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Expression of tight junction proteins and transporters for xenobiotic metabolism at the blood–CSF barrier during development in the nonhuman primate (*P. hamadryas*)

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ABSTRACT

The choroid plexus (CP) is rich in barrier mechanisms including transporters and enzymes which can influence drug disposition between blood and brain. We have limited knowledge of their state in fetus. We have studied barrier mechanisms along with metabolism and transporters influencing xenobiotics, using RNAseq and protein analysis, in the CP during the second-half of gestation in a nonhuman primate (*Papio hamadryas*). There were no differences in the expression of the tight-junctions at the CP suggesting a well-formed fetal blood–CSF barrier during this period of gestation. Further, the fetal CP express many enzymes for phase I–III metabolisms as well as transporters suggesting that it can greatly influence drug disposition and has a significant machinery to deactivate reactive molecules with only minor gestational changes. In summary, the study suggests that from, at least, midgestation, the CP in the nonhuman primate is restrictive and express most known genes associated with barrier function and transport.

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1. Introduction

The choroid plexus, which is situated in the ventricles of the brain, is the main blood–CSF interface. It contains a range of mechanisms to either prevent or facilitate transport of compounds across the choroid plexus epithelium. One important mechanism of the barrier is to protect the brain from potentially harmful exogenous or endogenous compounds. The plexus is well adapted for high transport of compounds from the blood to the cerebrospinal fluid. It contains a richly vascularized core with fenestrated vessels for promoting flux of fluid and compounds. Outside of the blood vessels is a continuous epithelium that is the main cellular barrier. These cells have highly complex tight-junctions insuring selective transfer by forcing flux of compounds across, rather than between, the epithelial cells that contain a battery of transporters and receptors. The tight-junctions have been shown to be restrictive already early in rodent development [1,2]. Many of the transporters have roles in the production of the cerebrospinal fluid or are important for the influx of nutrients to the central nervous system as well as the homeostasis of the brain.

The epithelial cells contain a range of enzymes and metabolic activities that act as a defence for the central nervous system to reduce xenobiotic loading on the brain [3]. Metabolism in the plexus can also make lipid-soluble compounds more hydrophilic and increases the time that they are available for further metabolisms or exclusion by the plexus. Drug metabolizing mechanisms have been shown to be present throughout the brain but are enriched at the blood–CNS interfaces, such as the blood–brain and blood–CSF barriers, where they can act as an outer defence against access to the brain [4]. Several drug metabolizing mechanisms are present in the developing plexus, including the energy-driven ATP-binding cassette proteins such as breast cancer related protein (BCRP) and multi-drug resistance related

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proteins in the ABCC family [5,6]. Transport by these pumps may be facilitated by phase II metabolism of compounds including conjugation of glutathione, sulfation and glucuronidation. A wide variety of other solute carriers are also present on choroidal epithelial cells belonging to the SLC- or SLCO-families that are indicated in drug transport. Previous studies have demonstrated that at least some of these mechanisms are working at a similar level in the fetus as in the adult [7]. A WHO sponsored investigation showed that women ingest an average of three prescription medications during pregnancy [8]. There is also a growing concern how environmental toxins affects the development of the brain [9]. An important question in regard to the potential harmful effects on the fetal brain in relation to xenobiotics such as drugs in the maternal circulation is therefore how well the defence mechanisms at the blood–CNS interfaces are developed in the fetus. Recently the gene changes of drug metabolizing and antioxidant systems were reported for the developing rat choroid plexus [10]. In order to further our understanding of these defence mechanisms at the choroid plexus in a translational model we sourced choroid plexus from a fetal nonhuman primate, the baboon (*Papio hamadryas*), which has very close development to the human. Our study was conducted during the second half of gestation when the majority of the cortical expansion is occurring in primates [11]. We firstly investigated the genes and proteins associated with cell-junctions at the blood–CSF barrier and secondly explored changes in genes related to drug transport, metabolism, antioxidant defence and CSF production.

