1A. Research Plan
2019-2020

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Title: The Role of Wnt/β-catenin Signaling in Blood-Brain Barrier Integrity in Experimental Autoimmune Encephalomyelitis (Multiple Sclerosis)

Category: Biomedical and Health Sciences

Rationale

Multiple Sclerosis (MS) is the most prevalent, nonfatal neurological disability among young adults (Dendrou, Fugger, & Friese, 2015; Wu & Alvarez, 2011). Unfortunately, there is no current cure for MS (Dendrou et al., 2015; Wu & Alvarez, 2011). One area of interest for treatment, research, and development is the blood-brain barrier (BBB) (Minagar & Alexander, 2003; Weiss, Miller, Cazaubon, & Couraud, 2009). Current drugs provide symptomatic relief. Understanding the repair mechanisms for a leaky BBB presents an opportunity to reverse disease progression. To explore the mechanisms of the BBB, experimental autoimmune encephalomyelitis (EAE) is a widely accepted disease animal model with pathological and clinical characteristics highly relevant to the study of MS (Constantinescu, Farooqi, O'Brien, & Gran, 2011a, 2011b; Miller, Karpus, & Davidson, 2010). EAE is an antigen-driven autoimmune model rather than a genetic model. EAE is induced through myelin oligodendrocyte glycoprotein 35-55 (MOG_{35-55}) which causes the infiltration of T cells into the CNS leading to myelin destruction, emulating MS disease pathology (Miller et al., 2010).

The BBB is a semi-permeable membrane that selectively restricts the blood-to-brain transport of substances across the epithelium preventing injury to the CNS (Begley, 2006; Dobbing, 1961; Palmela, Brites, & Brito, 2012). This selective restriction is necessary for cerebral homeostasis and proper neuronal function (Weiss et al., 2009).
A common feature of MS is compromised BBB integrity. Disrupted tight junction organization or degraded tight junction proteins lead to a leaky BBB and an increase of immune cell infiltration. Immune cells attack the nervous system along the brain, spinal cord, and optic nerve, resulting in inflammation (Frischer et al., 2009; Glass, Saijo, Winner, Marchetto, & Gage, 2010; Sospedra & Martin, 2016). Demyelination slows, and even stops, nerve impulses (Lubetzki & Stankoff, 2014). Immune cell infiltration, implicated in BBB degradation, results in the symptomatic expression of MS (Glass et al., 2010; Sospedra & Martin, 2016).

A potential repair mechanism for the BBB in MS is the Wnt/β-catenin signaling pathway. Throughout embryonic and postnatal development, Wnt signaling contributes to both blood-brain barrier formation and angiogenesis, which is the development of new blood vessels (Daneman et al., 2009; Liebner et al., 2008; Stenman et al., 2008; Zhou et al., 2014). In healthy adults, Wnt/β-catenin signaling is reduced to low levels which is still necessary to maintain BBB integrity (Liebner et al., 2008; Suryawanshi et al., 2015). Dysregulated activation of Wnt signaling has been related to the pathology of many autoimmune diseases, including rheumatoid arthritis (RA) and inflammatory bowel disease (IBD). Understanding Wnt signaling in autoimmune diseases may provide a new diagnostic marker and therapeutic target for developing novel treatments (Shi et al., 2016).

My mentor's lab initially hypothesized that the upregulation of Wnt signaling would help maintain BBB integrity and improve EAE disease progression. EAE mice with upregulated Wnt signaling were expected to be protected against EAE. Unexpectedly, these mice exhibited a significant increase in peak clinical scores compared to wild-type mice. These results indicated that worse EAE neurological outcomes were associated with the

hyperactivation of Wnt/β-catenin signaling. The explanation for why Wnt/β-catenin activation fails to restore/repair BBB function in EAE remains unknown.
To explore the worsened clinical progress, the *Zic3, FoxQ1, FoxF2* are three transcription factors that play a role in BBB formation in brain endothelial cells at distinct stages of development were selected. These transcription factors were shown to increase the expression of genes encoding markers of brain endothelial cell differentiation, so they are considered important in BBB formation (Hupe et al., 2017). After the activation of the Wnt pathway, these transcription factors begin to transcribe DNA to form the BBB, which makes them downstream targets of the Wnt pathway (Hupe et al., 2017). Thus, understanding the presence of these factors during EAE may better explain the role and mechanisms of Wnt/β-catenin signaling in maintaining BBB integrity.

