP1) that mediates Na+/independent transport of organic anions in the rat has been expressed cloned in a Xenopus laevis oocyte expression system utilizing rat liver mRNA (17, 18, 39). This transporter mediates bidirectional organic anion/HCO3− exchange (33) and is localized to the basolateral surface of the hepatocyte (4, 39). In recent studies, we found that oatp1 is also located in the choroid plexus, where it resides on the apical surface of the choroid plexus epithelial cell (1). Whether oatp1 mediates organic anion entry into or exit from the CSF is not as yet known. However, alteration of choroid plexus oatp1 function during development could play an important role in the pathobiology of maturation of the central nervous system. Immaturity of the transport mechanism for organic anions in the liver has been recognized for many years (3, 6, 22, 23). This may play a role in the predisposition of newborns to jaundice and cholestasis.

Recent studies by Dubuission et al. (10) indicate that expression of oatp1 mRNA in the developing rat liver does not reach adult levels until at least 28 days after birth. However, this study did not examine oatp1 protein expression or transport function. In the present study, we have examined expression, subcellular distribution, and transport function of oatp1 during development in the rat liver and choroid plexus.

METHODS

Animals. Adult male (200–225 g), neonatal, and timed pregnant female Sprague-Dawley rats were obtained from Taconic Farms (Germantown, NY) and were housed under standard conditions. All investigations followed the institutional criteria for the care and use of laboratory animals in research.

Isolation and short-term culture of rat hepatocytes. Rat hepatocyte isolation and culture were performed as previously described (25, 32, 41). In short, hepatocytes were isolated from neonatal rats, 12- to 23-day-old, 40- to 47-day-old, and adult male rats after perfusion of the liver with collagenase (Sigma, St. Louis, MO). Cells were suspended in Waymouth's 752/1 media (GIBCO, Grand Island, NY) containing 25 mM HEPES, pH 7.2, 5% heat-inactivated fetal bovine serum (Gemini Bio-Products, Calabasas, CA), an additional 1.7 mM CaCl2, 5 µg/ml insulin (Sigma), 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cell viability was assessed by trypan blue exclusion. Approximately 1.5 × 106 cells in 3 ml were plated on 60-mm Lux culture dishes (Nunc, Naperville, IL) and cultured for 2 h at 37°C in 5% CO2. Neonatal hepatocytes, isolated from rats as young as 12 days of age, had viability rates comparable with those seen in hepatocytes prepared from adult rats (85–95%). Light microscopic evaluation of hepatocytes that were prepared from neonatal rats and cultured for 2 h revealed that they were firmly attached to the dishes and had the appearance of hepatocytes rather than those of erythropoietic or nonparenchymal cells.

Uptake of [35S]bromosulfophthalein by cultured hepatocytes and choroid plexus tissue ([35S]brromosulphophthalein ([35S]BS)) at a specific activity of 3,000 mCi/mmol, was prepared as previously described (30) and was diluted with unlabeled BSP to a concentration of 0.8 µM containing 105 dpm/ml (25). Initial uptake of [35S]BS by hepatocytes was determined as previously described (25, 41). In brief, after 2 h of culture, cells were washed three times with 1.5 ml of...
serum-free medium (SFM) consisting of (in mM) 135 NaCl, 1.2 MgCl2, 0.81 MgSO4, 27.8 glucose, 2.5 CaCl2, and 25 HEPEs, pH 7.2. The washed cells were incubated for 15 min at 37°C in 1.5 ml of SFM. Cells were then incubated for 5 min at 37°C or 4°C with 1 ml of SFM containing [35S]BSP and 0.1% BSA. BSP transport is linear over this time. Cells were then washed with SFM three times at 4°C and incubated with 1.5 ml of 5% BSA in SFM at 4°C for 5 min to remove surface-bound radioactivity. The plates were washed three times more with SFM at 4°C. Cells were harvested with a rubber policeman and placed in 10 ml of Hydrofluor solution (National Diagnostics, Atlanta, GA), and radioactivity was measured in a Rackbeta model 1217 liquid scintillation counter (LKB Instruments, Gaithersburg, MD). Replica plates were washed with SFM and harvested in 0.1 N NaOH for determination of protein content by the method of Lowry et al. (24).

