SPARC EXPRESSION DURING DEVELOPMENT AND INFLAMMATION AT THE BLOOD-BRAIN BARRIER

by

Samir Alkabie
B.Sc., Wilfrid Laurier University, Waterloo, Ontario, Canada, 2008.

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)
March 2011
© Samir Alkabie, 2011
ABSTRACT

SPARC (secreted protein acidic and rich in cysteine) is a cell-matrix modulating protein involved in angiogenesis and endothelial barrier function, yet a potential role in cerebrovascular repair and inflammatory responses in the central nervous system (CNS) has not previously been characterized. The inflammatory demyelinating disease, multiple sclerosis (MS) is characterized pathologically by inflammatory infiltrates, demyelination and axonal damage/loss and aberrant alterations in blood-brain barrier (BBB) integrity. We hypothesize that SPARC expression may be influenced by inflammatory or repair processes during MS, and that SPARC itself may influence BBB integrity. This study examined SPARC expression in cultured human cerebral microvascular endothelial cell (hCMEC/D3), an in vitro model of the BBB, under steady state conditions or those modeling an inflammatory milieu by immunoblotting and immunocytochemistry. hCMEC/D3s constitutively express SPARC during proliferative growth and downregulate SPARC as cells establish a BBB phenotype. SPARC expression in cerebral endothelia directly correlated with the cell proliferation marker Ki-67, consistent with a role for SPARC in CNS angiogenesis. Proinflammatory cytokines associated with inflammation and immune activation differentially regulate SPARC expression in cerebral endothelia. Tumor necrosis factor alpha (TNF-α) cytokine or lipopolysaccharide (LPS) endotoxin treatment significantly increased SPARC protein levels. TNF-α and interferon gamma (IFN-γ) cotreatment abrogated SPARC induction compared to TNF-α alone, suggesting divergent roles for each cytokine in regulating SPARC expression in cerebral endothelia. Compared to cultures replenished with media lacking exogenously supplied SPARC, addition of a physiological SPARC concentration observed in healthy individuals (0.1µg/ml) increased tight junction protein
expression of zonula occludens 1 (ZO-1) and occludin by approximately thirty percent, suggesting a role in BBB maintenance. Paradoxically, functional assays show recombinant human SPARC applied exogenously increased the transendothelial permeability of hCMEC/D3 monolayers. In agreement, barrier hCMEC/D3s exposed to increased SPARC concentrations (1-10 μg/ml) associated with pathological conditions in vivo, reduced ZO-1 and occludin by one-third. Together, these data support a role for SPARC in BBB maintenance under normal physiological conditions and BBB alterations during inflammatory conditions. In this regard, SPARC levels may play a key role in regulating BBB integrity and serve to alter processes of CNS inflammation and repair.
# TABLE OF CONTENTS

Abstract .................................................................................................................................................. ii

Table of contents .................................................................................................................................. iv

List of tables .......................................................................................................................................... vii

List of figures .......................................................................................................................................... viii

List of abbreviations .............................................................................................................................. x

Acknowledgments ..................................................................................................................................... xi

Dedication ................................................................................................................................................ xii

## CHAPTER 1 Introduction ...................................................................................................................... 1

1.1 Multiple sclerosis pathology: past and present .............................................................................. 1

1.2 Prevalence of MS in Canada ............................................................................................................. 3

1.3 Public healthcare utilization and expenditure treating MS .............................................................. 3

1.4 MS etiology ....................................................................................................................................... 4

1.5 Genetic susceptibility in MS .............................................................................................................. 5

1.6 Non-infectious environmental factors .............................................................................................. 8

1.7 Infectious environmental factors in MS ............................................................................................. 10

1.8 The immune privileged CNS ............................................................................................................ 12

1.9 Immunopathogenesis of MS ............................................................................................................ 13

1.10 MS disease course and diagnostic criteria ....................................................................................... 16

1.11 MS lesions and associated neurological dysfunction ......................................................................... 18

1.12 Neuropathology of MS lesions ........................................................................................................ 19

   1.12.1 Chronic MS lesions .................................................................................................................. 19

   1.12.2 Active MS lesions ................................................................................................................... 20
CHAPTER 2 Materials and Methods

2.1 Cell culture and treatments
   2.1.1 hCMEC/D3 and astrocytoma cell lines
   2.1.2 Cell culture conditions
   2.1.3 Growth and development of hCMEC/D3s
   2.1.4 Proinflammatory treatment of hCMEC/D3s
   2.1.5 hCMEC/D3 exposure to SPARC

2.2 Antibodies and reagents

2.3 Immunocytochemistry

2.4 Image acquisition and analysis

2.5 Conditioned media and cell lysis
2.6 Immunoblotting .................................................................................................................. 49
2.7 Statistical analysis ............................................................................................................. 51

CHAPTER 3 Results ............................................................................................................. 52
3.1 Human cerebral endothelial cell (hCMEC/D3) model of the BBB ....................... 52
3.2 SPARC expression of hCMEC/D3s during growth and development ............... 53
   3.2.1 Localization of SPARC in hCMEC/D3s ................................................................. 53
   3.2.2 SPARC expression is greater in subconfluent than confluent hCMECs ... 54
   3.2.3 SPARC expression correlates with Ki-67, a marker of proliferation ...... 55
   3.2.4 Unchanged SPARC and reduced percentage of Ki-67 positive nuclei in
       VEGF treated hCMEC/D3 cultures ........................................................................... 56
3.3 Proinflammatory molecules regulate SPARC expression in hCMEC/D3s ........ 57
3.4 Effect of SPARC on tight junction protein expression in hCMEC/D3s .......... 58

CHAPTER 4 Discussion ....................................................................................................... 87
4.1 Summary .......................................................................................................................... 87
4.2 SPARC expression during BBB establishment ......................................................... 88
4.3 Regulation of SPARC expression during infection and inflammation .............. 92
4.4 Tight junction regulation at the BBB ........................................................................... 96
4.5 SPARC: friend or foe in CNS development and response to injury or repair ..... 104
4.6 Future directions .......................................................................................................... 105

References .......................................................................................................................... 107
LIST OF TABLES

Table 1. SPARC immunoreactivity assessment scale for hCMEC/D3 in culture........61
LIST OF FIGURES

Figure 1. Phase contrast images of hCMEC/D3 propagated on various culture surfaces and substrates..................................................................................................................62

Figure 2. The human astrocytoma cell line ccf-sttg1 expresses high levels of SPARC detectable by immunoblotting and immunocytochemistry ..............................................64

Figure 3. Localization of SPARC in cytoplasm, ECM, and conditioned media of hCMEC/D3 cultures detectable by immunoblotting and immunocytochemistry.................65

Figure 4. Immunocytochemistry images show subconfluent hCMEC/D3 cultures with higher SPARC expression than confluent monolayers......................................................66

Figure 5. Individual cells in confluent monolayers express less SPARC than cells in subconfluent cultures..................................................................................................67

Figure 6. Subconfluent hCMEC/D3 regions exhibit more intense SPARC expression than confluent regions by regional immunocytochemistry analysis........................................68

Figure 7. Percentage of cells positive for the proliferation marker Ki-67 in subconfluent and confluent hCMEC/D3 cultures..............................................................................69

Figure 8. Immunocytochemistry images of subconfluent cultures show higher SPARC and ki-67 expression than confluent cultures sharing less intense SPARC and Ki-67 expression............................................................................................................70

Figure 9. SPARC levels positively correlated with the percent of Ki-67 positive cell nuclei in subconfluent hCMEC/D3 cultures.............................................................................71

Figure 10. Intensity of SPARC staining on individual cells is similar in untreated and VEGF treated hCMEC/D3 cultures.......................................................................................72

Figure 11. VEGF treatment reduces percent of Ki-67 positive nuclei and cell numbers in subconfluent hCMEC/D3 cultures.................................................................................73

Figure 12. Percent of Ki-67 positive cells decreased within cells expressing negligible levels of SPARC in the presence of VEGF...........................................................................74
Figure 13. Immunocytochemistry shows discordance in intranuclear Ki-67 staining in subconfluent hCMEC/D3 exposed to VEGF compared to untreated controls............75

Figure 14. Western blotting confirms the nuclear translocation of NFκB secondary to hCMEC/D3 treatment with TNF-α compared to untreated controls..........................76

Figure 15. TNF-α increases SPARC expression in confluent hCMEC/D3 cultures.......77

Figure 16. Proinflammatory molecules differentially regulate SPARC expression by confluent hCMEC/D3s monolayers..................................................................................79

Figure 17. Phase contrast micrographs of TNF-α and SPARC treated cerebral endothelia cultured in the presence or absence of hydrocortisone.............................................81

Figure 18. Hydrocortisone protected against TNF-α reduction in hCMEC/D3 tight junction expression.............................................................................................................83

Figure 19. SPARC regulation of cerebral endothelia tight junction expression.........84

Figure 20. SPARC and TNF-α regulation of cerebral endothelia tight junction expression in the presence or absence of hydrocortisone..................................................85

Figure 21. Physiological SPARC regulation of cerebral endothelia tight junction expression in the presence and absence of hydrocortisone..............................................86
<table>
<thead>
<tr>
<th>ABBREVIATION</th>
<th>FULL NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebral spinal fluid</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminesence system</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FN</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>hCMEC/D3</td>
<td>Human cerebral microvascular endothelial cell</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>ILK</td>
<td>Integrin linked kinase</td>
</tr>
<tr>
<td>LN</td>
<td>Laminin</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal goat serum</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer solution</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SPARC</td>
<td>Secreted protein acidic and rich in cysteine</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight junction</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zonula occludens 1</td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENTS

My sincerest gratitude goes out to Dr. Jacqueline Quandt, whose creative scientific energy and keen intellect encouraged me to ask the right questions and stay focused. Jacquie’s training has surely improved my research abilities. My time in Jacquie’s lab was preceded by the privilege of researching in the Labs of Drs. Jane Roskams and Peter Rieckmann, who carried a clear vision and expertise on CNS pathobiology. Jane, my co-supervisor, conveyed the necessity of experimental controls and the potential for innovative data analysis in immunocytochemistry, while Peter ‘my original supervisor’ introduced paradigms of inflammation, development and regeneration that were paramount throughout my thesis.

A special thanks to post-docs Jayasree Basivireddy, Sam Lloyd Burton, and research technician Olena Preobrazhenska, whose selfless assistance, technical proficiency, and encouragement helped me through many experimental quandaries. I want to thank my lab ‘buddy’ Ash for engaging MS-related discussions and many laughs. Numerous thanks to Dr. Vince Duronio for being a great lecturer, Program Director, and member of my supervisory committee mediating helpful lines of support and suggestions throughout my time in the Experimental Medicine, masters program at UBC. Without the assistance and support of the aforementioned people, this work would not have been possible.

This research was supported by a Canadian Institute for Health Research (CIHR)—masters award, the Department of Pathology and Laboratory Medicine, the Michael Smith Foundation for Health Research, and an Experimental Medicine entrance scholarship.
DEDICATION

For loving parents Hisham and Suhair; brothers Sermed and Sinan; niece Samiya daughter of Silvia; friends and teachers.
CHAPTER 1

Introduction

1.1 Multiple sclerosis pathology: past and present

From pioneering work by Carswell in 1838 and Cruveilhier in 1841 and later elaborated by Charcot in 1868, the pathological hallmarks of multiple sclerosis (MS) were described as perivascular inflammation associated with damage and loss of myelin (demyelination) and neuronal tissue (neurodegeneration) along with pronounced meshing of, non-neuronal, glia (gliosis) and immune infiltrates in response to CNS injury or disease (Dutta and Trapp 2007). Examinations further revealed MS lesions have a spatial predilection for the optic nerve, brainstem, spinal cord, cerebellum, and periventricular white matter corresponding to clinical presentations of nystagmus of the eye, intention tremor, and scanning speech (Lucchinetti, Parisi et al. 2005; Dutta and Trapp 2007). Building upon these mid-19\textsuperscript{th} century descriptions of MS pathology, modern neural biology seeks to understand the mechanisms responsible for lesion development and progression.

MS can be considered a prototypical autoimmune, neuroinflammatory disease of the CNS involving aberrant peripheral immune responses leading to loss of myelin and neuronal structures, yet the precise pathogenic cascade is unknown. Because of the limited pathogenic data in MS, the pathogenic cascade has also been studied in an animal model of MS, experimental autoimmune encephalomyelitis (EAE) that exhibits similar CNS pathology (Abdul-Majid, Wefer et al. 2003; McFarland and Martin 2007). Many immune cells are implicated in the MS disease process including myelin reactive CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells which carry the peripheral immune response to the central
nervous tissue; CD25+ regulatory T cells which can control autoreactive CD4+ cells; B cells which produce the humoral response of myelin reactive autoantibodies; and macrophages and resident CNS microglia which respond to inflammatory cytokines and start to destroy myelin (Ziemssen 2005).

MS, at the level of central nerve fibres, involves the destruction of paranodal myelin leading to ineffectual, slow impulse propagation along denuded axolemma instead of quick, nodal, saltatory signal conduction along myelinated axons (Wilkins and Scolding 2008). Loss of myelin can occur via damage to the myelin producing glial cell (oligodendrocytes and precursors); direct neuroimmune myelin sheath insult; and/or disrupted axon-glial interactions (Chang, Tourtellotte et al. 2002). Demyelination of axons not only slows impulse propagation, but also decouples axon-glial trophic cross-talk required for survival and functional differentiation of neurons and oligodenroglia (Nave and Trapp 2008; Nave 2010). Failure of repair, loss of neurotrophic support, and chronic CNS inflammation in response to perpetual damage are postulated to underlie progressive neurological disability in MS (Rieckmann and Maurer 2002).

Two clinical phenomena, relapse and progression, define the natural disease course of MS (Hafler 2004). In modern medical opinion, relapses—periodic worsening and improvement of neurological function— are the clinical expression of autoimmune inflammation leading to focal demyelination and axonal damage disseminated throughout the CNS, while progression—progressive permanent loss of neurological function—signifies chronic demyelination, axonal loss, and astrogliotic scar formation in place of lost nervous tissue (Lucchinetti, Parisi et al. 2005). Brain imaging studies correlate a breakdown of the blood-brain barrier (BBB) with the initial inflammatory events and neurological disabilities in patients with MS (Soon, Altmann et al. 2007). In
these respects, MS reflects the progressive involvement of varying degrees of inflammation and structural degeneration and, eventually, functional loss of nervous tissue. Consequential to these subclinical pathological processes, MS patients endure a myriad of clinical symptoms including motor weakness, sensory disturbance, visual loss, gait ataxia, sphincter dysfunction, spasticity, cognitive impairment and depression (Poser 1994).

1.2 Prevalence of MS in Canada

MS is among the most common causes of neurological disability in young adults in western countries and affects 2.5 million people worldwide (Higginson, McCrone et al. 2009). In Canada where over 70% of the 32 million population are between the ages of 15 and 64 (Poppe, Wolfson et al. 2008), MS prevalence is known to be one of the highest in the world with estimated rates ranging from 55 to 350 per 100,000 people. Epidemiological studies also demonstrate geographic variability in MS prevalence across Canada. Systematic reviews of prevalence in Canada report a range from as low as 55.2 per 100,000 in Newfoundland and Labrador to a high of 350 per 100,000 in the Atlantic and “Prairie” (Alberta, Saskatchewan, and Manitoba combined) provinces (Beck, Metz et al. 2005). Other regions joining the ranks of the world’s five highest MS prevalent regions are the US, Northern Europe, Australia, and New Zealand (Pohar, Jones et al. 2007).

1.3 Public healthcare utilization and expenditure treating MS

While persons with MS may represent a minor proportion of the total population, each patient can develop a wide range of disabilities and clinical characteristics that burden patient quality of life requiring substantial rehabilitation and therapeutic
resources (Pohar, Jones et al. 2007). Healthcare expenditure on MS is remarkable, recorded to exceed $35,000 annually for severely affected individuals in Canada (1998). In chronic diseases like MS, cost of care rises with the progression of disease disability and the scope of treatment (Lad, Chapman et al. 2010). People with MS are the subjects of high rates of healthcare spending on immunomodulatory and anti-inflammatory drugs and rehabilitation services for an average of 40 to 50 years (Higginson, McCrone et al. 2009; Lad, Chapman et al. 2010). The chronicity of MS is essentially what makes MS such an economic burden.

Although targeted pharmacotherapeutics against MS disease can reduce inflammation in the CNS and rate of relapses, progressive accumulation of neurological deficits continue in spite of therapy (Rieckmann and Smith 2001). The majority of MS patients present a relapsing-remitting disease course of intermittent illness and recovery, although about 15% show a progressive decline in neurological function from onset (Higginson, McCrone et al. 2009). Current therapeutic options for treating MS often benefit patients with relapsing-remitting but not progressive forms of MS for unknown reasons (Peterson and Trapp 2005). Efficacious and cost-effective therapeutics are likely to be generated only subsequent to a better understanding of the pathogenesis and disease modifying factors influential in MS.