Additionally, Wnt signaling has been found to play a role in angiogenesis during development (Daneman et al., 2009; Dejana, 2010; J. Lengfeld et al., 2014). The epidermal growth factor-like 7 (*Egfl7*) can be used to observe neoangiogenesis in EAE. *Egfl7* expression is the highest when the endothelium is in an active, proliferating state which shows neoangiogenesis. In exploring worsened clinical scores associated with increased Wnt signaling, neoangiogenesis during EAE can be better understood by observing marker of *Egfl7*.

Early and persistent BBB damage plays an important role in the infiltration of immune cells during EAE, leading to pathological outcomes (Kermode et al., 1990; Minagar & Alexander, 2003; Palmela et al., 2012). Similarly, neoangiogenesis generates leaky blood vessels that may exacerbate EAE pathogenesis. This study explores novel molecular pathological mechanisms to help explain the dysfunctional BBB in EAE and reasons for worsened disease progression. The goal of this study was to better explain the initial unanticipated findings of increased (worse) clinical EAE scores with activated Wnt signaling in EAE observed by my mentor in a previous experiment.

Research Question and Hypothesis

Research Question (s):
Research Question 1
How does the hyperactivation of Wnt/β-catenin signaling in CNS endothelial cells in EAE affect the presence of transcription factors that act downstream of Wnt/β-catenin signaling to promote BBB formation?

Research Question 2
How does the hyperactivation of Wnt/β-catenin signaling in endothelial cells worsen affect neoangiogenesis, measured by *Egfl7*, leading to worse EAE neurological outcomes?

Hypothesis (es):
Hypothesis 1
The expression of several key transcription factors necessary for BBB maintenance (*Zic3*, *Foxq1*, *Foxq2*) would be reduced or absent in EAE.

Hypothesis 2
Egfl7 expression, indicating neoangiogenesis, is upregulated during peak EAE.

Methods

Performed by Mentor

Induction of EAE and Clinical Observations (Performed by Mentor)

To upregulate Wnt/β-catenin signaling, the mice used in this experiment will be *Tcf/Lef1*::H2B-eGFP Wnt reporter mice. EAE was induced in ten to twelve-week old Wnt-reporter mice, which will be observed for clinical symptoms for sixteen days after EAE induction. All experimental procedures involving live animals will be performed by the supervising scientists and were approved by the IACUC at the research institution. Clinical expression of EAE is evaluated on a standard scale: score 0 for no observed effects, score 1 for flaccid tail, score 2 for hind limb paresis, score 3 for bilateral hindlimb paralysis, score 4 for tetraplegia, and score 5 for moribund.

Collection of Tissues (Performed by Mentor)

Tissue samples will be collected from four experimental groups of Wnt reporter mice. For P3 mice, cerebellum tissue samples were collected at day 3 from healthy, developmental mice. Since Wnt/β-catenin signaling is very high at embryonic and early postnatal stages, P3 mice served as a positive control. For healthy adult mice, spinal cord tissue samples were collected at day 90. The healthy adult mice served as a negative control because Wnt/β-catenin signaling is reduced in late postnatal and adult stages. For acute EAE mice, spinal cord samples were collected at day 14 with an EAE clinical score of 2.5. For chronic EAE mice, spinal cord samples were collected at day 74 with an EAE clinical score of 2.

Tissue Analysis Performed by Student

Evaluation of Transcription Factors (Student Experiment)

To understand where and when three transcription factors exist in cells, fluorescence in situ hybridization (FISH) will be performed to label the transcription factors selected for their BBB-forming properties: *Zic3, Foxq1,* and *Foxf2*. FISH is a technique that uses fluorescent probes that are nucleic acid labeled with fluorescent groups and can bind to specific DNA/RNA sequences. The collected cross sections of the spinal cord and brain will be obtained from acute EAE, chronic EAE, P3, and healthy mice (n=3) and will be stained using FISH. An immunofluorescence with Anti-Cav-1 antibody will be used to label blood vessels. Lastly, slides will be mounted using VectaShield with DAPI (Vector Labs) and will be viewed using a confocal laser scanning microscope (LSM 510 Zeiss, Germany).