Uptake of [35S]BSP by choroid plexus was determined using freshly extirpated choroid plexus tissue washed with SFM and placed in 100 µl of SFM containing 0.8 µM [35S]BSP in individual wells of 96-well plates. For choroid plexus from younger animals, two pieces of tissue were used per well. After incubation at 37°C or 4°C, the tissue was washed three times with SFM at 4°C. The tissue was then solubilized in 0.1 N NaOH. From each sample, 75% was used for scintillation counting as described above and 25% was used for protein assay by the method of Lowry et al. (24).

Immunoblot analysis of oatp1. Livers and freshly extirpated choroid plexus were extracted with 0.1 M Na2CO3 to enrich integral membrane proteins (4). Tissue was homogenized in four volumes of ice-cold 1 mM NaHCO3 containing 50 µM phenylmethylsulfonyl fluoride (PMSF), with the use of 20 strokes of a loose Dounce homogenizer. NaHCO3 (1 mM) containing 50 µM PMSF was added to a final volume of 10.6 ml/g tissue. This homogenate was dialyzed 1:40 with 0.1 M Na2CO3, rotated for 15 min at 4°C, and centrifuged at 100,000 g for 60 min. The pellet was resuspended in water and was used for immunoblot analysis, utilizing a peptide antibody prepared to a 13-amino acid sequence near the carboxyl terminus of oatp1, as previously described (4).

Analysis of mRNA expression. RNA was extracted from adult, neonatal, and fetal rat liver by a modified guanidine isothiocyanate procedure (9). RNA was electrophoresed on a 1% agarose gel and was transferred to GeneScreen (DuPont-NEN, Boston, MA). After cross-linking occurred in a vacuum oven, the membrane was hybridized with 32P end-labeled linearized pGEX Kglid (14) containing a 2.2-kb oatp insert. After 17 h of hybridization, the membrane was washed at high stringency conditions. This included washing twice with 2× standard sodium citrate (SSC) for 5 min at room temperature, 2× SSC containing 0.5% SDS for 30 min at 65°C, 0.1× SSC for 30 min at room temperature, and 0.1× SSC containing 0.1% SDS and 0.1% sodium pyrophosphate for 15 min at room temperature. Oatp transcripts were identified by autoradiography (26). Densitometry of autoradiography bands was performed using an Ultrascan XL densitometer (Pharmacia, Uppsala, Sweden). RT-PCR was used to detect the presence of oatp mRNA as previously described (1), using primers specific for oatp1 based on the sequences of the known members of the oatp1 family (18, 20, 31). The 22-bp 5′-primer corresponded to nucleotides 1925–1946 of the cDNA sequence, TCTGCGTCCTCTTCCATTCTCG. The 24-bp 3′-primer corresponded to nucleotides 2349–2364 of the mRNA sequence, GTAGTGTTGGGTCACCTAAGA. The expected product of these primers is 425 bp. Enzymes and the thermal cycler were from Perkin-Elmer, Norwalk, CT.

Confocal microscopy. Immunolocalization of oatp1 and actin in choroid plexus and liver was performed as previously described (4). The specificity of reaction was determined using controls in which tissue was incubated with preimmune serum or with immune serum before and after absorption or purification on a peptide affinity column (4). In some studies in liver, the canalicular protein dipeptidyl peptidase IV (DPPIV) was immunolocalized using a specific antibody (Endogene, Woburn, MA). Briefly, tissue was immersion fixed in 4% paraformaldehyde for 3 h at 4°C. Whole choroid plexus fixed tissue or 30-µm liver sections cut on a vibratome (Lancer 1000, Warrington, PA) were first exposed to a sequence-specific anti-oatp1 rabbit serum dilute 1:100 (4) and were later detected with goat anti-rabbit IgG-Cy3 (Jackson Immunoresearch, West Grove, PA). Liver sections were incubated with anti-rat DPPIV monoclonal antibody (1:50) as well, followed by goat anti-mouse IgG-Cy5. Choroid plexus tissue was also incubated with the FITC derivative of phallolidin (Molecular Probes, Eugene, OR). Tissues were blocked with sodium borohydride and with a mixture of goat serum, BSA, and Tween detergent (4). The tissue was examined with an inverted Nikon fluorescence microscope attached to the Bio-Rad Mrc 600 confocal laser imaging system equipped with a krypton/argon laser. Single optical section and x and z axis analysis were performed through the entire depth of the tissue.