1.4 MS etiology

The precise etiology of MS is currently unknown. MS disease susceptibility, as inferred by epidemiological studies, is widely considered to be determined by an interaction of genetic and environmental risk factors.
1.5 Genetic susceptibility for MS

Several large population based genome-wide association studies of MS twins implicate a strong genetic basis for MS (Hafler 2004). Through advancements in high-throughput genotyping and sequencing technology a number of gene haplotypes are now identified to associate with increased risk of MS. Based on the possibility that MS is driven by autoimmunity, genetic studies sought to identify polymorphisms in candidate genes which regulate immune responses or myelin production (Kalman and Lublin 1999). After more than sixty years of study, the major histocompatibility complex (MHC) molecule human leukocyte antigen (HLA) remains the strongest susceptibility locus for MS and lends to the involvement of immunological processes (Jersild, Svejgaard et al. 1972). Genome wide association studies (GWAS) of MS populations also identified polymorphisms at the IL-7 receptor gene and other genes outside the HLA gene loci, each again largely related to immune function (Hafler, Compston et al. 2007).

Monozygotic twins with 100% genetic sharing have the highest recurrence rate of 35%. Consistent with the genetic association connecting genetic sharing and age-adjusted lifetime risk of MS, half siblings risk is lower than full-siblings (Compston and Coles 2002). Children of a conjugal pair with MS exhibit a 10 times higher risk for MS than in offspring of single affected parents, and 20-40 times higher risk than the general public (Compston and Coles 2002; Alcina, Vandenbroucke et al. 2010). Altogether, familial recurrence rates suggest MS has a measurable degree of heritability.

MS is considered a polygenic disease in that each gene alone applies a modest effect on MS susceptibility. In combination, however, multi-factorial gene risk interactions could conceivably ascribe alterations in immunological and neurovascular
physiology that increase risk for MS. The involvement of T cells which carry the immune response to the nervous system are believed to mediate autoimmune processes in MS consistent with the association between risk for MS and immune activation molecules such as HLA class II molecules (McFarland and Martin 2007). Many case-control studies have confirmed HLA class II haplotypes DRB1*15 and DRB1*11 are consistently associated with a relative increase and decrease respectively in conferred risk for MS (Kalman and Lublin 1999; Handel, Giovannoni et al. 2010; Handel, Handunnetthi et al. 2010). Caucasians of western European decent with MS often retain the two high risk HLA-DRB genes of the HLA-DR15 haplotype (DRB1*1501 and DRB5*0101) (McFarland and Martin 2007). Other important members of the HLA allele family are the susceptibility allele HLA-DRB1*03 associated with an increased risk for MS and the protective alleles HLA-DRB1*01 and HLA-DRB1*14 correlating with a less severe disease course (Handel, Handunnetthi et al. 2010).

Other non-HLA gene variants are being identified with biological functions critical in immune responses or other biological functions relative to MS disease pathology. Differential allele frequencies within genes of interleukin-1 (IL-1), IL-2, IL-4, intercellular adhesion molecule (ICAM), interferon gamma (IFN-γ), and transforming growth factor beta (TGF-β) have been identified in MS cohorts relative to controls (Kalman and Lublin 1999; ANZgene 2009). Moreover, MS disease associated allele variants exist for the cytokine receptors IL2RA (CD25), a receptor involved in apoptosis and immune responses; and IL7R (CD127), crucial in cell survival and immune responses (Hafler, Compston et al. 2007; Oksenberg, Baranzini et al. 2008).

Disease-associated gene alleles that confer risk in MS are also observed in other diseases with autoimmune components, such as rheumatoid arthritis (RA) and type-1
diabetes (T1D). The single nucleotide polymorphism (SNP) rs1678542/KIF5A, known to be a risk allele in RA, was more frequently genotyped in a cohort of 2864 MS cases than in 2930 controls; likewise, rs763361/CD226 and rs3184504/SH2B3 SNPs confer risk for both T1D and MS (Alcina, Vandenbroeck et al. 2010). Apolipoprotein E (APOE), responsible for receptor interactions essential for normal triglyceride-rich lipoprotein metabolism, is implicated in the prevention of neurotoxicity and repair processes in several neurological disorders (Martino, Adorini et al. 2002; Kantarci and Wingerchuk 2006). Of relevance to MS, APOE suppresses T-cell proliferation, reduces macrophage activation, and reduces CD1 lipid antigen presentation to natural killer T cells and may indeed be immunomodulatory (Zhang, Wu et al. 2010). APOE allele variants e3 and e4 have been associated with neuronal loss as measured by magnetic resonance spectroscopy, whereas e2 allele is associated with lesser disease severity in patients with familial MS (Kantarci and Wingerchuk 2006). Advanced sequencing and genotyping investigations in parallel with investigations of gene and gene product function are ongoing to further elucidate associations between genetic polymorphisms in the genome of MS patients and the pathobiology of MS.

MS cannot be explained by population genetics alone. An environmental contribution to MS disease etiology is implied by variations in disease incidence and prevalence according to geographic location. Indeed, a person’s risk of developing MS is subject to change when migrating from low-to-high or high-to-low prevalence areas. Interestingly, some isolated regions have reported epidemics of MS, for example on the Faroe Islands and Iceland. Above all, the most irrefutable evidence suggesting MS is not singularly a genetic disease and contains an environmental component is incomplete concordance in monozygotic twins (Kantarci and Wingerchuk 2006).
Environmental impact on risk for MS is largely divided into two categories, non-infectious (i.e. sunlight exposure/vitamin D, smoking, and pregnancy) or infectious (attributed to one or more infectious agents).

**1.6 Non-infectious environmental factors**

Migration studies of the geographic distribution of MS reveal the risk of acquiring MS in migrants is between that of their birthplace and final destination; closer to the latter when migration occurs earlier in life (Ascherio and Munger 2007). Beyond cultural genetic predispositions contributing to geographical variations, migration studies ascribe prevalence of MS to regional environmental risk factors of which the strongest correlate is duration and intensity of sunlight (Giovannoni and Ebers 2007). Across regions with temperate climate, MS incidence and prevalence increased with latitude, both north and south of the equator (Ascherio and Munger 2007) and have implicated environmental influences in disease susceptibility. In this regard, sunlight and vitamin D have been associated with a reduced risk of MS. Sunlight exposure is a major source of vitamin D production, and a major problem of sun-derived vitamin D supply exists beyond the 40th parallel north or south. The increase in serum concentration of 25(OH)D (the active vitamin D hormone) after one whole body erythemal (slight pinkness of the skin) exposure is similar to that of taking a single dose of 10,000 to 25,000IU vitamin D supplements (Holick 2005). Early ecological studies of veterans found annual hours of sunshine and the average December daily solar radiation at place of birth were inversely correlated with MS (Norman, Kurtzke et al. 1983). Further, altitude is a direct marker of sunlight intensity where higher altitudes experience increased sunlight exposure. In Switzerland MS prevalence and altitude are inversely correlated (Ascherio and Munger 2007). The effect of vitamin D on the immune response is thought to be its
main protective feature against MS. Vitamin D enhances innate immune response and regulates multifaceted aspects of adaptive immunity (Pierrot-Deseilligny and Souberbielle 2010). Macrophages, T-cells and B-cells all express vitamin D receptors, with which receptor mediated anti-inflammatory effects are achieved in the presence of vitamin D. Specifically, vitamin D treatment of CD4+ T cells in vitro reduces proliferation and promotes regulatory T cell phenotype. These experimental findings strongly implicate an immunomodulatory role for vitamin D in MS.

There is growing evidence to suggest that smoking increases the risk of developing MS. Smoking before disease onset was associated with a 1.8 fold increase in risk of MS (Kantarci and Wingerchuk 2006). Smoking may also worsen MS symptoms immediately in the immediate time after smoking. More recently, smoking habits were related with transition to a secondary progressive phase of MS compared with non-smoker controls (Ascherio and Munger 2007). The putative role of smoking in MS pathogenesis has been proposed in several competing hypotheses, although it is likely the detrimental effects of smoking are experienced in combination. One hypothesis is based on the aberrant effects of cigarette components (i.e. nicotine and cigarette glycoprotein) on the immune system (Hernan, Jick et al. 2005). Others propose that vascular effects, increased nitric oxide (NO) free radical production, increased respiratory infections, and neurotoxic effects of cyanides and other components of cigarette smoke contribute to disease (Ascherio and Munger 2007; Healy, Ali et al. 2009).

Reproduction also influences the risk for MS. A two thirds majority of MS patients are women and MS in women statistically coincides with child-bearing ages (Devonshire, Duquette et al. 2003). Most available information on hormones and
reproductive risk factors in MS patients indicate partial remission during pregnancy followed by severe relapses post partum (Dwosh, Guimond et al. 2003; Alonso and Clark 2009). Estrogen appears to be protective by mediating a shift from the more proinflammatory CD4+ T helper profile (Th-1, producing IFN-γ, IL-2, TNF-α) to a more immunosuppressive (Th-2, producing IL-4, IL-5, and IL-13) class of neuroimmunity. Elevated estrogen and progesterone levels may explain the decreased incidence of relapses during pregnancy (Ascherio and Munger 2007). Placenta-derived hormones released during pregnancy likely ascribe a number of neuroprotective effects in MS patients; such as, promotion of suppressive regulatory T lymphocytes expansion and suppression of damaging natural killer (NK) proliferation. Clinical improvements during pregnancy are followed by temporary exacerbation of MS symptoms post-partum, once the hormones previously providing protection subside (Nicot 2009).

1.7 Infectious environmental factors in MS

Interestingly, at a London lecture in 1868, the French neurologist Jean-Martin Charcot stated, “...we can attribute disseminated sclerosis to acute infection incurred in the past” (Haahr and Hollsberg 2006). In 1963, Poskanzer and colleagues noted similarities in the epidemiology of poliomyelitis and MS, proposing that the neurological manifestation of MS, like poliomyelitis, could result from infectious origins (Poskanzer, Schapira et al. 1963). According to the ‘hygiene hypothesis’, risk for MS should increase with age of infection, as late age infection is more common in individuals with early hygienic practices and interferes with robust development of adaptive immunity (Giovannoni and Ebers 2007). Indeed, in principle this was supported by studies indicating increased MS incidence with increased sanitation in Israel and with an increase in socioeconomic status in America (Leibowitz, Antonovsky et al. 1966).
Recent investigations found infection with intestinal helminths benefit individuals with MS, purportedly by supporting Th-2 and regulatory T cell rather than Th-1 cellular immunity. Fleming and Cook (2006) used global prevalence of the parasite *Trichuris trichiura* to confirm MS prevalence was lower in regions with adequate threshold exposure to *Trichuris trichiura* (Fleming and Cook 2006). Leading candidates for microbial infection that increase risk for MS are Epstein–Barr virus (EBV), human herpes virus (HHV) type 6, multiple sclerosis-associated human endogenous retrovirus (HERV) and Chlamydia pneumonia, although, most attention is currently focused on EBV (Giovannoni and Ebers 2007).

EBV is a double-stranded DNA-containing virus with a tropism for resting B cells. Within B cells, EBV can either establish a latent infection, expressing only a limited set of genes, or lytic cycle, in which new EBV virion particles are made. Primary EBV infection is commonly asymptomatic early in life, but can cause mononucleosis at puberty and in adults (Haahr and Hollsberg 2006). The epidemiological trends of mononucleosis and MS are similar, and a hypothesis exists relating late EBV exposure to increased risk for MS (Ascherio and Munger 2007). The group of Francesca Aloisi, has recently described the abundant presence of EBV-containing B cells in tertiary lymphoid follicle aggregates in the meninges, but also in active lesions of MS patients. BLFR-1, an EBV-encoded protein marker of lytic viral replication, was present in B cells of the meninges which indicates active expansion of the EBV virus in the secondary progressive phase of MS (Meinl, Krumbholz et al. 2008). Furthermore, EBV could contribute to MS through molecular mimicry with myelin antigens. T-cells targeting EBV nuclear antigen 1 (EBNA-1) can also cross react with myelin antigens, which elicits co-expression of IFN-γ and IL-2, proinflammatory cytokines that promote expansion of Th-
1 lineage of CD4+ T cells (Lunemann, Huppke et al. 2008; Lunemann, Jelcic et al. 2008; Lunemann and Munz 2008).

1.8 The immune privileged CNS

Several CNS characteristics contributed to its consideration as an ‘immune privileged’ state. Foremost, the BBB formed by cerebral endothelia that line cerebrovasculature between blood and brain interstitial fluid (ISF) provides a regulatory barrier that limits exchange of hydrophilic humoral molecules, peripheral immune infiltration, and actively extrudes circulating drugs or other neurotoxic factors which otherwise elicit immune reactions in the CNS. Low expression of MHC class II in the CNS reduces the potential for T cell mediated immunity; yet, even if immune reactions initiate, glial cells including astrocytes and microglia anergize (inactivate) infiltrating lymphocytes and condition the local environment with soluble factors that inhibit peripheral immune responses (Schwartz, Moalem et al. 1999; Hohlfeld, Kerschensteiner et al. 2000; Hohlfeld, Kerschensteiner et al. 2007). Resident microglia comprise approximately 12% of the brain’s total mass and have immune-like phagocytotic and antigen presenting functions that compensate for an apparent lack of peripheral immune surveillance in the CNS (Schwartz, Moalem et al. 1999). BBB endothelia mature apposed to astrocytic end feet. Astrocytes of the CNS support neural ion and fluid homeostasis and support astroglial-endothelial induction required to establish and maintain the BBB. Astrocytes along with other BBB associated cells such as pericytes secrete a range of biochemical agents including transforming growth factor beta (TGF-β), a known morphogen that suppresses proliferative growth and induces endothelial differentiation and basic fibroblast growth factor (bFGF), a morphogen and a mitogen which promotes both growth and BBB properties in cerebral endothelial cells.
Other factors at the neurovascular niche with similar BBB inducing properties include glial derived neurotrophic factor (GDNF) and angiopoetin (ANG1) (Abbott, Ronnback et al. 2006). Reciprocally, one study showed convincingly that endothelial-derived leukaemia inhibitory factor (LIF) induced astrocyte differentiation in vitro (Mi, Haeberle et al. 2001). Inflammatory induced activation of astrocytes also enables the hyperproliferation of a protective perivascular astrogliotic mesh that restricts the robust indiscriminant infiltration of leukocytes following CNS injury (R.R. Voskuhl and Sofroniew 2009).

1.9 Immunopathogenesis of MS

Evidence from research using an animal model of MS, EAE; histopathology of MS tissue; and assessment of clinical responses to disease modifying therapies suggest MS is mediated immunologically (McFarland and Martin 2007; Dhib-Jalbut 2010). In a typical MS autoimmunity paradigm, autoreactive myelin specific Th-1 cells, activated by antigen presenting cells (APCs), produce proinflammatory cytokines and MMPs that disrupt the BBB and allow mass recruitment of leukocytes (T cells, B cells, and monocytes) into the CNS (Dhib-Jalbut 2007; Dhib-Jalbut 2010). The associated disruption of the BBB observed in patients can last approximately a month according to MRI imaging (McFarland and Martin 2007).

Circulating peripheral blood mononuclear cells (PBMCs) and reactive astrocytes produce cytokines, such as IL-1, IL-12 TNF-α and IFN-γ that upregulate the expression and surface presentation of adhesion molecules and their ligands that facilitate inflammatory CNS trafficking of leukocytes. Two lymphocyte surface antigens—very late antigen 4 (VLA-4) and lymphocyte function-associated antigen 1 (LFA-1)—bind
cognate cerebral endothelia adhesion molecules—vascular cell adhesion molecule 1 (VCAM-1) and intracellular adhesion molecule 1 (ICAM-1)—to facilitate adhesion and extravasation of leukocytes into the CNS (Dhib-Jalbut 2007). In parallel, MMPs -1, -2, -3 and -9 capable of digesting basement membrane components and vascular interstitial elements, are upregulated by leukocytes under inflammatory conditions and play a key roles, in the proteolytic cleavage of BBB tight junctions promoting recruitment of leukocytes into the CNS past the BBB (Hummel, Kallmann et al. 2001).

TNF-α, a classical proinflammatory cytokine, upregulates VCAM-1 and ICAM-1 on human cerebral endothelial cells and adhesion counter ligands on immune cells initiating immune responses in the CNS. In addition, TNF-α induced MMP secretion cleaves/releases VCAM-1 and ICAM-1 from the endothelial cell surface into a soluble state. Soluble VCAM and ICAM are then free to neutralize adhesion proteins on PBMCs blocking further entry of immune cells. An increase in soluble adhesion molecule concentration in the presence of TNF-α mediates immunomodulatory effects and is further enhanced by IFN-γ. Taken together increased soluble VCAM-1 and ICAM-1 detected in MS patient indicates a feedback inhibition of leukocyte adhesion and migration into the CNS and is protective against excess leukocyte infiltration observed in MS (Kallmann, Hummel et al. 2000; Hummel, Kallmann et al. 2001; Kallmann, Wagner et al. 2002).