The same process to perform FISH will be repeated to stain for each transcription factor, along with an immunofluorescence with Anti-Cav-1 antibody to label blood vessels. Slides will be mounted using VectaShield with DAPI (Vector Labs) and will be viewed using a confocal laser scanning microscope (LSM 510 Zeiss, Germany).

Additional Staining for Zic3 (Student Experiment)

To further investigate the expression of *Zic3*, immunofluorescence will be performed. Cross sections from the spinal cord and brain will be obtained from P3 (three-days old) and acute EAE mice (n=3). The lumbar, thoracic, and cervical regions of the spinal cord, along with the cerebellum, will be removed after perfusion with PBS solution and 4% PFA, postfixed overnight, and then transferred into 20% sucrose in PBS solution. The samples will then be sectioned using superfrost plus slides. Immunofluorescence with Anti-Cav-1 antibody will be used to label blood vessels, and slides will be mounted using VectaShield with DAPI (Vector Labs) to view with a confocal laser scanning microscope (LSM 510 Zeiss, Germany).

Evaluation of Neoangiogenesis (Student Experiment)

To understand where and when neoangiogenesis exists in cells, FISH will be performed to label *Egfl7* (a marker of neoangiogenesis). The obtained cross sections from acute EAE, chronic EAE, P3, and healthy mice (n=3) will be stained for *Egfl7*. The same process to perform FISH will be repeated, along with an immunofluorescence with Anti-Cav-1 antibody to label blood vessels. Slides will be mounted using VectaShield with DAPI (Vector Labs) and viewed using a confocal laser scanning microscope (LSM 510 Zeiss, Germany).

Confocal Microscopy (Performed by the Student)
For laser scanning confocal microscopy, labeled sections will be examined using an LSM 510 Zeiss, Germany. All sections will initially be surveyed at low magnification at various levels so that overall patterns of blood vessels could be compared in cells oriented at different angles with respect to the section plane. Images will be collected from the lumbar region of the spinal cord. Since EAE is characterized by ascending motor paralysis, the lumbar region is the first area of the spinal cord to be affected in the mouse. Three sections of the lumbar spinal cord will be imaged per slide. For each section, an image will be taken of the dorsal funiculus, ventral funiculus, and four lateral funiculi. This will be completed routinely done using a 40x objective with water magnification. These images can be viewed individually or projected together to reconstruct the image of the blood vessel. The reconstruction of each image will be processed using the ImageJ program (which is freely available from NIH), and then analyzed for quantification of expression.

Data Analysis

Statistical analysis will be performed on ImageJ to quantify the expression of the three transcription factors and *Egfl7* using the processed images. The quantitation of expression will be calculated using two methods. The first will be calculating Pearson's R for the colocalization of the red and green channels in the image. The second is calculating the mean fluorescent intensity of the red channel (expression of transcription factor of *Egfl7*) in the area of the green channel (blood vessel). To do this, the mean fluorescent intensity of the red channel will be calculated on ImageJ. The intensity of the red channel will be divided by the selected area of the green channel. ImageJ will be used to calculate the Pearson's R of the fluorescent intensity. Student t-tests will be used to compare the mean fluorescent intensity among the groups of mice.

Risk and Safety

Some potentially hazardous biological agents included concentrated hydrochloric acid, Phenol/Chloroform/Isoamyl Alcohol, acetic anhydride, paraformaldehyde, triethanolamine, hydrogen peroxide. Safety precautions taken were wearing lab coat, gloves, full-length clothes, and closed toed shoes, laminar flow hood. Harmful chemicals were disposed of in appropriate containers, sharps were disposed of in sharps containers, and the rest of the materials were disposed of in red biological dumpster bins.

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