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Spatial and temporal recruitment of the neurovascular unit during development of the mouse blood-retinal barrier

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ABSTRACT

The inner blood-retinal barrier (BRB) is made up by the neurovascular unit, consisting of endothelial cells, pericytes and glial cells. The BRB maintains homeostasis of the neural retina, but in pathological eye conditions the neurovascular unit is often disrupted, causing BRB loss. Here, we investigated in detail temporal and spatial recruitment of the neurovascular unit in the neonatal mouse retina from postnatal day (P)3 to P25 employing immunohistochemical staining of vascular endothelium (isolectin B4), pericytes (α-SMA and NG2) and astrocytes (GFAP). In addition, we investigated gene expression of polarized astrocytic end-feet markers aquaporin-4 and laminin α2 chain with qPCR. We observed GFAP-positive cells migrating ahead of the retinal vasculature during the first postnatal week, suggesting that the retinal vasculature follows an astrocytic meshwork. From P9 onwards, astrocytes acquired a mature phenotype, with a more stellate shape and increased expression of aquaporin-4. NG2-positive cells and tip cells co-localized at P5 and invaded the retina together as a vascular sprouting front. In summary, these data suggest that recruitment of the cell types of the neurovascular unit is a prerequisite for proper retinal vascularization and BRB formation.

1. Introduction

The neural retina is protected by the inner blood-retinal barrier (BRB), which maintains homeostasis by selectively regulating the entry of molecules into the retina and controlling vascular permeability (Klaassen et al., 2013). The BRB consists of endothelial cells lining the retinal microvessels, pericytes, their basal lamina and glial cells, together forming the neurovascular unit. The presence and crosstalk between these cell types in the neurovascular unit is essential for the maintenance of a tight BRB (Wisniewska-Kruk et al., 2012).

In humans, the retinal vasculature is completed around mid-gestation (Gariano, 2003), but in mice, retinal vascular development starts in the first postnatal week. The retinal vasculature provides oxygen and nutrients to the inner retina, which has several (anatomically tightly-arranged) cellular layers that contain interneurons, ganglion cells and glial cells. Vessels grow from the optic disc into the ganglion cell layer (containing ganglion cells, amacrine cells and (peri-)vascular cells) and radiate outwards to the periphery, that is reached around postnatal day (P) 7. From this superficial vascular plexus, collateral sprouting of capillaries into the inner plexiform layer (where photoreceptor input is processed through bipolar cells, amacrine cells and ganglion cells) and into the inner nuclear layer up to the boundaries of the outer plexiform layer (where photoreceptor cells form connections with bipolar cells and horizontal cells) initiates generation of the interconnected intermediate and deep capillary layers (Fruttiger, 2007). The retinal pigment epithelium and photoreceptors are part of the outer retina and are supplied with oxygen and nutrients by a different vascular bed, the choriocapillaris, which is not discussed here.

Initially, the retinal vasculature lacks a functional barrier during development, as is demonstrated by leakage of plasma proteins and small molecules from the lumen of the vessels (Chow and Gu, 2017; van der Wijk et al., submitted). Recruitment of pericytes and astrocytes to the retina to form the neurovascular unit may be crucial for final maturation of the endothelium and for the formation of a functional BRB.

Previously, it has been shown that astrocytes serve as a meshwork for growing vessels in the retina, thereby guiding endothelial cell migration (Dorrell et al., 2002;O'Sullivan et al., 2017). In addition, pericytes may be involved in the early formation of the BRB by providing a

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suitable microenvironment for retinal endothelial cells to develop barrier characteristics, as was shown in blood-brain barrier development (Daneman et al., 2010). Moreover, permeability of the blood-brain barrier in neonatal mice inversely correlated with pericyte coverage (Armulik et al., 2010; Daneman et al., 2010), confirming the essence of pericytes in barrier integrity. As the pericyte-to-endothelial ratio is relatively high in the retina (1:1), when compared to brain (1:3) and other microvascular beds (1:10) (Stewart and Tuor, 1994) such a function of pericytes may be particularly important in the BRB.

In the present study, we investigated in detail the temporal and spatial composition of the neurovascular unit in the neonatal mouse retina using gene and protein expression of selected markers of retinal pericytes and astrocytic end-feet.

2. Materials and methods

2.1. Animals

Animal experiments were performed with the approval of the Animal Ethics Committee of the University of Amsterdam and in compliance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. To study the development of the BRB, neonatal (wildtype) mice were killed on P3, P5, P7, P9, P11, P13, P15, P17 and P25 with an intracardial injection of ketamine-medetomidine-atropine for young mice (until P13), whereas older mice were euthanized with CO_2 asphyxiation. Eyes were enucleated and either snapfrozen in liquid nitrogen (for qPCR and immunohistochemistry) or processed immediately for retinal wholemount staining.