Excess proinflammatory cytokines influence T cell polarization towards an inflammatory Th-1 phenotype. Upon exposure to IL-12, naive T cells (Th0) polarize to an inflammatory Th-1 phenotype and begin producing TNF-α and IFN-γ; if exposed to IL-4, Th0 cells polarize towards a regulatory Th-2 phenotype (Kasper and Shoemaker et al. 2010). Cytokine levels of TNF-α and IFN-γ are significantly higher in MS patients
supporting Th-1 mediated pathogenesis (Kahl, Kruse et al. 2002; Kahl, Kruse et al. 2002).

Th-17 cells, a subset of T helper cells discovered in 2003, are responsible for initiating and sustaining T cell-mediated tissue damage in a range of autoimmune diseases (Steinman 2007). Th0 cells are polarized towards Th-17 lineage by the presence of TGF-β, IL-1, IL-2, IL-6, IL-21 or IL-23 in multiple combinations (McFarland and Martin 2007). Knockout mice lacking components of the IL-23/Th-17 axis were resistant to EAE. Moreover, Th-17 cells are more abundant in RRMS patients during relapse and in acute clinically isolated syndrome patients who express acute symptoms of MS. IL-17 may carry higher pathogenic potential as they are more proliferative, more capable of migrating across the BBB, and more difficult to suppress than Th-1 subsets of T cells (Brucklacher-Waldert, Steinbach et al. 2009).

In EAE and MS, pro-retention and prosurvival factors propagate chronic inflammation on a background of BBB disruption and continual recruitment of lymphocytes into the CNS (Martino, Adorini et al. 2002). Prosurvival inflammatory cytokines (eg. TNF-α and IL-1β) and proretentive chemokines (eg. macrophages inflammatory protein 3a, secondary lymphoid tissue chemokine, and B-lymphocyte chemoattractant) have been identified in active MS plaques. The net effect of prosurvival and proretention proteins is an accumulation of blood-borne mononuclear cell, T cells, and B cells in the CNS (Martino, Adorini et al. 2002). In most MS patients, lymphocytes and monocytes persist in perivascular cuffs. B cells form ectopic tertiary lymphoid aggregates in the meningeal spaces that contain local immune APCs, astrocytes and migroglia (Hohlfeld, Meinl et al. 2008; Meinl, Krumbholz et al. 2008). These follicle-like structures perpetuate a local inflammatory response associated with
cortical pathologies in the forms of demyelination and neuronal loss (Meinl, Krumbholz et al. 2008).

1.10 MS disease course and diagnostic criteria

The evolution of MS in individuals follows heterogeneous disease courses varying in severity of symptoms and rates of accumulating disability. MS disease course is characterized by two clinical phenomena, relapse of acute neurological symptoms ending in complete or partial remission, and progression in which symptoms steadily worsen without resolution over ≥6 months (Confavreux and Vukusic 2006). The majority of patients (~85%) experience a relapsing-remitting disease course of MS (RRMS) identified by episodic alternations of neurologic disability and recovery. Within 10 years, more than 50% and by 25 years 90% of RRMS patients experience a conversion to a secondary progressive phase of MS (SPMS). After onset, an estimated 10% of patients encounter a steady decline in neurologic function without functional recovery, characterizing the primary progressive disease course of MS (PPMS). The fourth clinical disease course occurs in 5% of MS patients and is termed progressive relapsing MS (PRMS) because steady progressive accumulation of disability is determined by acute relapses with and without recovery (Peterson and Trapp 2005). Therefore, PRMS and PPMS share a similar trend toward a progressive accumulation of neurological disability from onset, but are distinct in that sporadic relapses and partial recovery punctuate PRMS but not PPMS disease course (Confavreux and Vukusic 2006).

The McDonald MS diagnostic criteria, revised in 2005, presents a scheme with which neurologists diagnose MS with high sensitivity and specificity. The criteria to confirm an MS diagnoses formally incorporates findings from magnetic resonance
imaging (MRI) scans, neurological examinations, and a number of paraclinical laboratory measures (Polman, Reingold et al. 2005). MS lesions visualized by MRI appear as ‘black holes’ in T1-weighted images (T1 MRI lesion) roughly corresponding to axonal loss or as hyperintensities in T2-weighted images (T2 MRI lesion) sensitive for the detection of lesions (Arnold and Matthews 2002; He, Han et al. 2010). Revised diagnostic criteria categorize individuals into MS, non-MS, or possible MS. The core concept in MS diagnosis relies on determining if lesions are disseminated in time and space by MRI (Compston and Coles 2002). Dissemination of space can be supported by MRI imaging and requires any three features of the following: 1) one gadolinium (Gd, a paramagnetic tracer typically excluded by the CNS vasculature and indicating lesion is of recent origin) enhancing lesion or nine T2 MRI lesions (hyperintense bright spots on an MRI scans indicative of demyelination and/or oedema); 2) ≥1 infratentorial lesions; 3) ≥1 juxtacortical lesion; or 4) ≥3 periventricular lesions if visual evoked potential (VEP) or cerebrospinal fluid (CSF) for oligoclonal bands are positive. Dissemination in time by MRI can be supported by the detection of one Gd positive lesion 3 or more months after disease onset; or Gd positive or new T2 lesion on a second scan repeated 3 months following onset. Patients exhibiting appropriate clinical presentation of MS, who do not meet the diagnostic criteria, can be classified as having possible MS (Nielsen, Korteweg et al. 2005; Polman, Reingold et al. 2005).

Gd has strong paramagnetic properties and is routinely used in diagnostic MRI imaging. Both size and intensity of the Gd enhancement provide information on intravascular concentrations and BBB permeability, which relatively correspond to increased vascularisation and permeability of microvasculature in the CNS. Increased permeability by opening of tight junctions and increased density of blood vessels are
important pathological features common in MS that would incite an increase in Gd enhancing signal (Martino, Adorini et al. 2002; Holley, Newcombe et al. 2010).

Oligoclonal bands (OCB) in the cerebral spinal fluid (CSF) of MS patients have been recognized for decades as an important clinical feature in MS diagnosis. Representing immunoglobulins produced by B cells residing in the CNS and distinguishable from those found in an individual’s serum, OCBs are prominent in the CSF of approximately 95% of MS patients (Harrer, von Budingen et al. 2009). They persist in CSF of MS patients for years but vary in electrophoretic pattern over time. The fact that OCB persist suggest that the CNS provides pro-retention factors for long-term survival of antibody producing plasma B cells populations. Longitudinal changes in banding pattern observed in MS may illustrate immunopathological shifts, alternative clonal expansion, and hypermutation in reactive B cell populations of the inflamed CNS (Martino, Adorini et al. 2002; Meinl, Krumbholz et al. 2008).

1.11 MS lesions and associated neurological dysfunction

Lesions in the brain and spinal cord manifest clinicopathologies. For example, lesions of the cerebrum present symptoms of cognitive impairment in the form of impaired attention, reasoning, and executive function. Painful unilateral loss of vision often suggests lesions in the optic nerve, while clumsiness, poor balance, limb incoordination and gait ataxia likely indicate lesions in the cerebellum or cerebellar pathways that mediate motor control (Compston and Coles 2002). Spasticity, palsy (weakness), paresis/paralysis, erectile impotence, and incontinence commonly present in patients with spinal cord lesions (Korteweg, Barkhof et al. 2005).
1.12 Neuropathology of MS lesions

In the past decade, new developments in immunohistopathology and morphological analysis of brain and spinal cord tissue have vastly refined topographical profiles of MS lesions. MS lesions are either chronic or active. Further, four distinct patterns of demyelination have been identified.

1.12.1 Chronic MS lesions

Demyelinating foci, termed plaques, in chronic MS cases often display features typical of chronic lesions. Chronic lesions appear throughout the CNS and vary in size and number in individual cases. Macroscopic plaques, visible after Luxol Fast Blue (LFB)-periodic acid Schiff (PAS) (LFB-PAS) staining of post-mortem MS brain, indicate loss of myelin and predominantly encircle the angles of the lateral ventricle in the periventricular white matter (Lucchinetti, Parisi et al. 2005). The optic nerve and retinal nerve fiber layer, deep white matter, brainstem, and spinal cord are also lesion prone. Chronic active plaques are hypocellular with no evidence of active demyelination. Fibrillary astrogliotic scars form and axonal networks are partially preserved. Few oligodendrocytes are visible and inflammation is localized to perivascular regions (Lucchinetti, Bruck et al. 2004; Lucchinetti 2005; Lucchinetti, Parisi et al. 2005; Peterson and Trapp 2005). Remyelination is rare in chronic lesions, typically localized to the peripheral border of plaques. Even so, remyelinated regions become new targets for demyelinating attacks in MS brain. Premyelinating oligodendrocyte progenitor cells intersperse in chronic lesions. Despite a lack of axonal receptivity to remyelinating signals and a paucity of precursor oligodendrocyte maturation, there appears to be no shortage of myelinating progenitor cells in demyelinated MS lesions (Chang, Tourtellotte et al. 2002).
1.12.2 Active MS lesions

Lesions of high inflammatory activity are often abundant in lipid-laden macrophages engorged on myelin and cellular debris; hypertrophic reactive astrocytes; and variable profiles of perivascular inflammatory infiltrates. Early affected regions show demyelination with preserved axonal integrity, although most severely affected lesions demonstrate axonal damage as indicated by tortuous-bulbous ended transected axons (Barnett and Prineas 2004; Dutta and Trapp 2007). Staging of lesions is possible by profiling myelin composition in macrophages. For example, myelin oligodendrocytes glycoprotein (MOG) or myelin-associated glycoprotein (MAG) is rapidly degraded in 1-2 days; in contrast proteolipid protein (PLP) lasts 6-10 days; and sudanophilic and periodic acid–Schiff (PAS)–positive granular lipids persist in active lesions for several months (Lucchinetti, Bruck et al. 2004; Lucchinetti 2005; Lucchinetti, Parisi et al. 2005).

1.13 Heterogeneous patterns of MS plaque demyelination

Patterns of demyelination are segregated based on variable plaque structural and cellular architecture, oligodendrocyte damage and progenitor reconstitution, immunoglobulin deposition and complement activation, and by the extent of myelin protein loss. All patterns display in common, however, T cell-mediated inflammation and microglial/macrophage activation. In 220 MS lesions, pattern incidence was 19% pattern I, 53% pattern II, 26% pattern III, and 2% pattern IV (Lucchinetti, Bruck et al. 2004).

Pattern I MS lesions resembles macrophage induced toxic damage seen in mouse models of autoimmune encephalomyelitis, in which products of activated macrophages ablate the myelin sheath (Taupin, Renno et al. 1997). Macrophages also
actively phagocytose myelin and myelin debris (Lucchinetti, Parisi et al. 2005). Myelin and oligodendrocytes have a special vulnerability to various immune and potentially neurotoxic mediators produced by activated macrophages including TNF-α, IFN-γ, matrix metalloproteases (MMPs), nitric oxide (NO) or reactive oxygen species (ROS) (Kornek and Lassmann 2003). Typically pattern I and II MS lesions are perivascular surrounding small veins and venules (Lucchinetti, Bruck et al. 2004; Lucchinetti, Parisi et al. 2005). This said, emerging evidence suggests proinflammatory cytokines like TNF-α and IFN-γ may afford some degree of neuroprotection via induction of neurotrophic factors among other regulatory roles related to the resolution of inflammation (Hohlfeld, Kerschensteiner et al. 2000). Antibodies against myelin proteins like MOG and MBP are found in a subset of pattern II MS lesions. Murine models of MS secondary to immunization with and autoreactivity to MOG show congruent evidence of demyelination by effect of encephalitogenic T cells and demyelinating MOG antibodies surrounding blood vessels (Kornek and Lassmann 2003). Inflammatory infiltrates in pattern III MS lesions do not surround blood vessels and are variably comprised of macrophages, activated microglia (resident antigen presenting cells of the CNS), and T cells. This pattern is associated with oligodendrocyte apoptosis, a marked reduction in oligodendrocytes, and minimal remyelination. Immune inflammation results in early selective loss of myelin-associated glycoprotein (MAG) indicative of distal dying-back of glia processes termed oligodendrogliopathy (Lassmann, Bruck et al. 2001; Lucchinetti, Bruck et al. 2004). Increased expression of heatshock proteins (HSP70) and hypoxia inducible factor (HIF) in pattern III MS lesions may indicate that immune mediated responses are secondary to ischemia, anoxia, hypoxic stress, or excess excitatory stimulation (Kornek and Lassmann 2003).

1.14 Neurodegeneration in MS

Neurodegeneration is the major cause of irreversible neurological disability in MS patients. Histology and proton magnetic resonance spectroscopy (MRS) provide methods with which to detect neuronal damage. Axonal transaction and loss in post-mortem MS brains (Trapp, Peterson et al. 1998; Trapp and Nave 2008); detection of brain atrophy in MS patients (Miller, Barkhof et al. 2002); and reduced MRS detection of the neuronal-specific marker, N-acetyl aspartic acid (NAA) (Fu, Matthews et al. 1998) are evidence of axonal damage in MS. Even patients at early disease stage (<3 years) have a reduced NAA to creatine (Cr, a baseline metabolite with which to compare NAA levels) ratio related to axonal loss, despite having low T2-weighted MRI lesion load. The NAA: Cr metabolic information provided by MRS analysis has proven a more sensitive predictor of EDSS score in RRMS patients than T2-weighted lesion volumes (Lisak 2007).

Axonal injury disrupts the transport of proteins down the axon. Continual anterotrograde transport leads to the accumulation of organelles, proteins, and other molecules in terminal ovoids bulbs, a defining feature of Wallerian degeneration. Ovoid swellings associated with transected axons have been identified in active lesions and at
the edges of chronic active MS lesions (Trapp, Peterson et al. 1998; Peterson and Trapp 2005; Trapp and Nave 2008).

One mechanism of direct axonal damage in MS is specific immune insult. Immune mediated axonal injury is suggested by the positive correlation between inflammatory activity and axonal damage (Dutta and Trapp 2007). Transected axons are often surrounded by macrophages and activated microglia in MS lesions. Neurofilament inclusions inside proximal macrophages and activated microglia further suggest engulfment of axons by these cells (Peterson and Trapp 2005). Even so, whether these immune cells engage and attack axons initially or remove debris following primary axonal degeneration remains equivocal.

Axonal damage could also be a non-specific adverse reaction to nearby inflammation. Proteolytic enzymes such as MMP, as well as cytokines or ROS produced by inflammatory cells and distressed cells of the nervous parenchyma, carry the potential to damage axons (Trapp and Nave 2008). Studies suggest chronic inflammation leads to aberrant glutamate homeostasis and excess NO production, both of which cause damage to axons (Lassmann 1999; Lassmann, Bruck et al. 2007). Inflammation may also instigate chronic immune mediated demyelination of axons compromising electrical conductance and structural integrity of axons, as well as supply of trophic factors between axons and glia (Harrer, von Budingen et al. 2009; Trapp and Stys 2009).

Oligodendrocyte-derived myelin sheaths around axons enable rapid nodal (saltatory) propagation of electrochemical impulses. Demyelination not only disrupts electrical conductance but also deprives trophic support at the axoglial interface (Leob
which is otherwise provided by oligodendrocytes. Exposure of neurons to oligodendrocyte conditioned media (OCM) increases the length of axons on each neuron, trophic effects mediated by glial-derived growth factor (GDGF) found to be an important prosurvival factor of myelinated axons (Wilkins, Majed et al. 2003). Several other glial-derived neurotrophic factors have been identified with similar important roles in protecting neurons including nerve-growth factor (NGF), insulin-like growth factor (IGF), and ciliary neurotrophic factor (CNTF) (Leob 2007).