RESULTS

Immunoblot analysis. Immunoblot of liver extracts obtained from fetal (19-day gestation), 1-day-old, 8-day-old, and 15-day-old rats revealed no immunoreactive oatp1 (Fig. 1). However, by 30 days of age, adult levels were observed (Fig. 1). In contrast to results in neonatal rat liver, immunoblot analysis of choroid plexus showed that oatp1 protein is abundant even at 1 day (Fig. 1).

Analysis of mRNA expression. Under high stringency conditions, the major 4.3-kb oatp1 transcript was not detectable in total RNA from 1-day-old and 8-day-old rat liver. A weak hybridization signal was seen at 15 days of age, and this oatp1 transcript reached adult levels at 30 days of age. Densitometry of data obtained from three sets of animals was quantified and expressed as densitometry readings of the oatp1 band relative to the 28S rRNA determined by methylene blue staining (Fig. 2). Because of the small amount of tissue available, RT-PCR techniques were previously used to identify the mRNA for oatp1 in adult rat choroid plexus

Fig. 1. Immunoblot detection of organic anion transport protein (oatp1) in rat liver (A) and choroid plexus (B) at different stages of postnatal development. Liver and choroid plexus obtained at the indicated times of development were homogenized and extracted with 0.1 M Na2CO3 to enrich integral membrane proteins. After SDS-PAGE of 25 µg of protein per lane, immunoblot analysis was performed as described in METHODS. Only the oatp1-specific band at relative molecular weight 80,000 is shown.
(1). With the use of this approach, the mRNA for oatp1 was also detected in neonatal rat choroid plexus (data not shown).

$[^{35}S]$BSP transport. The initial rate of $[^{35}S]$BSP uptake in hepatocytes obtained from rats in the first 3 wk of age was markedly decreased compared with that in adult hepatocytes obtained at 100 days of age. At 6–7 wk postnatally, $[^{35}S]$BSP transport by hepatocytes was $67 \pm 8\%$ of adult values (Fig. 3). In contrast, accumulation of $[^{35}S]$BSP by choroid plexus obtained from neonatal rats (0–8 wk) was two- to threefold higher than that seen in the adult (Fig. 4). Adult levels were attained by 10 wk of age.

Confocal microscopy. In previous experiments with adult rat choroid plexus, oatp1 immunoreactivity was found exclusively on the apical surface. In contrast, fluorescence confocal microscopy of the neonatal choroid plexus epithelium showed that a preponderance of the oatp1 immunoreactivity was intracellular, localized to vesicular-appearing structures (Fig. 5a). Fluorescence confocal microscopy experiments were also performed on choroid plexus tissue from animals of 1, 2, 3, 4, 5, 6, 7, 8, and 12 wk of age. At 10 wk of age, a partial shift in the distribution of staining to the apical surface of the choroid plexus epithelium is observed (Fig. 5b). By 12 wk, essentially all of the oatp1 antigen is localized to the apical plasma membrane (Fig. 5c). A few positive vesicles are evident in the cytoplasm. The apparent thickening and intensification of the fluorescence observed is consistent with the morphometric studies of the developing choroid plexus, which show the development of the microvilli in the first 2 wk of life in the rat (21). This process appears to be complete by 10–12 wk of age. Distribution of oatp1 in adult rat liver is seen in Fig. 5d, in which localization of oatp and DPPIV, a bile canalicular apical marker, was determined. These studies reveal that oatp1 is exclusively localized to the basolateral surface of hepatocytes (Fig. 5d), with no canalicular distribution.

DISCUSSION

Transport of various organic anions is an important function of both liver and choroid plexus (1, 5, 27, 34, 40). In recent studies, an organic anion transport protein (oatp1) has been expression cloned utilizing rat liver mRNA (18). Computer analysis reveals that oatp1 is the first member of a unique family of transport proteins that have diverse substrate specificities and tissue distributions (18, 20, 31, 39). In the liver, oatp1 is limited to the basolateral surface of hepatocytes (4, 39). In the adult choroid plexus, it is expressed on the apical surface of the choroid plexus epithelial cell (1).