2. Material and methods

2.1. Animals

Female baboons (*P. hamadryas*) from the Southwest National Primate Research Center (San Antonio, TX, USA) were used for this study. All procedures were approved by the Institutional Animal Care and Use Committee of the Texas Biomedical Research Institute and conducted in AAALAC-approved facilities. Feeding and management have previously been explained in detail [12]. Food was withdrawn for 16 h before surgery. Cesarean sections were performed at 90 (50% gestation), 120 (67% gestation) and 165 days (90% gestation) of gestation (term 184 days) under general anesthesia as previously reported [13]. Following cesarean section maternal analgesia was provided with buprenorphine hydrochloride 0.015 mg/kg per day (Hospital, Inc., Lake Forest, IL, USA) for three days post-surgery. When cesarean section was performed to obtain samples from multiple areas of the brain and other fetal tissues, fetal telencephalic choroid plexus (lateral ventricular plexus) was collected and frozen in liquid nitrogen at gestation day (GD) 90 (2 males/1 female), GD120 (2 males/2 females) and GD165 (3 males/3 females). Choroid plexus was also obtained from two non-pregnant adult females at 9.4 and 10.2 years of age and two pregnant baboons at 9.4 and 11.3 years of age. These adult animals were necropsied as part of a wider study of aging. Where euthanasia was performed on fetuses and adult animals it was conducted by exsanguination while the animal was under general anesthesia as approved by the American Veterinary Medical Association.

2.2. RNA preparation and RNA sequencing

A detailed description of RNA sequencing has been published elsewhere [14]. Briefly, frozen tissues were homogenized and RNA extracted with an RNAeasy mini-kit (Qiagen) according to manufacturer's specifications. RNA quality was analyzed by Biorad Experion electrophoresis. RNA-seq was performed at the Genomics Core Facility at University of Gothenburg. Only high quality RNA was used for library preparation (RIN > 8). Libraries were created using the TruSeq™ RNA Sample Preparation v2 kit and the library was subjected to 50 bp single end read cycles of sequencing on an Illumina HiScan SQ.

2.3. Analysis of sequencing data

RNA-seq reads were mapped to the current baboon genome assembly (https://www.hgsc.bcm.edu/content/baboon-genome-project) using Tophat [15]. Cufflinks [16] was used for transcript assembly of individual samples. All assemblies were merged to create a reference transcript, which was used to calculate gene counts with HTSeq (http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html). DESeq [17] was then used to find differentially expressed genes. Annotation of genes was done through blasting (blastx) [18] each transcript against the Swiss-Prot database (Uniprot Consortium). Differential expression of transcripts was considered between GD90 and GD165 with *p*-value considered significant when less than 0.05 after false discovery rate corrections. The expression levels for genes were divided into five levels for the Results section whereby normalized raw counts between 1 and 10 were considered very low expression, 10–100 low expression, 100–1,000 medium expression, 1,000–10,000 high expression, and >10,000 very high expression. The cut-off detection level was set to 1 (i.e. all transcripts where the raw read count was less than 1 across all ages were removed). In addition, we have not further deliberated on significant changes in genes for which expression was limited to the very low expression range (<10 counts) across all developmental ages. Changes in genes were visualized in heatmaps generated in the statistical software R along with cluster analysis. We have used GO terms for human proteins annotated to tight-junction and cell–cell adhesion to make relevant heatmaps of genes associated to these terms, however, genes were also manually collated with duplicates removed.

2.4. Immunohistochemistry (IHC)

After fixation for 24 h in 4% paraformaldehyde, plexuses from fetuses at GD120, GD165 and adults (no GD90 animals were available) were dehydrated in increasing concentrations of alcohol and embedded in paraffin. Serial sections were cut on a microtome and prepared for IHC. Paraffin was removed from sections in xylene, sections were rehydrated in decreasing concentrations of ethanol and gently boiled in 0.01 M citrate buffer or protease digestion (Streptomyces griseus, Sigma; 1 mg/ml at 37 °C) for 10 min. Blocking steps included H_2O_2 treatment (3% in methanol for 10 min) and appropriate serum (5%) or DAKO serum-free protein block for 1 h. Sections were then incubated in the following primary antibodies toward three key plexus tight-junctional proteins: ZO-1 (1:100; Cat#61-7300, Invitrogen), occludin (1:100; Cat#71-1500, Invitrogen) and claudin-1 (1:200; Cat#71-1500, Invitrogen). They were then incubated in biotinylated secondary against the appropriate species followed by the ABC kit (Vector) or HRP-labeled secondary antibody (BrightVision poly HRP-anti-rabbit IgG from ImmunoLogic) and developed with DAB. In between all steps, sections were washed in PBS with 0.1% tween20. After 5 min in DAB solution, sections were washed in water, dehydrated and coverslipped. Blank sections were obtained when primary antibody was omitted.

2.5. Protein analysis

Choroid plexus tissues (from the same samples as for RNAseq) were homogenized by sonication in ice-cold PBS containing 5 mM

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