Ion imbalance and mitochondrial aspects of axon damage may be consequential compensatory changes to restore impulse conduction after demyelination in MS. Redistribution of sodium (Na) channels along the denuded axon (axolemma) increase Na influx. Restoration of ionic gradients is necessary for neurotransmission accomplished through active transport of Na/K ATPases. Increased Na influx leads to greater consumption of ATP by Na/K ATPases causing an energy imbalance leading to axonal vulnerability (Dutta and Trapp 2007). After CNS injury or in any energy-limited environment, vulnerable neurons unable to meet their high energy demand are subject to degeneration and death (Peterson and Trapp 2005; Trapp and Stys 2009). In MS, mitochondrial energy production might be compromised by genetic defects in the electron transport chain, as well as by increased ambient nitric oxide (NO) and peroxynitrite levels. Increased glutamate concentrations might also inappropriately activate ion influx, further exacerbating damage due to excess neuronal energy demands (Lassmann 2003; Lassmann, Bruck et al. 2007; McFarland and Martin 2007; Trapp and Stys 2009).
1.15 Disease modifying treatment for MS

Several disease modifying drugs (DMDs) are available for MS treatment, others are in development, each assessed for its efficacy, safety profile, and varied mechanisms of action (Dhib-Jalbut 2010). Current treatment paradigms for MS target CNS inflammation and in limiting these processes reduce the rate and duration of clinical relapses, albeit only patients with ongoing relapse or MRI disease activity typically benefit from DMDs such as recombinant interferon-beta (IFN-β) (Li, Zhao et al. 2001) or glatiramer acetate (Johnson, Brooks et al. 2000). The administration of anti-inflammatory steroids for the management of acute attacks, or more recently immunomodulatory recombinant IFN-β or glatiramer acetate for MS patients with a relapsing course reduces relapse duration and frequency and even, in some cases, slows disease progression. However, patients with a progressive disease course accumulate progressive neurological deficits in spite of anti-inflammatory/immunomodulatory treatments, suggesting MS is not caused by inflammation alone (Rieckmann and Smith 2001). Where recent studies have established that several inflammatory components are potentially beneficial, it remains to be determined whether non-specific inhibition of inflammation may actually impair the potential for such factors to promote repair after CNS injury associated with MS (Rieckmann and Maurer 2002).

1.16 Regenerative neuroinflammatory components

Despite contributing to early MS pathology, inflammation in the CNS is not exclusively detrimental. Some inflammatory components beneficially promote repair and growth activity after CNS injury (Rieckmann and Smith 2001). The molecular rationale...
for this paradigm is that the immune system and CNS engage in cross-talk. A range of cytokines that affect the immune system are CNS derived; reciprocally, growth factors for the CNS can be secreted by immune cells (Hohlfeld, Kerschensteiner et al. 2000; Hohlfeld, Kerschensteiner et al. 2007).

Growing evidence suggest certain inflammatory components protect against permanent neuronal loss and glial dysfunction in MS. Furlan et al. (2001) found that morbidity rates in mice with EAE are four fold higher when animals are treated with monoclonal antibodies against IFN-γ. Furlan and colleagues further showed recombinant IFN-γ administered by intrathecal route before the onset of EAE protected mice by improved clearance of encephalitogenic T cells via an apoptotic pathway dependent on upregulation of TNF receptor 1 (Furlan, Brambilla et al. 2001). Similarly, preliminary clinical trials using neutralizing monoclonal anti-TNF antibody therapy to treat MS resulted in monophasic CNS demyelination and worsening of MS disease activity (Titelbaum, Degenhardt et al. 2005).

Immune cell interactions with the CNS can ascribe neuroprotective effects in lieu of neuroinflammation. CD4+ T cells can induce microglial production of prostaglandin E2, a molecule that inhibits IL-12, in support of an anti-inflammatory Th-2 shift in neuroimmune responses (Martino, Adorini et al. 2002). Macrophages enhance remyelination by removing myelin debris, which otherwise inhibit processes of repair and clean-up occurring in the reparative process. In experiments by Harrer et al. (2009) removal of myelin debris after antibody-mediated demyelination of organotypic CNS cultures induced spontaneous remyelination (Harrer, von Budingen et al. 2009). Macrophages also stimulate remyelination through TNF-α secretion, shown in vitro to
promote oligodendrocyte precursor proliferation via TNF receptor II signalling (Martino, Adorini et al. 2002).

Some evidence suggests inflammatory mediators in the CNS increase the expression and secretion of bioactive neurotrophic factors. Bayas et al. (2002) found that TNF-\(\alpha\) treatment of human cerebral endothelial cells (HCEC) increased mRNA and protein levels of brain derived neurotrophic factor (BDNF), a protein with neuroprotective activity shown to rescue neurons following axon transaction. BDNF produced by HCEC supported motoneuronal survival and function (Bayas, Hummel et al. 2002). Other studies show administered BDNF protein or viral BDNF gene transactivation rescue damaged neurons and induce neurite outgrowth and regeneration after CNS injury. Several neurotrophic factors reduce MHC class II on microglia reducing antigen presentation and immune activation (Leob 2007). Current aims for treating MS focus on immune suppression and reconstitution of glia and neuronal populations, both of which are promoted by trophic factors derived from inflammatory processes (Hohlfeld, Kerschensteiner et al. 2000).

1.17 The vasculature in MS

Blood vessels and MS lesions share an inherent connection. First recognized 130 years ago by Edward Rindfleish, MS lesions frequently circumscribe to abnormal central blood vessels on which lesions project longitudinally along post-capillary venules of the periventricular white matter (Kirk et al. 2004). Histological studies show perivascular finger-like projections of infiltrates associated with myelin pallor and axonal loss, termed ‘Dawson’s fingers’ (Lucchinetti et al. 2005). The extent of microvascular abnormalities and their precise spatiotemporal association with MS lesions in vivo was
elusive until recent remarkable advancements in ultra-high field seven tesla (7-T) MRI. By virtue of an increased signal to noise ratio, 7-T MRI images reveal microvascular abnormalities as primary evidence of lesion development in RRMS patients. Early lesions by 7-T MRI appear angiocentric, sometimes even with the BBB still intact (Ge, Zohrabian et al. 2008).

1.17.1 New vessel formation in MS

Angiogenesis, the process of forming new blood vessels from pre-existing ones, is a major axis for damage and repair in several physiological and pathological conditions ranging from embryogenesis, to wound healing, to tumour neovascularization (Kirk et al. 2004). Increased tissue vascularity restores homeostasis by providing oxygen, nutrients, trophic factors, and waste removal, but can also promote inflammatory damage by providing a larger surface area for leukocyte infiltration (Chen and Easton 2010). Angiogenesis involves endothelial cell proliferation and migration (Lee, Han et al. 2009), two endothelial functions routinely used in laboratories to assess angiogenesis (Chen and Easton 2010).

Neovascularization frequently demarks the edge of MS lesions and is indicative of focal acute inflammation (Shauna Kirka 2004). Blood vessels at the penumbra of MS lesions appear tortuous and irregularly oriented (Singh and Zamboni 2009). Vascular aberrations observed in MS tissue often correlates with increased MHC class II antigen presentation on perivascular microglia and macrophages, perivascular fibrin deposition, increased vessel permeability, and vessel wall hyalinization (Holley, Newcombe et al. 2010). Perivascular correlates of MS pathology are present in three of four MS lesion patterns (Lassmann, Bruck et al. 2001). Pattern three, not observed around veins or
venules, express HIF-1 and HSP70 which are essential for hypoxia induced angiogenesis as cells must be within 100-200A of a blood vessel to survive (Hafler 2004; Shauna Kirka 2004). In concordance, pattern III lesions may either degenerate or progress to a perivascular pattern.

A reduction in endoneural blood flow may be another determinant of hypoxia-induced stress in the CNS. The peptide endothelin-1 (ET-1) is one of the most potent vasoconstrictors and levels of ET-1 are higher-than-normal in cerebral spinal fluid (CSF) bathing the brain and spinal cord, and sera of MS blood samples. Chronic vasoconstriction exacerbates MS by the manner in which it restricts blood flow to nervous tissue with already high energy demands (Haufschild, Shaw et al. 2001).

Increased vessel wall thickness and capillary proliferation are frequently seen in autopsied MS lesions. Vascular endothelial growth factor (VEGF), a major regulator of angiogenesis which mediates various aspects of endothelial cell growth and responses to inflammation, is highly expressed in MS and EAE lesions and is found at higher-than-normal levels in MS sera (Seabrook, Littlewood-Evans et al. 2010). Along with a role in angiogenesis, VEGF is also an autocrine and paracrine factor which disrupts tight junctions between cerebral endothelia inducing BBB breakdown when produced by local astrocytes and pericytes activated by a developmental or inflammatory environment (Minagar and Alexander 2003; Argaw, Gurfein et al. 2009). VEGF levels in sera correlate with MRI activity and clinical disease activity in MS patients (Su et al 2005). VEGF-treated endothelial cells express higher-than-normal levels of MMPs, VCAM-1, and ICAMs, operative during angiogenesis in response to CNS inflammation such as that in MS (Hummel, Kallmann et al. 2001; Lee, Han et al. 2009).
Post-mortem MS brain had significantly higher blood vessel density than non-MS controls (Holley et al. 2010). Vessels positive for endoglin (CD105), a specific marker of proliferating endothelial cells, were prevalent in normal appearing white matter (NAWM), acute, subacute, and chronic lesions. CD105 positive microvessel staining was greatest in the NAWM implicating endothelial proliferation early in lesion development (Holley, Newcombe et al. 2010). Holley et al. (2010) hypothesized that ‘virtual hypoxia’ caused by increased energy demands of demyelinated axons coupled with inflammation instigates higher rates of CNS angiogenesis in MS patients. The consequence of CNS vascularity on MS disease pathogenesis and progression is a current hot topic of MS research.

1.18 Blood-neural barriers

The brain is one of the most sensitive organs in the body, whereby concentrations of ions such as Na\(^+\), K\(^+\), and Ca\(^{2+}\) must be maintained within very narrow ranges to preserve efficient neurological function (Hawkins and Davis 2005). The CNS is tightly sealed from dynamic hemodynamic fluctuations by blood-neural barriers, two of which are the BBB formed by cerebral endothelia between blood and neural parenchyma and the blood-CSF barrier (BCSFB) formed by choroid plexus epithelia between blood and CSF compartments.
1.18.1 BBB structure and function

Early studies by Paul Ehrlich (1885) demonstrate a barrier separating vascular and neural compartments under normal conditions. Ehrlich showed trypan blue injected into the vascular system did not penetrate brain tissue but did permeate peripheral tissue (Lee, Han et al. 2009). Tightly apposed cerebral endothelial cells (CMEC) form the restrictive interface separating vascular and neural compartments that is designated the BBB and line 99% of brain vasculature (Abbott, Ronnback et al. 2006). Cerebral microvasculature classically lack fenestrations maintaining a physically restrictive blood-neural interface. BBB endothelia also have reduced numbers of pinocytotic and endocytotic vesicles and express a variety of polarized transporters and receptors that regulate uptake of nutrients yet restrict access and actively extrude circulating drugs or other neurotoxic products (Minagar and Alexander 2003).

CMECs are joined together by tight junctions (TJ) that restrict paracellular permeability and form the anatomical basis of the BBB (Martino, Adorini et al. 2002; Persidsky, Ramirez et al. 2006). Low paracellular permeability and high transendothelial electrical resistance (TEER) of the BBB are attributed to interendothelial TJs of which claudins, occludins, and junctional adhesion molecule-A (JAM-A) are the major molecular contributors. TJs are transmembrane proteins with extracellular binding domains that interact homophilically and heterophilically to join cells together. TJs anchor the cytoskeleton for cytoarchitectural support via submembranous adaptor proteins, including zonula occludens (ZO)-1 and ZO-2, cingulin, AF-6, and 7H6 (Engelhardt and Sorokin 2009). ZO-1 was the first TJ associated protein identified and later was discovered to colocalize with adheren junctions (AJ) and TJ complexes as a cytoarchitectural scaffolding protein that anchor junctional molecules to the cytoskeleton.
(Minagar and Alexander 2003; Abbott, Ronnback et al. 2006). AJs facilitate interendothelial adhesions, enable contact inhibition of growth, and even regulate TJ expression (Persidsky, Ramirez et al. 2006; Gavard and Gutkind 2008). ZO-1 translocates into the nucleus functioning as a signal transduction molecule in proliferating endothelial cells in response to injury (Hawkins and Davis 2005).

Between CMECs and other brain cells is a basement membrane composed of extracellular matrix (ECM) molecules, such as collagen, fibronectin, laminin, proteoglycans, vitronectin, and tenascin that network to stabilize microvasculature of the brain (Persidsky, Ramirez et al. 2006; Engelhardt and Sorokin 2009). ECM degradation strongly correlates with increased BBB permeability (Hawkins and Davis 2005). Similarly, loss of agrin, a basement membrane protein spatiotemporally associated with endothelial barrierogenesis during development, results in paracellular BBB permeability (Wolburg and Lippoldt 2002). CMEC-ECM interactions are predominantly determined by integrins, heterodimeric transmembrane receptors composed of an alpha and beta subunit that binds ECM in a selective and specific manner providing angioarchitectural support for CNS microvessels. For example, fibronectin (FN) in the ECM is engaged by CMEC bound FN integrin receptor, α5β1 (Mardon and Grant 1994), and this cell-ECM interaction stabilize CNS microvasculature during angiogenesis in response to CNS injury (Hawkins and Davis 2005).

A number of factors promote a barrier phenotype or “barriergenesis” in CMECs. Conventionally, factors that induce barriergenesis increase expression and peripheral localization of TJs and tighten the BBB as measured by increased transendothelial electrical resistance (TEER) and reduced macromolecular transport or diffusion (Lee, Han et al. 2009). The wnt family of proteins have recently been highlighted as key
developmental factors in CNS angiogenesis and barriergenesis. The wnt/β-catenin axis regulates glucose transporter 1 (GLUT-1) and claudin-3 and -5 expression at the BBB (Lammert 2008; Daneman, Agalliu et al. 2009). Other proteins that support BBB development and maintenance are Src-suppressed C kinase substrate (SSeCKS), angiopoietin-1 (ANG-1), and TGF-β (Lee, Han et al. 2009). Neural mechanisms of BBB tightening are considered to be protective under conditions of stress such as hypoxia or traumatic injury by preventing vasogenic edema and excessive leukocyte infiltration following CNS injury. In brain endothelium, increased intracellular cAMP levels, histamine, and ATP resulting from increased neuronal energy demands under various stress conditions increases TEER and upregulates P-glycoprotein (Pgp) and glucose transporter activity as a form of protective neurobarrier coupling after CNS injury (Abbott, Ronnback et al. 2006). The defect in BBB structure and function appears to result from either a loss of barrier-inducing factors (i.e. TGF-β, GDNF) or excess of permeability inducing (i.e. VEGF, TNF-α) factors at the BBB.

Restrictive angioarchitecture at the BBB preserves the precise ionic homeostasis required for reliable neuronal signalling and limits transendothelial flux of immune infiltrates following CNS injury. Under pathological conditions, however, BBB dysfunction, associated with downregulation and disrupted localization of cerebral endothelial TJs, increases BBB permeability permitting leukocyte trafficking into the CNS (Stamatovic, Keep et al. 2008). BBB disruption and leukocyte CNS trafficking are among the earliest preclinical cerebrovascular events in MS pathogenesis (Minagar and Alexander 2003). BBB breakdown disrupts the precisely regulated CNS milieu and promotes inflammatory neuroimmune reactions that damage vulnerable myelin and neuronal components. BBB breakdown in neuroinflammatory CNS diseases, such as
MS, result because of the downregulation, proteolytically degradation, and internalization of TJs between cerebral endothelia that comprise the BBB (Minagar and Alexander 2003; Hawkins and Davis 2005; Stamatovic, Keep et al. 2008; Stamatovic, Keep et al. 2009). Markers of hypoxia-like damage and repair are present in pattern III MS lesions with evident distal oligodendrogliopathy and apoptosis (Lassmann, Bruck et al. 2007). Expression of HSP70 and HIF are elevated in pattern III MS lesions and are suggestive of ischemia, anoxia, hypoxic stress, or excess excitatory stimulation (Aboul-Enein, Rauschka et al. 2003; Lassmann 2003). Levels of Endothelin-1 (ET-1), a potent vasoconstrictor, is elevated in CSF and sera of MS patients compared to controls. ET-1 overexpression exacerbates MS by restricting endoneural flow to nervous tissue with already high metabolic demand (Timoshin, Sazonova et al. 2000). Sheer stress from blood flow is necessary for endothelial barrier tightness. hCMEC/D3 cultures, a model of the BBB comprised of immortalized cerebral endothelial cells (CMEC), establish a tighter monolayer with lower transendothelial permeability under flow conditions compared to static conditions (Cucullo, Couraud et al. 2008). In this regard, intraluminal flow contributes to BBB maintenance.

Histological studies of active MS lesions reveal fibrinous and proteinaceous regions around blood vessels associated with immune infiltrates, venulitis, with vessel wall damage and BBB breakdown (Soon, Altmann et al. 2007; Soon, Tozer et al. 2007). BBB abnormalities are not only observed in active lesions, they are also found in inflamed microvessels in NAWM (Martino, Adorini et al. 2002). The proinflammatory cytokines (TNF-α and IFN-γ abrogate tight and adheren junctions of CMECs and activate inducible nitric oxide synthase (iNOS) accounting in part for some of the BBB damage observed in MS (Minagar and Alexander 2003). On the other hand, BBB
disruption may not be singularly pathogenic, but could enhance passive and immune mediated repair and removal of nervous tissue debris after injury (Schwartz, Moalem et al. 1999; Rieckmann and Smith 2001; Rieckmann and Maurer 2002). Transiently opening the BBB in response to histamine released from nerve terminals permits the passage of neuro protective growth factors and antibodies into the brain from plasma or CNS sampling of plasma composition to elicit appropriate conditional neurophysiological and behavioural responses (Abbott, Ronnback et al. 2006). The extent to which BBB opening and accessibility to the CNS enables repair is to date unclear. The rationale for therapeutically targeting the BBB in MS treatment is that if BBB damage can be reduced, halted, or reversed this could help treat neurological conditions in which neuronal damage, the source of disability, is secondary to or exacerbated by BBB damage.