Computer modeling of the derived amino acid sequence of oatp1 suggests that it is a hydrophobic protein with 12 transmembrane domains (18, 39). The molecular determinants of organic anion transport by oatp1 have not as yet been elucidated. Functional characteristics of oatp1 transport have been examined in a HeLa cell line that was stably transfected with a plasmid encoding oatp under regulation of a metallothionein promoter (33, 35). These studies indicated that oatp1-mediated transport is bidirectional, does not require Na$^+$ for activity, and exchanges organic anions with HCO$_3^-$.

In the hepatocyte, oatp1 most likely mediates uptake of organic anions. In the choroid plexus epithelial cell, it is not as yet known whether oatp1 mediates organic anion entry into or exit from the CSF.

The development of liver function in the fetus and the neonate has been the subject of investigation for many years (3, 6, 22, 23). In particular, immaturity of hepato-
cyte transport of organic anions in the neonatal period has been well described (3, 15). Dubuisson and co-workers (10) have shown that hepatic expression of the mRNA encoding oatp1 undergoes upregulation with age, requiring >28 days to reach adult levels. In the present study, these observations have been extended to quantify hepatocyte expression of oatp1 protein as well as transport function in the neonate. These studies reveal little expression of oatp1 protein for the first month of life, with adult levels attained by ~40 days of age. Quantification of BSP transport by hepatocytes reveals a similar developmental pattern.

Development of oatp1 expression and organic anion transport function in the choroid plexus is in marked contrast to that of the liver. In the choroid plexus, oatp1 protein is expressed abundantly at birth. Scanning confocal microscopy reveals a large intracellular vesicular-appearing distribution of oatp1 in the choroid plexus epithelial cell during the initial 8–10 wk of development, far outshadows that on the apical plasma membrane. This intracellular localization is a minor portion of the oatp1 expression in adult choroid plexus. That this intracellular protein may be functional is suggested from our studies that indicate a two- to threefold increase per milligram of protein of [35S]BSP accumulation in choroid plexus tissue obtained as late as 8 wk of age compared with that in choroid plexus tissue obtained from animals 10 wk of age or older.

The choroid plexus serves as a major determinant of permeability of molecules into and out of the CSF (36). Studies of developmental regulation of this barrier have been limited, although it appears to represent a tight barrier even in the fetus. Many compounds have low permeability across neonatal choroid plexus. Transport activity of the choroid plexus epithelium for substances such as K⁺, Cl⁻, or glucose is low at birth and increases significantly with time thereafter (11, 28, 29). Protein content of the glucose transporter GLUT-1 (16), as well as Na⁺-K⁺-ATPase (28), is low at birth and increases markedly in the choroid plexus within 2–3 wk. Uptake of the organic cation tetraethylammonium is low at 1 day of life, with levels reaching those of the adult by 15 days of age (19). In contrast, several molecules have increased permeability across the neonatal choroid plexus. Tauc and co-workers (38) demonstrated that impermeability to peroxidase of the blood-CSF barrier in the choroid plexus reaches adult levels by the 14th day of fetal development in the rat. Studies by Jakobson (19) demonstrated that uptake of bilirubin by the choroid plexus obtained from rabbits as old as 21 days was elevated compared with results in adults. This transport was inhibited by BSP.

Similar to results previously reported for bilirubin, the present study shows that BSP transport by the choroid plexus of the newborn is high and is not reduced to adult levels until the animal is ~10 wk old. These results are in contrast to those obtained in hepatocytes in which transport of BSP is low at birth and does not attain adult levels until ~8 wk of age. Little is known regarding the molecular basis of regulation of oatp1 transcription, although the large differences seen in liver and choroid plexus are intriguing and suggest the presence of potent organ-specific transcription factors (42). The mechanism by which the large amount of intracellular oatp1 seen in the neonatal choroid plexus mediates increased accumulation of
BSP is unknown. As the uptake assay is performed over 1 h, what is measured is total tissue accumulation, not initial uptake, and could represent sequestration of the ligand in these putative intracellular vesicles.

The physiological role of increased oatp1 transport activity in the neonatal choroid plexus is not known. It may be speculated that it functions to remove organic anions from the CSF. A disorder such as kernicterus may be speculated that it functions to remove organic anions by the choroid plexus.


Samuelson, A. C., R. J. Stockert, A. B. Novikoff, P. M. Novikoff, J. S. Saez, D. C. Spray, and A. W. Wolkoff. Influence...