2.2. RNA isolation and mRNA quantification

Retinas (at least 6 to 8 retinas per group) were treated by hypotonic lysis to enrich for retinal vessels (Kowluru et al., 1998). Each retina was incubated in 1 ml sterile water for 2 h at 4 °C. Next, retinas were spun down and sterile water was replaced with sterile water containing 40 µg DNase I (Life Technology, Breda, The Netherlands) and left for 5 min at room temperature. Retinas were spun down, supernatant was removed and the retinal vessels were resuspended in 500 µl TRIzol reagent (Invitrogen, Bleiswijk, The Netherlands) and stored at −20 °C until further processing. Total RNA was isolated according to the manufacturer's protocol and dissolved in RNAse-free water. RNA yield was measured using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE) and 1 µg of RNA was treated with DNAse-I (Invitrogen) and reverse transcribed into first strand cDNA with a Maxima First Strand cDNA Synthesis Kit (ThermoFisher). Real-time quantitative PCR was performed on 20× diluted cDNA samples using a CFX96 system (Bio-Rad, Hercules, CA) as described previously (Klaassen et al., 2009). Specificity of the primers was confirmed by NCBI BLAST. The presence of a single PCR product was verified by both the presence of a single melting temperature peak and detection of a single band of the expected size on 3% agarose gel. Non-template controls were included to verify the method and the specificity of the primers. Relative gene expression was calculated using the equation: $R = E^{-Ct}$, where E is the mean efficiency of all samples for the gene being evaluated and Ct is the cycle threshold for the gene as determined during real-time PCR. Primer efficiencies (E) were determined with LinRegPCR software (Ruijter et al., 2009) and ranged from 1.631 to 1.973. PCR products that did not show a single melting temperature peak were excluded from analysis. Expression data was normalized by the global mean normalization method (Mestdagh et al., 2009) using expression data of 30 genes in total.

2.3. Retinal wholemount staining

Enucleated mouse eyes were washed in PBS and fixed in 4% paraformaldehyde for 5 min, transferred to 2× PBS for 10 min and retinas were dissected in PBS. Isolated retinas were post-fixed in methanol and stored in −20 °C until further use. For immunofluorescence staining, retinas were briefly washed in 2x PBS and incubated in wholemount-blocking buffer (1% fetal calf serum, 3% TritonX-100, 0.5% Tween20, 0.2% sodium azide in 2x PBS) for 2 h at room temperature. Next, retinas were incubated overnight with the following antibodies: rabbit anti-glial fibrillary acidic protein (diluted 1:400, Cat # Z0334; GFAP; Dako, Heverlee, Belgium), mouse monoclonal anti-alpha-smooth muscle actin (α-SMA) antibody (diluted 1:200, Cat # C6198; Cy3 labeled, Sigma-Aldrich, Zwijndrecht, The Netherlands), isolectin B4 (diluted 1:30, Cat # I21411; Alexa Fluor-488 labeled, Invitrogen) or rabbit polyclonal anti-NG2 antibody (diluted 1:100, kindly provided by prof. W. Stallcup from the Burnham Institute, La Jolla, CA) diluted in wholemount blocking buffer. GFAP was used as a marker for astrocytes, α-SMA as marker for smooth muscle cells and pericytes, isolectin B4 was used to detect endothelial cells and NG2 was used as marker for pericytes. After 3 wash steps (3 times 30 min in wholemount blocking buffer), secondary antibody was added and wholemounts were incubated for 2–3 h (goat-anti-rabbit Alexa Fluor-633 or goat-anti-rabbit Cy3; Invitrogen, diluted 1:100 in wholemount blocking buffer). After overnight washing in wholemount blocking buffer, retinas were mounted on glass and covered in Vectashield (Vector Laboratories, Burlingame, CA). All staining procedures were performed under gentle agitation at room temperature.

For staining of the cryostat sections, samples were fixed for 10 min using 4% paraformaldehyde, permeabilized for 10 min with 0.2% Triton X-100 and blocked for 1 h with 10% normal goat serum. Staining was performed in wholemount blocking buffer with anti-rabbit Apq4 (diluted 1:100, Cat # AB2218; Millipore, Amsterdam, The Netherlands) for 2 h, followed by secondary antibody incubation (goat-anti-rabbit Cy3) for 1 h.

Images of retinal wholemounts were taken at the central retina (at the site of the optic disc), the middle retina and the peripheral retina and were captured using a confocal laser scanning microscope SP8 (Leica Microsystems, Wetzlar, Germany) with a 20x or 63x objective at the Cellular Imaging Core Facility of the Academic Medical Center. Specificity of the staining was checked by absence of fluorescent signal in samples that were stained in the absence of primary antibody.

2.4. Transmission electron microscopy (TEM)

Eyes were harvested and immersion fixed in McDowell fixative in phosphate buffer. To facilitate access of the fixative into the eye, eyes were punctured with a 29 G (which equals a diameter of 0.287 mm) needle and the cornea was dissected. Samples were processed for routine TEM, as described previously (Wisniewska-Kruk et al., 2016). Ultrathin sections of 80 nm were examined with a Technai-12 G2 Spirit Biotwin microscope (FEI, Eindhoven, The Netherlands) and micrographs were captured with a Veleta TEM camera (Emsis; Münster, Germany) using Radius acquisition software (Emsis) at a magnification of 11.000–30.000×, at the Electron Microscopy Center of the Academic Medical Center.

3. Results and discussion

3.1. Ultrastructure of the neurovascular unit

The close association of endothelial cells and pericytes, which share their basal lamina, and glial cells, which envelope the retinal vessels with their end-feet and a second basal lamina, is referred to as the neurovascular unit of the BRB in the mature retina (Fig. 1A–C). The endothelial cells of the BRB have no fenestrations, few pinocytotic vesicles and a continuous array of inter-endothelial tight junctions, which seal the intercellular space between endothelial cells (Klaassen et al., 2013)(Fig. 1B and b).