1.18.2 The BCSFB and CSF production

The BCSFB between blood and CSF compartments is primarily determined by choroid plexus epithelia sealed together by TJ molecules, analogous to how cerebral endothelia form the BBB (Engelhardt et al. 2001). Choroid plexus epithelia outline choroid plexuses, branched structures with multiple villi protruding into ventricular cavities. Choroid plexus villi contain a core of connective tissue and blood capillaries with a blood supply 10 times that of cerebral cortex blood supply. Unlike capillaries in the majority of the cerebral circulatory system possessing a BBB, choroid plexus capillaries are fenestrated, allowing passive movement of water and ions (Brown, Davies et al. 2004).
Choroid plexus epithelium produce CSF which bathes the brain ventricles, spinal cord canal, and subarachnoid compartments of the CNS (Brown, Davies et al. 2004; Engelhardt and Sorokin 2009). CSF is not ultrafiltrate of blood, but the result of precise active transport of ions and osmosis. The total volume of CSF is replenished four times daily (Brown, Davies et al. 2004). The composition of CSF and plasma are similar, except plasma has more protein than CSF. Secreted protein and other molecules circulate in CSF to multiple periventricular targets, including the caudate, hippocampus, specialized circumventricular organs, hypothalamus, pia-glia and arachoid membranes. Therefore, CSF transports soluble factors, including micronutrients, neurotrophic factors, hormones, neuropeptides, and growth hormones that likely afford tissue protective effects in MS or other CNS diseases (Johanson, Duncan et al. 2005). CSF drains at distal subarachnoid villi, facial lymphatics, or into venous blood of the superior sagittal sinus (Johanson, Duncan et al. 2005).

1.19 **Secreted protein acidic and rich in cysteine (SPARC)**

SPARC is a secreted matricellular protein that modulates cell-ECM interactions during development and in response to injury. SPARC modulates the configuration of the cytoskeleton and ECM, yet does not subserve a structural component of the ECM (Brekken and Sage 2001). Many cell types, including endothelia, fibroblast, macrophages express SPARC in tissue regions undergoing high rates of remodelling, repair, or proliferative turnover (Bradshaw and Sage 2001). SPARC acts both intracellularly, regulating the secretion of and responses to components of the ECM, and extracellularly, through growth factor and ECM interactions, all of which elicit variable alterations in cell phenotype (Bornstein and Sage 2002). These bioactivities
enable SPARC to regulate tissue repair by altering adhesion, migration, proliferation, and differentiation of cells (Bradshaw and Sage 2001).

SPARC is highly expressed in the developing nervous system during embryogenesis and postnatal development, but becomes restricted in the adult CNS to neurogenic subventricular zones, and specialized glia in regions undergoing high rates of remodelling and repair (Bradshaw and Sage 2001; Vincent, Lau et al. 2008). Mendis et al. (1994) reported that developing cerebral blood vessels express high SPARC mRNA levels whereas developmentally mature blood vessels had undetectable levels, consistent with a role for SPARC in CNS angiogenesis and a temporal expression coinciding with BBB establishment (Vincent, Lau et al. 2008). SPARC expression remains largely restricted to microglia and astrocytes in the healthy adult CNS (Vincent, Lau et al. 2008) yet alterations in response to CNS injury suggest SPARC may be operative in damage and repair processes in the CNS. Cortical laceration increased SPARC mRNA expression in vasculature proximal to the wound day 3 to 10 post injury (Mendis and Brown 1994; Mendis, Ivy et al. 1998). Nasal (lamina propria) olfactory ensheathing cells (LP-OECs) transplanted into spinal cord lesions promoted repair by limiting gliotic scar and cavity formation, stimulating axonal outgrowth, and directing angiogenesis (Ramer, Au et al. 2004). Subsequent proteomic screens of LP-OEC conditioned media identified SPARC as the key secreted protein supporting neural tissue repair after damage. In fact, SPARC promoted axonal outgrowth in vivo and in vitro by enhancing Schwann cell mediated neurite outgrowth (Au, Richter et al. 2007). Moreover, SPARC-null OECs transplanted into contused rat spinal cord reduced outgrowth of specific subsets of sensory and supraspinal axons and impaired immune
response to injury confirming SPARC’s role in propagating neurite outgrowth and immune responses after CNS injury (Au, Richter et al. 2007).

Targeted gene disruption of most matricellular proteins results in a grossly normal or mild phenotype exacerbated by injury (Bornstein and Sage 2002). SPARC-null mice express a mild phenotype of osteopenia, abnormal dermal collagen fibrils, cataractogenesis, adiposity, aberrant wound closure, and impaired immune response to injury (Bradshaw, Reed et al. 2001; Bradshaw and Sage 2001; Kelly, Allport et al. 2007). SPARC null mice are immune compromised in their attenuated response to lipopolysaccharide (LPS) derived from the cell walls of bacteria. SPARC augments the rate of naive T cell priming and accelerates dendritic cell migration in response to pathogens (Rempel, Hawley et al. 2007). SPARC is known to have multiple binding partners, some of which are relevant to immune responses. Kelly et al. (2007) discovered SPARC is a novel VCAM-1 ligand inducing intercellular gap formation in lieu of actin filament reorganization and morphological rounding, effects conducive for leukocyte trafficking. The study reports exogenous recombinant SPARC increased the paracellular permeability of microvascular endothelial monolayers in vitro. These finding were corroborated in vivo as immune recruitment to an inflamed peritoneum was impaired in SPARC null mice suggesting SPARC promotes endothelial permeability and leukocyte infiltration in response to inflammation (Kelly, Allport et al. 2007).

SPARC is associated with angiogenesis, but plays both pro-angiogenic as well as anti-angiogenic roles varying with the conditions and environment. At sites of development, hypoxia, wound healing, and tissue remodelling, microvascular endothelial cells participate in angiogenesis and express high levels of SPARC (Iruela-Arispe, Hasselaar et al. 1991; Bradshaw and Sage 2001; Brekken and Sage 2001).
Confocal immunofluorescence microscopy of developing chicken chorioallantoic membranes depicted specific SPARC expression localized in endothelial cells of growing blood vessels. Immunoreactivity for SPARC was notably higher in smaller newly formed blood vessels in comparison to larger developmentally mature vessels (Iruela-Arispe, Diglio et al. 1991; Iruela-Arispe, Lane et al. 1995). The authors further revealed plasmin proteolysis of SPARC yields a pro-angiogenic cleavage fragment localized at the tip of growing vessels. Plasmin cleaved SPARC fragments contain the Cu$^{2+}$-binding amino acid KGHK motif associated with angiogenic activity (Iruela-Arispe, Lane et al. 1995). Conversely, recombinant SPARC exogenously applied to microvascular endothelial cells decreased proliferation in vitro (Kato, Lewalle et al. 2001). Hence, the effect of SPARC on angiogenesis depends on the conditions under which SPARC is endogenously expressed by or exogenously applied to endothelial cells.

SPARC is known to bind multiple growth factors, such as TGF-β, VEGF, and PDGF-AB and their receptors with either anti-angiogenic or pro-angiogenic effects. In proliferative endothelia, SPARC gates VEGF signal transduction through pro-angiogenic VEGF receptor 2 (VEGFR2) by blocking anti-angiogenic VEGF receptor 1 (VEGFR1) (Nozaki, Sakurai et al. 2006; Uehara, Luo et al. 2010) to support angiogenesis. Nozaki et al. (2006) found that laser induced injury of the choroid depletes SPARC at injury sites permitting VEGF binding to VEGFR1 with a net anti-angiogenic effect. In fact, pre-treatment with a SPARC-specific neutralizing monoclonal antibody inhibits laser induced choroidal neovascularisation in mice consistent with a pro-angiogenic role for SPARC in VEGF signalling (Nozaki, Sakurai et al. 2006; Uehara, Luo et al. 2010). A similar paradigm applies to PDGF-AB in which SPARC binds to
PDGF and its receptor reducing proliferation and angiogenesis (Raines, Lane et al. 1992). In a reciprocal manner, SPARC expression depends on the same growth factors that SPARC can bind, including platelet derived growth factor (PDGF), insulin-like growth factor (IGF), fibroblast growth factor-2 (FGF-2), TGF-β, and VEGF which have all been shown to alter SPARC mRNA and protein expression in endothelial cells (Kupprion, Motamed et al. 1998; Brekken and Sage 2001; Kato, Lewalle et al. 2001). Altogether, SPARC is an integral regulator of growth factors relevant to repair processes. A baseline level of SPARC can be detected in plasma and sera of healthy individuals (range 0.1 to 0.8 μg/ml in plasma) and is increased during neoplastic and specific inflammatory conditions (1.5 to 10 μg/ml in plasma) (Malaval, Fournier et al. 1987; Malaval, Ffrench et al. 1990; Serebruany, Murugesan et al. 1999). Increased SPARC secretion has been associated with various types of diseases including carcinoma (Porte, Triboulet et al. 1998) and other tumours (Porter, Sage et al. 1995) such as gliomas (Yunker, Golembieski et al. 2008), and glomerulonephritis, an inflammatory renal disease (Pichler, Bassuk et al. 1996; Shankland, Hugo et al. 1996).

The main goals in developing new therapies for inflammatory demyelinating diseases such as MS focus on reducing damage while enhancing repair processes and identifying molecules relevant to these processes. Proteomics analysis of CSF identified four proteins unique to MS patients which were absent in CSF from healthy individuals. The four proteins were CRTAC-IB (cartilage acidic protein); tetranectin (a plasminogen-binding protein); autotaxin t (a phosphodiesterase); and SPARC-like 1 (a calcium binding cell signalling glycoprotein, hevin or SPARCL1) (Hammack et al. 2004). SPARCL1, a member of the SPARC family of matricellular proteins, shares 62% homology with human SPARC, and while their spatiotemporal expression after CNS
injury are currently under investigation, they appear to be distinct (Roskams unpublished data).

The role of SPARC in mediating inflammation and repair in inflammatory or demyelinating diseases such as MS is largely unknown, yet its proposed roles in modulating vascular repair and development along with its ability to influence immune infiltrates and BBB properties make it a molecule of particular interest. Experiments in both Quandt and Roskams laboratories are characterizing the expression of SPARC in MS and its animal model, EAE, to investigate the role of SPARC in influencing the integrity of the BBB, disease pathogenesis and associated repair processes.

1.20 HYPOTHESIS

SPARC expression at the BBB is altered during BBB development and under inflammatory conditions. We hypothesize that SPARC expression is influenced by inflammatory or repair processes during MS, and that SPARC alters the integrity of the BBB through altering tight junction expression in cerebral endothelial cells.

1.21 SPECIFIC AIMS

i. To characterize SPARC expression in cultured human cerebral microvascular endothelial cells, an in vitro endothelial model of the BBB, distinguishing alterations in SPARC expression as cells transition between developing (proliferative) and barrier (quiescent) phenotype and as cells are cultured under steady state conditions or those modeling an inflammatory milieu.

ii. To characterize the potential for SPARC to alter BBB integrity by changing tight junction protein expression in cerebral endothelia.
CHAPTER 2

Materials and Methods

2.1 Cell culture and treatments

2.1.1 hCMEC/D3 and human astrocytoma cell lines

This study used a well characterized *in vitro* BBB model comprised of immortalized human cerebral microvascular endothelial cells (hCMEC/D3), obtained under license from INSERM, France. The hCMEC/D3 cell line was originally established from brain tissue surgically excised from the temporal lobe of a female with epilepsy, and immortalized with a lentivirus vector transducing the catalytic unit of telomerase (hTERT) and SV-40T antigen. hCMEC/D3s retain BBB-specific tight intercellular junctions such as claudins 1, 3, 5, and 12, occludin, JAM-A, and ZO-1 localized at cell borders that reduce paracellular permeability. hCMEC/D3s express a panel of chemoattractant cytokine (chemokine) receptors involved in leukocyte recruitment including CXCR 1-5 and CCR 3-5 and efflux transporters such as Pgp and multidrug resistance-associated protein (MRP1) consistent with primary cerebral endothelial cells isolated fresh at autopsy (Weksler et al. 2005). hCMEC/D3s in the current study were between passage 28 and 32, although providers have described use until passage 35 without loss of BBB properties (Miller and Couraud 2009). The human astrocytoma cell line (CCF-STTG1) used as positive control for immunoblotting and immunocytochemistry was acquired from Cheryl Wellington’s lab at the Child and Family Research Institute, Vancouver, BC, originally sourced from the American Type Culture Collection (ATCC).
2.1.2 Cell culture conditions

For all experiments excluding initial localization immunoblotting, hCMEC/D3 cells were grown as most recently recommended by the cell provider, in EBM-2 media supplemented with 5% fetal bovine serum (FBS) (PAA Laboratories inc., ON), 1% penicillin-streptomycin (Sigma, MO), 1.4μM hydrocortisone (Sigma), 5μg/ml L-ascorbic acid (Sigma), 1:100 chemically defined lipid concentrate (Invitrogen-Gibco, CA), 10mM HEPES (Sigma), and 1ng/ml bFGF (Invitrogen-Gibco, CA). Cells were cultured in an incubator at 37°C with 5% CO₂, 95% air and saturated humidity. Media was replenished every 2 to 3 days. For immunoblotting, cells were grown on 25cm² flasks and 6 well plates (Corning) coated with type I rat tail collagen (150μg/ml; Sigma). To test hCMEC/D3 growth on various culture substrates, glass coverslips were coated with collagen I (150μg/ml; Sigma) and fibronectin (100μg/ml; R&D systems, MN), alone and combined. For immuncytochemistry, cells were grown on collagen coated glass coverslips or collagenous membrane inserts attached to a circular plastic support separating the upper/inner region from the bottom/outer surface creating “upper” and “lower” chambers (Cellagen discs, ICN Biomedicals, CA). Individual cellagen discs placed in wells of a 24 well plate were pre-wet for 2 hours in growth media before use. Cells were seeded on cellagen discs at 1.2x10⁴ cells/cm², with 200μl and 350μl media in the upper and lower chambers respectively. hCMEC/D3 grown on cellagen disc membranes form confluent, contact-inhibited monolayers with BBB properties.

Astrocytoma cells (CCF-STTG1; ATCC #CRL-1718) were cultured in RPMI-1640 media (Sigma cat #8758) supplemented with 10mM HEPES (Sigma), 1mM sodium pyruvate (Invitrogen-Gibco, CA), 2500mg/l D-glucose (Sigma). Media was replaced
fresh every 2 to 3 days. Cells were seeded at a density of 50,000 cells/cm² onto culture-treated 25cm² flasks or 50ng/ml poly-L-lysine (Sigma) coated glass coverslips (Fisher Scientific, ON). Cells were incubated at 37°C with 5% CO₂, 95% air and saturated humidity. For subculturing, confluent cell cultures were rinsed with PBS and 0.25% trypsin-EDTA (Sigma) to remove all traces of serum containing trypsin inhibitors. Cultures were then treated with 0.25% trypsin-EDTA for 5 minutes at 37°C and subsequent inactivation with growth media. Cell suspensions were centrifuged at 250xg for 8 minutes at 8°C, supernatant removed, and cell pellets resuspended in media for haemocytometer count and seeding onto culture surfaces.

For initial localization studies only, hCMEC/D3s were cultured according to a previous recommended protocol by F. Miller, Institut Cochin, France (2008). hCMEC/D3 were subcultured in growth factor rich “proliferation media” and maintained at confluence in growth factor reduced “differentiation media”. Proliferation media consisted of EBM-2 supplemented with 2.5% FBS (PAA Laboratories inc.), 1% penicillin-streptomycin (Sigma), 1.4μM hydrocortisone (Sigma, MO), 10mM HEPES (Sigma, MO), bFGF, VEGF, IGF, and EGF (EGM-2 MV SingleQuots from Clonetics; cat.#CC-4147). At confluence (typically day 3 after seeding 25,000 cells/cm² onto wells of a 6 well plate) proliferation media was replaced with differentiation media, which was serum-free EBM-2 with 1% penicillin-streptomycin, 1.4μM hydrocortisone, 10mM HEPES, and bFGF.

2.1.3 Growth and development of hCMEC/D3s

hCMEC/D3s cultured on cellagen disc inserts were fixed in 4% paraformaldehyde (PFA)-PBS when subconfluent (50-70% confluent) or confluent, then
probed with monoclonal antibodies against SPARC, ZO-1, and Ki-67 for immunocytochemical analysis. VEGF (12.5ng/ml; R&D systems, MN) treatment of cells was used to drive endothelial SPARC expression as previously described (Kato, Lewalle et al. 2001) as a positive control for immunoblots and immunocytochemistry assays.

2.1.4 Proinflammatory treatment of hCMEC/D3s

Confluent monolayers grown on cellagen discs were treated for 24h and 48h with 10U/ml and 100U/ml recombinant TNF-α (Invitrogen), 100U/ml recombinant IFN-γ (Invitrogen), and 50ng/ml LPS (Sigma), alone and in combination. Culture media was replenished with and without inflammatory molecules which were added to the top chamber alone. SPARC protein expression was then determined by immunocytochemistry and immunoblot analysis.

2.1.5 hCMEC/D3 exposure to SPARC

hCMEC/D3 cultured on collagen I coated 6 well plates until confluence were treated one day later with 0.1, 1, 10μg/ml recombinant human SPARC (R&D systems, MN) and TNF-α (200U/ml) for 24 hours in fresh serum reduced (1% FBS) complete media. The protein levels of endothelial TJ were determined by western blotting analysis.

2.2 Antibodies and reagents

The following primary antibodies (Abs) were used for immunoblots and immunocytochemistry: monoclonal mouse anti-human SPARC IgG1 (2.5μg/ml; Haematologic Technologies Inc., cat.#AON-5031, VT); polyclonal rabbit anti-ZO-1 IgG
(1\(\mu\)g/ml; Invitrogen, cat.#40-2200); monoclonal mouse anti-ZO-1 (2.0\(\mu\)g/ml; cat.#33-9100, CA); monoclonal mouse anti-occludin IgG\(_1\) (0.5\(\mu\)g/ml; Zymed, cat.#33-1500, CA), polyclonal rabbit anti-claudin-5 (0.5\(\mu\)g/ml; Zymed, cat.#34-1600), monoclonal mouse anti-Ki-67 IgG\(_1\) (1:100; Millipore cat.#HCS206, MA), polyclonal rabbit anti-NF\(\kappa\)B IgG (1:1000; Santa cruz biotechnology inc., Heidelberg); and monoclonal mouse anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:10,000; Santa cruz biotechnology inc., Heidelberg). Secondary abs included alexa fluor 568 goat anti-mouse IgG (1:200); alexa fluor 488 goat anti-rabbit IgG (1:200); alexa fluor 488 actin phalloidin (1:500; all from Invitrogen, CA), peroxidase conjugated goat anti-mouse IgG (1:5,000; Jackson Immuno Research, ON). Isotype matched control abs included mouse IgG\(_1\) (2.5\(\mu\)g/ml; Invitrogen) and rabbit IgG (1\(\mu\)g/ml; Invitrogen). RhSPARC was used in the antigen adsorption test for specificity and as a positive control for immunoblots. Recombinant human (Rh) laminin and Rh fibronectin were included as controls for dot blots to confirm cellular and ECM fraction isolation.

2.3 Immunocytochemistry

Cultures grown on cellagen disc membranes were processed for immunocytochemistry (ICC) in volumes of 200\(\mu\)l in the upper chamber and 350\(\mu\)l in the surrounding well, while glass coverslips were stained in 90\(\mu\)l volumes by adhesion on parafilm. Cultures were washed twice with warmed (~37\(^\circ\)C) PBS and fixed at room temperature (RT) in 4\% PFA-PBS for 10 minutes. Cultures were directly stored in PBS-0.01\% sodium azide at 4\(^\circ\)C until stained. For staining, cultures were washed twice with PBS and incubated for 10 minutes in permeabilization-blocking buffer (0.1\% Triton-X100 and 4\% normal goat serum (NGS) in PBS). Cultures were incubated twice for 10
minutes in blocking buffer (4% NGS-PBS) then incubated with primary abs diluted in blocking buffer for 1 hour at RT or overnight at 4°C. After primary ab incubation, cultures were washed three times for 5 minutes with PBS, and then incubated in secondary ab in blocking buffer for 50 minutes in the dark. Cultures were washed twice in PBS, stained with DAPI nuclear stain (1:10000) in PBS for 5 minutes, and again washed three times in PBS. Membranes were excised from cellagen discs using a scalpel, drained of excess PBS, and embedded in 10μl of ProLong Gold anti-fade reagent (Invitrogen) underneath a glass coverslip. Cultures on glass coverslips were mounted cell-side down on ProLong Gold (Invitrogen).

2.4 Image acquisition and analysis

Fluorescence images were captured by an Axioplan2 imaging epifluorescent microscope (Zeiss, Jena, Germany) and Axiovision 4 software (Gottingen, Germany). Confocal micrographs were captured by an Olympus Fluoview 1000 laser scanning confocal microscope with Nomarski optics and FV1000 Fluoview software. Fluorescence images were captured consistently with a 40X objective. Analysis was performed using Adobe Photoshop extended CS3 version 10.0 or Image J 1.42i software (National Institute of Health (NIH), MD), or assessed by SPARC immunoreactivity scale. Adobe Photoshop measurements of nuclear Ki-67 intensity were performed by outlining individual nuclei on a DAPI/blue filter image using the ‘quick selection tool’, and measuring Ki-67/green filter mean pixel intensity (MPI)—the average grey scale value of all pixels within a region—within delineated nuclear regions. Ki-67 positivity was relative to a threshold determined by blindly screening 3 images of confluent and subconfluent images each and denoting those cells negative for Ki-67. A threshold limit was set by averaging the Ki-67 MPI of those nuclei visually deemed Ki-
67 negative (n=59) plus three standard deviations. Ki-67 positive nuclei were those exceeding this pre-defined Ki-67 threshold. Quantification for SPARC immunofluorescence was performed either by regional analysis where regions for analysis were demarcated by thresholding the cell edge such that only cell covered surface area of a field/image was assessed for MPI using Image J or assessed based on a SPARC immunoreactivity scale defined in Table 1. Cell counting of DAPI-positive nuclei was performed using the Image J ‘analyze particles’ tool and automated using Image J macroscript. Representative images were corrected for sharpness, contrast, and brightness.

2.5 Conditioned media and cell lysis

Conditioned media (CM) was collected from hCMEC/D3 cultures and concentrated with salt removal for improved immunoblot detection. CM was concentrated at 4000 x g for 10 minutes with a centrifugal filter device (Amicon Ultra-4, Millipore, MA) that excludes proteins <10kDa but retains concentrated CM containing larger proteins such as SPARC (~43kDa).

For cell lysis, hCMEC/D3 cultures grown on 6 well plates were washed twice with cold PBS and incubated in situ on ice for 10 minutes in 125μl of ice cold NP-lysis buffer (50mM Tris-HCl, 150mM NaCl, 5mM EDTA, and 1% NP-40, adjusted to pH 8.0), fresh protease inhibitor and phosphatase inhibitor cocktails added before use (EMD Biosciences Inc., Darmstadt, Germany). Cultures were then scraped and collected into tubes and triturated on ice through a 28 Gauge needle 5 times and centrifuged at 13500rpm for 12 minutes at 4°C. Supernatants were collected as whole cell lysate fraction and stored at -80°C.
hCMEC/D3 lysis for TJ protein analysis required a lysis buffer with stronger detergents. Cultured cells in 6 well plates were washed twice with cold PBS, then incubated for 10 minutes on ice in situ in 125 µl of ice cold radio-immunoprecipitate assay (RIPA) buffer containing 20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS, 0.5% Sodium deoxycholate, adjusted to pH 7.5, with fresh protease inhibitor and phosphatase inhibitor cocktails added before use (EMD Biosciences Inc., Darmstadt, Germany). Cultures were then scraped and collected into tubes on ice for further 10 minute incubation, triturated on ice through a 28 G needle 6 times, and centrifuged at 13500 rpm for 12 minutes at 4°C. Supernatant were collected and stored at -20°C.

ECM fractions were isolated in 10 mM ethylenediaminetetraacetic acid (EDTA) aqueous solution, adjusted to pH 8. Confluent hCMEC/D3 cultures grown in 6 well plates were first washed twice with PBS then incubated in 10 mM EDTA solution for 4 minutes at 37°C, then 2 minutes on a shaker at RT, and finally 1 minute on ice. Next, cultures were lifted by cell scraper (Corning, NY), collected and triturated into a single cell suspension, before centrifugation at 10,000 rpm for 5 minutes at 4°C. ECM-enriched supernatant was removed and stored at -80°C. The remaining unlysed cell pellet was fractionated into nuclear and cytoplasmic components using the NE-PER nuclear and cytoplasmic extraction kit (Thermo Fisher Scientific, cat# 78833, ON) according to manufacturer instructions.

2.6 Immunoblotting

Protein concentrations were determined by bicinchoninic acid (BCA) protein assay kit (Sigma). Briefly, BCA and copper (II) sulphate solutions were combined at a
relative 50:1 ratio for the assay diluent. Bovine serum albumin (BSA) standards (0-10 μg/ml) and samples were diluted in assay diluent, incubated at 37°C for 30 minutes, and left on ice for 1 minute before absorbance spectroscopy. Absorbance was detected at 562nm by a DU700 spectrophotometer (Beckman Coulter, CA) and protein estimations based on the BSA standard absorbance curve.

Equal quantities of lysate (40μg) were diluted 1:1 in reducing 2X laemmli sample buffer (Bio-Rad, CA) containing 0.05% β-mercaptoethanol and heated at 100°C for 5 minutes. Samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE 6% gel for ZO-1; 12% gel for SPARC; 15% gel for occludin and claudin-5) for 1.5 hours at 80-100V and transferred to nitrocellulose membranes in wet transfer buffer at 90mA overnight at 4°C. For dot blots, ECM-enriched EDTA was applied directly onto nitrocellulose membranes (35μg loaded, 5μl per application) parallel to recombinant human laminin (50 ng/ml; Chemicon, Temecula, CA) and fibronectin (50ng/ml), known ECM components employed as controls.

Immunoblots for detection of SPARC, claudin-5, occludin and GAPDH were blocked in 5% skim milk in Tris-buffered saline (TBS: 1.37M NaCl, 27mM KCl, 0.25M Tris, adjusted to pH 7.4) for 1 hour and then incubated overnight in primary antibody in 2% skim milk-TBST (TBS+0.01% Tween 20 (TW20)) blocking at 4°C (or 2 hours at RT for GAPDH) on a rocker. Subsequently, SPARC, claudin-5 and GAPDH immunoblots were washed three times for 5-7 minutes in TBST (0.05% TW20) or TBST plus 0.1% milk for occludin blot washes, then incubated for 1.5 hours in HRP conjugated-secondary ab in 2% skimmed milk-TBST (0.01% TW20). After secondary ab incubation, blots were washed again three times for 5-7 minutes in TBST (0.05% TW20). Immunoblots for detection of ZO-1 were blocked in 1% BSA-TBST (0.05% TW20) for 4
hours at RT and then incubated in monoclonal anti-ZO-1 primary ab (1:250) in TBS overnight at 4°C. Next day, ZO-1 blots were washed 4 times 30 minutes in TBST (0.05% TW20) then incubated in HRP conjugated-secondary ab for 2 hours at RT. ZO-1 immunoblots were again washed 4 times 30 minutes in TBST (0.05% TW20). Immunoblots were developed in enhanced chemiluminescence substrate (ECL; Thermo Fisher Scientific, ON). Band signals were detected using a Versadoc imaging system (Bio-Rad, CA) and band densities quantified by Image J 1.42i software (NIH, MD).

2.7 Statistical analysis

GraphPad Prism version 5.01 was used for graph synthesis and statistical analysis. Parametric data expressed as mean ± standard error of the mean (mean±SEM) were statistically analysed by one way ANOVA and a Newman Keul multiple comparison test. Non-parametric data expressed as mean ± standard deviation (mean±SD) were analysed by a Kruskall Wallis and Dunns multiple comparison test. Mann Whitney comparison was performed on data comparing two non-parametric groups/treatments.
CHAPTER 3

Results

3.1 Human cerebral endothelial cell (hCMEC/D3) model of the BBB

Cultured hCMEC/D3 cells form confluent monolayers with intercellular tight junctions characteristic of endothelial BBB models comprised of primary brain microvessel endothelial cell cultures (Weksler, Subileau et al. 2005; Forster, Burek et al. 2008; Poller, Drewe et al. 2010). In our study, hCMEC/D3 (Fig. 1A) cells grown on collagen I coated flasks (Fig. 1A) had fusiform morphology and were tightly apposed forming monolayers with minimal overlap upon reaching confluence. Cultures grown on collagen discs (Fig. 1B) were most comparable in morphology and growth rate to that of cells grown on collagen I coated flasks with limited overgrowth and similar spindle-type morphology.

Growth of hCMEC/D3 cells in terms of morphology of an intact monolayer, and consistent growth rate and BBB properties (i.e. TJ formation) was highly dependent on the culturing surface and substrate. In longer term cultures, several days post confluence, there was a tendency for hCMEC/D3s to form patches of overcrowded-disorganized cells, especially on rat tail collagen type I-coated glass coverslips (Fig. 1C). Moreover, cells grown on glass coverslips had irregular morphology with numerous fine extending processes in several directions, different than the smooth fusiform shape of cells grown on plastic or collagen disc inserts. To facilitate immunocytochemistry, alternative culture substrates were tested including glass coverslips coated with fibronectin (Fig. 1D) and a combination of both collagen and fibronectin (Fig. 1E). Cells on fibronectin exhibited slower growth and more consistent morphology across cultures.
(Fig. 1D-D’), whereas cells cultured on collagen I expanded faster with more evident morphological irregularities and overcrowding (Fig. 1C-C’ and D-D’). Cells grown on collagen disc inserts consisting of a collagen membrane (type I and IV collagen) (fig. 1b) were most comparable in morphology and growth rate to that of cells grown on collagen I-coated plastic flasks (Fig. 1A) and thus became our preferred culturing surface for immunocytochemistry experiments carried out parallel to immunoblotting experiments.

3.2 SPARC expression of hCMEC/D3s during growth and development

SPARC was constitutively expressed in cerebral endothelia during growth and downregulated with maturity as cells developed a BBB phenotype of continuous ZO-1 staining at cell-cell borders. Increased SPARC expression during subconfluent stages of growth positively correlated with Ki-67, a marker of proliferation.

3.2.1 Localization of SPARC in hCMEC/D3s

Antibodies to SPARC from different commercial sources and protocols for both immunocytochemistry and immunoblotting experiments were tested using an astrocytoma cell line known to express SPARC (Raghu, Lakka et al. 2010). The astrocytoma cell line (ccf-sttg1) was strongly positive by both immunoblotting and immunocytochemistry (Fig. 2A and B) consistent with previous studies. Using established immunoblotting protocols, SPARC was detected in cytoplasm, ECM, and conditioned media of hCMEC/D3 cultures in different preparations. SPARC from all preparations resolved on immunoblots at the molecular weight observed for RhuSPARC (~43kDa) (Fig. 3). ECM-enriched EDTA isolates revealed SPARC at 43kDa (Fig. 3A), and were positive for the ECM rich protein recombinant laminin (rLN) but devoid of α-actin confirming efficient ECM fractionation (Fig. 3B). Intense bands in both the whole
cell lysate and the conditioned media also represented high levels of SPARC protein associated with cell membranes or intracellularly and secreted forms. Merged differential interference contrast (DIC) and immunofluorescence images of subconfluent hCMEC/D3 cells revealed SPARC subcellular localization was perinuclear and cytoplasmic, enriched in perinuclear regions containing structures that resemble the golgi-endoplasmic reticulum organelles (Fig. 3C).

3.2.2 SPARC expression is greater in subconfluent than confluent hCMEC/D3s

To investigate SPARC expression during hCMEC growth and as they form a confluent monolayer and develop an in vitro BBB phenotype, cultures were fixed when ~50-70% confluent (subconfluent) and confluent and probed with a monoclonal antibody against SPARC for immunocytochemistry. Confluency and an intact monolayer were visually assessed by phase contrast for absence of intercellular gaps and subsequently confirmed by subcellular localization of ZO-1, a marker of TJs. Subconfluent cultures covered 50-70% of culture surface, growing in small patches with spaces visible. These cells exhibited irregular and often discontinuous peripheral ZO-1 bands. Conversely, confluent cells exhibited tightly apposed monolayers with minimal overlap, and continual ZO-1 bands delineating the cytoplasmic membrane of the majority of cells (Fig. 4).

In analysis of hCMEC/D3 cell preparations, incubation of cultures with control abs, including isotype mouse IgG₁, goat IgG, and buffer with no primary antibody, produced no significant staining. SPARC levels were heterogeneous across hCMEC/D3 cultures. The intensity of SPARC expression (negligible, low, moderate, and high) in subconfluent and confluent cultures was assessed on confocal micrographs. Distribution analysis was performed in a blinded fashion where cells were distinguished and coded numerically on a DAPI image to avoid duplication and evaluated for level of
SPARC expression. SPARC immunoreactivity levels were assigned to individual cells according to the classification scheme and representative images in Table 1. Subconfluent cultures had a higher proportion of cells with low, medium, and high SPARC levels than confluent cultures that predominantly express negligible and low SPARC levels (Fig. 4 and 5).

SPARC expression was also examined on high resolution (1024x1024 pixels) confocal micrographs by global measurement of mean pixel intensity for a defined area of cell growth (Fig. 6). SPARC intensity was measured in subconfluent and confluent cultures within a 300µm x 300µm ocular field on images pooled from replicate wells reported as relative mean pixel intensity ± standard deviation (MPI±SD). SPARC intensity was greater in regions of subconfluent culture (3.85±0.492) than regions of confluent culture (2.83±0.426) (Fig. 6). Representative confocal micrographs illustrate subconfluent cultures, those with low and discontinuous ZO-1 staining at cell borders, had greater SPARC immunoreactivity than confluent cultures which showed intense ZO-1 bands and staining at cell-cell contacts (Fig. 4).

3.2.3 SPARC expression correlates with Ki-67, a marker for proliferation

The Ki-67 protein (also known as MKI67) is a cellular marker for proliferation present during all active phases of the cell cycle (G1, S, G2, and mitosis), but absent from resting cells (G0) (Verheijen, Kuijpers et al. 1989; Cheutin, O'Donohue et al. 2003). Consistent with a quiescent versus proliferative state, cells from confluent cultures had a significantly reduced percentage of Ki-67 positive cell nuclei than subconfluent cultures (Fig. 7, P=0.0143). The immortalization of this cell line is consistent with lower but not negligible incidence of Ki-67 positive staining in quiescent/confluent cultures. Concomitantly, cells in subconfluent cultures with negligible, low, moderate to high
levels of SPARC expression correlated with increased frequency of Ki-67 positive cell nuclei (Fig 8 and 9; P<0.0001). Conversely, cells in confluent monolayers were primarily categorized as low in SPARC and Ki-67 expression (Fig. 5,6,7).

3.2.4 Unchanged SPARC and reduced Ki-67 positive nuclei in VEGF treated hCMEC/D3 cultures

Kato et al. (2001) reported VEGF increased SPARC expression in a dose dependent manner in human microvascular endothelial cells (HMEC) and human umbilical vein endothelial cells (HUVEC) under serum-reduced conditions. Accordingly, VEGF was chosen as a treatment to increase SPARC expression as a positive control for immunocytochemistry. Preliminary studies showed that VEGF treatment of subconfluent hCMEC/D3 in the presence of 5% FBS did not alter SPARC expression. An analysis of individual cells in untreated or VEGF-treated subconfluent cultures, showed no significant difference in distribution across descriptive categories of SPARC expression (Fig. 10).

Exposure to VEGF treatment did, however, significantly (P<0.0001) reduce the percentage of Ki-67 positive nuclei in VEGF treated cells (47.5±9.3) compared to untreated cells (76.9±4.1) (Fig. 11A). Consistent with a reduction in cellular proliferation, enumeration of total DAPI positive cells showed cell counts in VEGF treated cultures were significantly (P<0.0001) lower (276.2±10.4 cells/ocular region (890x660μm) than untreated cultures (334.1cells/image ±7.6) (Fig. 11B,C).

Interestingly, the percent of Ki-67 positive cells was reduced in those cells expressing negligible levels of SPARC in the presence of VEGF. In untreated cultures SPARC expression levels increased in accordance with percentage of Ki-67 positive cells. However, after VEGF treatment, the incidence of Ki-67 positive cells that were
negligible for SPARC expression was significantly lower than in other untreated or VEGF treated cultures (Fig. 12). Representative images of untreated and VEGF treated subconfluent hCMEC/D3s in preliminary studies show SPARC staining and distribution of intensity for cells in culture was comparable in untreated and VEGF treated conditions (Fig. 13). Ki-67 staining, however, is noticeably reduced despite no apparent effect on the distribution of SPARC immunoreactivity showing that full concordance is not always observed between proliferative status of cells and SPARC expression.

3.3 Proinflammatory molecules regulate SPARC expression in hCMEC/D3s

Because the translocation of nuclear factor kappa B (NFκB) is an immediate signalling event secondary to TNF-α cytokine stimulation, TNF-α activity and appropriate stimulation of hCMEC/D3 was confirmed first by immunoblot measurement of activated NFκB nuclear translocation (Fig. 14). Nuclear (Nuc) fractions of TNF-α treated (100ng/ml, 350U/ml) cultures contained higher levels of NFκB than cells from untreated cultures (Fig. 14).

In phase contrast and immunofluorescence images, untreated confluent cells on cellagen disc membranes were tightly apposed with minimal overlap at the time of treatment and maintained regular fusiform morphology for the 48 hour incubation period. Incubation of cells with proinflammatory mediators resulted in elongated morphology and whirling (data not shown) with a visually intact monolayer. On an observational basis, the peripheral ZO-1 localization and banding at the cell-cell interface otherwise observed in cells of untreated monolayers, is disrupted and often completely absent following treatment with 10 or 100 U/ml of TNF-α (Fig. 15).

SPARC staining was negligible or low in untreated confluent monolayers, but increased when cultures were incubated with 10U/ml or 100U/ml TNF-α for 24 or 48
hours. 100 U/ml TNF-α treatment increased levels of SPARC 1.42 fold after 24 hours (P<0.0001) and 1.33 fold after 48 hours (P<0.0001), whereas 10 U/ml TNF-α did not significantly increase SPARC levels until 48 hours (1.29 fold, P<0.001). In a subsequent experiment, TNF-α (100U/ml) and LPS (25ng/ml) treatments alone increased SPARC levels 1.76 fold and 1.70 fold respectively after 24 hours (P<0.0001); the increase following TNF-α treatment was maintained at 48 hours (1.56 fold, P<0.001) (Fig 16). Compared to untreated controls, IFN-γ did not significantly increase SPARC levels until 48 hours (1.48 fold, P<0.05). Interestingly, IFN-γ and TNF-α cotreatment abrogated the effect observed for TNF-α alone at both 24 and 48 hours.

3.4 Effect of SPARC on tight junction protein expression in hCMEC/D3s

Parallel work in our laboratory suggests that the addition of exogenous RhSPARC to hCMEC/D3 disrupts barrier function leading to a decreased TEER and increased permeability of confluent monolayers to macromolecules 3kDa in size and greater (Basivireddy, unpublished data). To test the ability of SPARC to alter BBB associated TJ protein expression, hCMEC/D3 monolayers were treated with RhSPARC (0.1, 1, 10µg/ml) or TNF-α (200U/ml) as a well characterized modulator of TJ expression in BBB endothelial cells. Phase contrast micrographs of the TNF-α and SPARC treated hCMEC/D3 monolayers showed that cultures remained intact under all treatment conditions and retained regular fusiform morphology and apposition with minimal overcrowding and no apparent lifting or dissociation (Fig. 17). Previous studies of BBB integrity and TJ expression have described a barrier-promoting/protecting property for hydrocortisone (Forster, Burek et al. 2008); hydrocortisone was included at doses recommended by the hCMEC/D3 providers based on publications to test its
influence on barrier phenotype. Cell morphology and growth appeared similar in the presence or absence of hydrocortisone after 24 hours (Fig. 17).

Addition of the classical proinflammatory cytokine TNF-α decreased total expression of all TJ proteins studied (Fig. 18 A-C). TNF-α treatment of hCMECs lowered the expression of ZO-1 (0.765, P=0.013), occludin (0.617, P=0.044), and claudin-5 (0.477, P=0.033) compared to controls. The addition of hydrocortisone to the media with TNF-α increased levels of both ZO-1 and claudin-5 expression, but was not effective at raising occludin levels in the presence of TNF-α.

Our experiments revealed that hCMEC/D3 in culture produce low to moderate levels of SPARC even under resting conditions, suggesting that levels of SPARC in culture closely model the physiological levels of SPARC detected in the plasma and serum of healthy individuals (ranging from 0.1 to 0.5 μg/ml). In this regard, it is difficult to assess the physiological relevance of comparing cells cultured in “0” or “no SPARC” conditions which would exist immediately following media replacement.

Compared to cells cultured in media without exogenously applied SPARC, it is interesting that cells in parallel cultured in media with a physiological plasma concentration of SPARC (0.1μg/ml) consistently showed higher levels of ZO-1 (1.33 fold; P<0.05) and occludin (1.31 fold; P<0.05) expression (Fig. 19, 20). However, as exogenous SPARC was applied in amounts observed in individuals with various inflammatory disorders in the range of 1 to 10 μg/ml, the expression of ZO-1 and occludin was significantly reduced concomitant to increased SPARC concentrations (Fig. 19A and B). Increasing concentrations of exogenously applied SPARC from 0.1 to 1 μg/ml lowered both ZO-1 (0.768, P<0.05) and occludin (0.796, P<0.001) expression (Fig. 19). Representative immunoblots and normalized quantifications (Fig. 20, 21)
depict trends observed. The presence of hydrocortisone did not appear to significantly alter the observed trends in SPARC regulation of TJ expression as it did in response to TNF-α (Fig. 19). These data reflect similar findings to functional studies within our laboratory using FITC-dextran (3 and 10 kDa) diffusion and TEER measurements to show RhSPARC-mediated increases in transendothelial permeability of hCMEC/D3 monolayers. Interestingly, the bi-modal trends observed for ZO-1 and occludin in response to SPARC were reversed for claudin-5. Compared to “no SPARC” conditions, the lowest physiological dose of SPARC always lowered levels of claudin-5 (0.70 fold; P<0.05). However, upon increasing the dose of SPARC in the media, claudin-5 expression was subsequently increased (1.62 fold; P<0.001) across 0.1 to 10 μg/ml SPARC treatments (Fig. 19). It was notable that the patterns of TJ alterations and degree of response to both SPARC and TNF-α by hCMEC/D3 were indeed different; the reduction in endothelial TJ expression after TNF-α treatment was consistent across all TJ proteins studied and was greater than that observed for SPARC (0.1-10 μg/ml) (Fig. 20), suggesting that different mechanisms of protein expression and regulation may be involved.
**Table 1.** SPARC immunoreactivity assessment scale for hCMEC/D3 in culture. Immunocytochemistry images were used to classify four distinct SPARC levels distinguished by pattern and intensity of SPARC staining in individual cells. Approximate MPI ranges were determined for reference from 10 measurements of individual cells assigned each SPARC level.

<table>
<thead>
<tr>
<th>SPARC Level</th>
<th>Staining</th>
<th>Representative Immunocytochemistry</th>
<th>Description</th>
<th>Approximate mean pixel intensity (MPI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Negligible</td>
<td><img src="image_url" alt="Image" /></td>
<td>Faint diffuse cytoplasmic staining.</td>
<td>1.9-2.3</td>
</tr>
<tr>
<td>1</td>
<td>Low</td>
<td><img src="image_url" alt="Image" /></td>
<td>Perinuclear staining.</td>
<td>2.3-2.9</td>
</tr>
<tr>
<td>2</td>
<td>Moderate</td>
<td><img src="image_url" alt="Image" /></td>
<td>Perinuclear and diffuse cytoplasmic staining.</td>
<td>2.9-3.5</td>
</tr>
<tr>
<td>3</td>
<td>High</td>
<td><img src="image_url" alt="Image" /></td>
<td>Perinuclear and intense cytoplasmic staining.</td>
<td>3.5-4.5</td>
</tr>
</tbody>
</table>
Figure 1. Phase contrast images of hCMEC/D3 propagated on various culture surfaces and substrates: A) collagen I coated, tissue culture treated, plastic flasks; B) cellagen disc inserts; C) collagen I coated glass coverslips; D) fibronectin coated glass coverslips; E) combined collagen I and fibronectin coated glass coverslips. Letters with prime superscript (‘) denote day 4 rather than day 3. Growth and morphology is most similar and preferred on (A) collagen coated flasks and (B) collagen discs.
Figure 1. Phase contrast images of hCMEC/D3 propagated on various culture surfaces and substrates.
Figure 2. The human astrocytoma cell line ccf-sttg1 expresses high levels of SPARC detectable by immunoblotting and immunocytochemistry. A) Immunoblots detected recombinant human SPARC (RhSP) and SPARC in ccf-sttg1 lysates (40, 20, 10, and 5μg loaded) at the correct molecular weight (~43kDa). B) Confocal micrographs and DIC image of ccf-sttg1 cultures on PLL-coated glass coverslips show intense SPARC immunoreactivity (red) localized to cells stained with DAPI (blue, insets). Stains performed with isotype antibodies or lacking primary antibodies served as negative controls and demonstrated negligible staining. Five images were acquired per each duplicate well from one experiment to optimize staining conditions. Scale bar (white)= 30μm.
Figure 3. Localization of SPARC in cytoplasm, ECM, and conditioned media of hCMEC/D3 cultures detectable by immunoblotting and immunocytochemistry. A) Recombinant human SPARC, in situ lysates, ECM-enriched EDTA, and conditioned media were all positive for SPARC immunoreactivity at the expected molecular weight (~43kDa) by western blotting. Results from independent blotting. B) ECM fractions were immunoblot positive for laminin (LN) and negative for actin by dot blot confirming efficient ECM and cytoplasmic fractionation. C) Merged differential interference contrast (DIC) and immunofluorescence images of hCMEC/D3 cells revealed SPARC subcellular localization was perinuclear and cytoplasmic.
**Figure 4.** Immunocytochemistry images show subconfluent hCMEC/D3 cultures with higher SPARC expression than confluent monolayers. Representative immunocytochemistry images show subconfluent hCMEC/D3 cultures had higher SPARC expression (red) than confluent cultures. Representative confocal micrograph images show greater SPARC staining in subconfluent cultures compared to confluent cultures. Subconfluent cultures exhibit discontinuous peripheral ZO-1 bands (green), whereas confluent cells were tightly apposed and organized into monolayers with continual ZO-1 bands at interendothelial borders. Data represents one of two replicate experiments. Scale bar=30μm.
Figure 5. Individual cells in confluent monolayers express less SPARC than cells in subconfluent cultures. Levels of SPARC immunoreactivity (negligible, low, moderate, or high) were assigned according to Table 1. A total of 605 subconfluent cells in n=18 images (6 images from each triplicate well) and 835 confluent cells in n=10 images (5 images from each duplicate well) were analysed. Bars represent the average of results from n=18 and n=10 images respectively. Error bars indicate SEM. Analysis revealed that subconfluent cultures (empty bar) had a significantly higher proportion of cells with low, medium, and high SPARC expression compared to confluent cultures (filled bar) that predominantly express negligible and low SPARC levels. Table below shows data from the graphed (Exp. A) and a second experiment (Exp. B) with similar results. One way ANOVA and Tukey multiple comparison test, *** P<0.0001.

<table>
<thead>
<tr>
<th></th>
<th>Neg</th>
<th>Low</th>
<th>Mod</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S</strong></td>
<td>20.5±1.8</td>
<td>44.7±2.5</td>
<td>23.1±2.2</td>
<td>23.1±2.2</td>
</tr>
<tr>
<td><strong>C</strong></td>
<td>78.7±1.7</td>
<td>19.0±1.6</td>
<td>2.0±0.4</td>
<td>0.4±0.2</td>
</tr>
<tr>
<td><strong>S</strong></td>
<td>25.7±2.3</td>
<td>51.8±2.1</td>
<td>15.9±1.6</td>
<td>6.6±1.5</td>
</tr>
<tr>
<td><strong>C</strong></td>
<td>74.3±1.8</td>
<td>24.9±1.7</td>
<td>0.8±0.6</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 6. Subconfluent hCMEC/D3 regions exhibit more intense SPARC expression than confluent regions by regional immunocytochemistry analysis. SPARC intensity was measured in mean pixel intensity (MPI±SD) for selected cell covered regions. SPARC staining in subconfluent cultures (3.85±0.49, n=21 images, 7 from each triplicate well) was greater than that in confluent cultures (2.83±0.43, n=14 images, 7 from each duplicate well). Bars represent the average of results from n=21 and n=14 images respectively, Error bars represent SD. Data were analyzed for regional SPARC MPI in one experiment. Mann Whitney comparison test, ***P<0.0001.
**Figure 7.** Percentage of cells positive for the proliferation marker Ki-67 in subconfluent and confluent hCMEC/D3 cultures. 727 subconfluent cells (20 images from triplicate wells) and 589 confluent cells (8 images from duplicate wells) grown on collagen discs were analysed. B) 355 subconfluent cells (8 images from duplicate wells) and 450 confluent cells (8 images from duplicate wells) grown on collagen I coated glass coverslips were analysed. Confluent hCMEC/D3 cultures in both experiments had significantly (A: P=0.0143; B: P=0.0017) lower incidence of Ki-67 positive nuclei than subconfluent cultures. Bars represent the average of results from n=20 and n=8 images from subconfluent or confluent cultures, respectively. Error bars indicate SD. Data represents two replicate experiments (A,B). Mann Whitney comparison test, *P<0.05, **P<0.001.
Figure 8. Immunocytochemistry images of subconfluent cultures show greater SPARC and Ki-67 expression than confluent cultures. Images represent SPARC staining (red) alone (upper panels) or with Ki-67 staining (green) in lower panels. Data represents one of two replicate experiments. Scale bar=30μm.
Figure 9. SPARC levels positively correlated with the percent of Ki-67 positive cell nuclei in subconfluent hCMEC/D3 cultures. 727 subconfluent cells in 20 images pooled from triplicate wells were analyzed. Ki-67 staining is significantly different between groups classified according to increasing levels of SPARC expression from negligible to low, through to moderate and high combined (P<0.0001). SPARC expression levels are positively correlated with increasing incidence of Ki-67 positive nuclei in subconfluent hCMEC/D3s. Bars represent the average of results from n=20 images. Error bars indicate SEM. Data represents one of two experiments with similar significant results. One way ANOVA and Tukey multiple comparison test, **P<0.001, ***P<0.0001.
Figure 10. Intensity of SPARC staining on individual cells is similar in untreated and VEGF treated hCMEC/D3 cultures. Levels of SPARC immunoreactivity (negligible, low, moderate, or high) were assigned to individual cells according to criteria outlined in Table 1. 355 untreated cells (8 images, 4 images per well run in duplicate) and 329 VEGF treated cells (8 images, 4 images per well run in duplicate) were analysed. Data represents one experiment.
Figure 11. VEGF treatment reduces percent of Ki-67 positive nuclei and cell numbers in subconfluent hCMEC/D3 cultures. A) 355 untreated cells (4 images per well in duplicate) and 329 VEGF treated (12.5 ng/ml) cells (4 images per well in duplicate) were examined after media replacement and incubation in media with or without VEGF for 24 hrs. VEGF treatment in preliminary studies reduced the percentage of Ki-67 positive nuclei. Unpaired t-test, *P=0.0117. B) Cell counts of VEGF treated hCMEC/D3 cultures were lower compared to untreated controls. DAPI positive nuclei of 8019 untreated (8 images per well in triplicate) and 6628 VEGF treated cells (8 images per well in triplicate) were counted using Image J software. Average cell counts in VEGF treated cultures (276.2 cells/image ±10.4) were lower than that in untreated cultures (334.1 cells/image ±7.6). Data represents one experiment. Error bars represent SEM. T-test comparison performed, ***P=0.0004. C) Representative DAPI images from cell count analysis. Scale Bar (white)= 50µm.
Figure 12. Percent of Ki-67 positive cells decreased within cells expressing negligible levels of SPARC in the presence of VEGF. 355 untreated cells (4 images per well in duplicate) and 329 VEGF treated cells (4 images per well in duplicate) were examined. Data represents one experiment. Error bars represent SEM. One way ANOVA and Newman Keul multiple comparison, **P<0.001.
**Figure 13.** Immunocytochemistry shows discordance in intranuclear Ki-67 staining in subconfluent hCMEC/D3 exposed to VEGF compared to untreated controls. Regional SPARC and Ki-67 expression were both increased in untreated cultures, while VEGF treatment reduced the percent of Ki-67 positive nuclei with no apparent effect on the distribution of SPARC immunoreactivity in preliminary studies. 4 images were acquired per well in duplicate. Data represents one experiment. Scale bar=30μm.
**Figure 14.** TNF-α mobilizes NFkB to the nucleus of hCMEC/D3 cells. Immunoblotting confirms the nuclear translocation (Nuc) of NFκB out of the cytoplasm (Cyto) secondary to hCMEC/D3 treatment of confluent monolayers with 100ng/ml (350U/ml) TNF-α. Lysates from three wells were pooled from one experiment.
Figure 15. TNF-α increases SPARC expression in confluent hCMEC/D3 cultures. The effect of TNF-α on SPARC expression of confluent hCMEC/D3 cultures was assessed by quantitative immunocytochemistry. Cultures were incubated for 24 and 48 hours with 10U/ml or 100U/ml TNF-α. (A,B) Mean pixel intensity of SPARC immunofluorescence was pooled from 10 images derived from duplicate wells. (C,D) SPARC staining (red) was minimal in untreated cultures, but increased when cultures were incubated for 24 and 48 hours with 10U/ml and 100U/ml TNF-α. 100 U/ml TNF-α treatment significantly increased levels of SPARC after 24 and 48 hours (P<0.0001) whereas 10 U/ml TNF-α did not significantly increase SPARC levels until 48 hours (P<0.001). Addition of TNF-α also reduced ZO-1 (green) localization to regions of contact (banding) between endothelial cells with similar kinetics to alterations in SPARC expression. Data shown represents one of two experiments with similar results and significance, error bars represent SEM. One way ANOVA and Tukey multiple comparison test vs. untreated controls, *** P<0.0001. Scale bar=30μm.
Figure 15. TNF-α increases SPARC expression in confluent hCMEC/D3 cultures.
Figure 16. Proinflammatory molecules differentially regulate SPARC expression in confluent hCMEC/D3 cultures. Effect of proinflammatory molecules on SPARC expression of hCMEC/D3s was assessed by quantitative immunocytochemistry analysis. hCMEC/D3 cultures grown to confluence on membranes of cellagen discs were incubated for 24 hours and 48 hours with 100U/ml TNF-α, 100U/ml IFN-γ, or 25ng/ml LPS, alone or in combination. (A,B) SPARC immunofluorescence quantification was pooled from 10 images of duplicate wells for each condition. No primary and isotype negative controls had negligible staining. (C,D) Untreated confluent cultures showed minimal SPARC staining at 24 and 48 hours. TNF-α and LPS treatment alone increased SPARC levels after 24 hours (P<0.0001), and the effect of TNF-α was maintained until 48 hours (P<0.001). Compared to untreated controls, IFN-γ did not significantly increase SPARC levels until 48 hours (P<0.05). Treatment with TNF-α and IFN-γ abrogated the effect observed for TNF-α treatment alone after 24 and 48 hours. Data represents one of two experiments with similar significant results, error bars represent SEM. One way ANOVA and Tukey multiple comparison test vs. untreated controls, *P<0.05, **P<0.001, ***P<0.0001. Scale bar=30μm.
Figure 16. Proinflammatory molecules differentially regulate SPARC expression in confluent hCMEC/D3s cultures.
**Figure 17.** Phase contrast micrographs of TNF-α and SPARC treated cerebral endothelia cultured in the presence or absence of hydrocortisone. Confluent hCMEC/D3 monolayers were treated with SPARC (0.1, 1, 10μg/ml) or TNF-α (100U/ml) in the presence or absence of hydrocortisone for 24 hours. Cultures remained intact under all treatment conditions. Cells retained regular fusiform morphology and apposition with minimal overcrowding. Cell morphology and growth were similar in the presence or absence of hydrocortisone after 24 hours.
Figure 17. Phase contrast micrographs of TNF-α and SPARC treated cerebral endothelia cultured in the presence or absence of hydrocortisone.
Figure 18. Hydrocortisone protects against TNF-α induced reduction in hCMEC/D3 tight junction expression. TNF-α treatment decreased total expression of all TJ proteins studied (A-C). TNF-α treatment lowered the expression of ZO-1 (0.765 fold, P=0.0130), occludin (0.617 fold, P=0.0441), and claudin-5 (0.477 fold; P=0.0332) in the absence of hydrocortisone. Hydrocortisone was protective against TNF-α reduction of ZO-1 and claudin-5 expression, but not occludin expression. Data is pooled from n=2 (occludin and claudin-5) and n=3 (ZO-1) experiments, the error bars represent SD. Kruskal-wallis and Dunns multiple comparison tests performed, * P<0.05.
Figure 19. SPARC regulation of cerebral endothelial tight junction expression. Confluent hCMEC cultures replenished with fresh media and varying doses of SPARC (0, 0.1, 1, 10 μg/ml) for 24 hours were analysed for tight junction (TJ) protein expression by immunoblotting. Specific antibodies against junctional proteins ZO-1, occludin, and claudin-5 as well as GAPDH were used. Relative TJ protein levels were normalized to GAPDH. (A,B) Compared to SPARC-null (0μg/ml) conditions, the lowest SPARC dose (0.1μg/ml) consistently increased levels of ZO-1 (1.329 fold, P<0.05) and occludin (1.305 fold, P<0.05), which was subsequently reduced with increasing SPARC concentrations. Increasing exogenous SPARC from 0.1 to 1 μg/ml lowered both ZO-1 (0.768 fold, P<0.05) and occludin (0.796 fold, P<0.001) expression. The bi-modal trends observed for ZO-1 and occludin was reversed for claudin-5. The lowest SPARC dose decreased the expression of claudin-5 (0.694 fold, P<0.05), which was subsequently increased (1.62 fold, P<0.001) between 0.1 and 10μg/ml. The bars represent the mean expression from 5 (ZO-1) and 4 (claudin-5 and occludin) different experiments, error bars represent the SEM. ANOVA (ZO-1, P=0.0360; occludin, P=0.0101; claudin-5, P=0.0033) and Newman Keul multiple comparison test,* P<0.05 and **P<0.001.
**Figure 20.** SPARC and TNF-α regulation of cerebral endothelial tight junction expression in the presence or absence of hydrocortisone. Confluent hCMEC/D3 cultures were treated with SPARC (0.1, 1, 10 μg/ml) or TNF-α (200U/ml) for 24 hours in newly replenished HC-enriched (conventional) and HC-free media. TJ content was quantified by immunoblot analysis of hCMEC/D3 lysates parallel to TJ-negative human astrocytoma (Astro.) cell lysate employed as a negative control. Specific antibodies against junctional proteins including ZO-1, occludin, and claudin-5 as well as GAPDH were used. Relative TJ protein levels were normalized to GAPDH. The reduction in endothelial TJ expression after TNF-α treatment was always greater than that observed for SPARC (0.1-10 mg/ml). Data shows blots and normalized expression data from one representative experiment.
Figure 21. Physiological SPARC regulation of cerebral endothelial tight junction expression in the presence and absence of hydrocortisone. Confluent hCMEC/D3 cultures were replenished with fresh media containing varying doses of SPARC (0, 0.1, 1, 10 μg/ml) for 24 hours in HC-enriched or HC-free media. TJ content was quantified by immunoblot analysis. Specific antibodies against junctional proteins including ZO-1, occludin, and claudin-5 as well as GAPDH were used. Relative TJ protein levels were normalized to GAPDH. Data (A-F) represents SPARC concentration dependent downregulation of ZO-1 and occludin, converse to an upregulation of claudin-5 expression. Data shows blots and normalized expression data from one representative experiment.
CHAPTER 4

Discussion

4.1 Summary

The current study describes the dynamics of SPARC expression in human cerebral endothelia during development and inflammation using an in vitro model of the BBB. SPARC is constitutively expressed in cerebral endothelial cells during proliferation and downregulated as cells become quiescent and establish a BBB phenotype. SPARC expression positively correlated with a marker of proliferation (Ki-67) in subconfluent cultures; however, concordance between SPARC and proliferation is not absolute as lower Ki-67 levels after exposure to VEGF did not affect SPARC levels in cerebral endothelia. At quiescence, SPARC downregulation in ‘barrier’ endothelia is consistent with in vivo observations where SPARC expression was diminished in developmentally mature blood vessels yet enriched in developing blood vessels.

Furthermore we investigated the influence of inflammatory mediators on SPARC expression in cerebral endothelia and the functional impact of SPARC on barrier properties, specifically tight junction proteins. Both endotoxin LPS and proinflammatory cytokine TNF-α upregulated SPARC in confluent monolayers at the protein level detected by immunoblotting and immunocytochemistry. SPARC induction by TNF-α was inhibited by cotreatment with IFN-γ, suggesting distinct roles for these two cytokines in regulating SPARC expression in cerebral endothelia during CNS injury and repair. Cells cultured in physiological levels of SPARC expected in healthy human plasma revealed higher levels of the tight junction proteins ZO-1 and occludin. Conversely, replenishment of media without SPARC or increasing SPARC to levels
consistent with inflammatory or neoplastic conditions decreased ZO-1 and occludin expression. In this regard, differential expression of SPARC during inflammation may influence BBB integrity modulating the response to injury and subsequent repair.

4.2 SPARC expression during BBB establishment

Over past decades, molecular research on the BBB has been facilitated by in vitro models of the BBB. An immortalized human brain endothelial cell line, hCMEC/D3, was adopted for current experiments because the cell line recapitulates most of the unique in vivo features of the BBB and hence constitutes a valid in vitro BBB model (Weksler, Subileau et al. 2005). Cerebral microvascular endothelial cells form an active yet restrictive blood neural barrier termed the BBB (Abbott, Ronnback et al. 2006). BBB endothelial cells maintain barrier integrity and low paracellular permeability through constitutive expression and membrane localization of interendothelial tight junctions (TJs). Consistent with previous reports, hCMEC/D3 in this study expressed a classical TJ marker, ZO-1, appropriately localized to cell borders as well as other TJ proteins, claudin-5 and occludin. Confluent hCMEC/D3s grown on culture treated plastic flasks and cellagen discs demonstrated a contact-inhibited morphology with minimal overlap after several days in culture as assessed by phase contrast microscopy. Together with a modest increase in TEER as cells establish confluent monolayers, these observations confirmed the suitability of the in vitro BBB model and distinguished two developmental stages in which to study SPARC expression at the BBB.

This report describes novel in vitro evidence that cerebral endothelia constitutively express SPARC during proliferative growth and down regulate SPARC as cells become quiescent and establish a BBB phenotype. By quantitative
immunocytochemistry, SPARC expression was greater in subconfluent than confluent hCMEC/D3s. Subconfluent hCMEC/D3s are expected to divide in contrast to confluent cells that assume a barrier phenotype and show contact inhibited morphology. *In vivo*, where endothelial cell migration is paramount to the establishment of new vessels (angiogenesis), SPARC produced by subconfluent cerebral endothelia may mediate focal adhesion disassembly and inhibit cell spreading conducive with cell migration and proliferation (Chlenski, Guerrero et al. 2010).

The present study examined the association between SPARC and a marker of proliferation, Ki67 in cerebral endothelia. SPARC levels positively correlated with Ki-67 in subconfluent hCMEC/D3 cultures where increased SPARC expression levels were associated with higher percent of Ki-67 positive nuclei measures on an individual cell basis. In similar respects, confluent monolayers shared low SPARC and low Ki-67 levels providing a direct correlation between proliferation and SPARC expression. Elevated expression of SPARC mRNA and protein has been observed in endothelial cells involved in angiogenesis (Reed, Puolakkainen et al. 1993; Kato, Lewalle et al. 2001). Bovine aortic and human microvascular endothelial cells grown from confluent cultures into morphogenic tube-like vessel structures revealed these new vessel branches have a high mitotic index and are SPARC enriched. Confocal microscopy of developing chicken chorioallantoic membranes (CAM), an *in vivo* model for studying angiogenesis, similarly show intense SPARC staining in small calibre, newly formed, blood vessels and negligible staining in larger developmentally mature vessels (Iruela-Arispe, Diglio et al. 1991; Iruela-Arispe, Hasselaar et al. 1991). SPARC coincides with blood vessel neovascularisation on aortic valves and not normal avascular valves (Charest, Pepin et al. 2006). Pulmonary arteries express high levels of SPARC during
pseudogladular rat lung development (Strandjord, Sage et al. 1995). SPARC expression was upregulated 4.2 fold in bovine aortic and 10 fold in rat cerebral (resistant vessel) endothelial cell (RVEC) cultures that spontaneously organized into capillary tubes in vitro (Iruela-Arispe, Diglio et al. 1991; Iruela-Arispe, Hasselaar et al. 1991; Iruela-Arispe, Lane et al. 1995), suggesting a role for SPARC in ‘de novo’ morphogenesis of new microvessels (vasculogenesis). SPARC highly expressed in developmentally new blood vessels undergoing angiogenesis in vivo is consistent with our findings in vitro that SPARC expression in human cerebral endothelia is associated with proliferation, a vital component of angiogenesis.

Despite SPARC’s spatiotemporal association in many instances with endothelial proliferation and new vessel sprouting, the role of SPARC in angiogenesis and vessel morphogenesis remains complex. Even though SPARC expression coincides with angiogenesis, extracellular SPARC can directly bind growth factors (PDGF, VEGF, TGF-β) and antagonize their interaction with cognate receptors, preventing ERK activation with subsequent anti-angiogenic effect (Raines, Lane et al. 1992; Kupprion, Motamed et al. 1998; Motamed and Sage 1998; Bradshaw, Reed et al. 2001; Brekken and Sage 2001). Interestingly, exogenous application of plasmin-cleaved SPARC peptides stimulated endothelial cell proliferation and radial angiogenesis in CAMs, whereas full length SPARC is inactive in the same assay (Iruela-Arispe, Diglio et al. 1991; Iruela-Arispe, Hasselaar et al. 1991; Iruela-Arispe, Lane et al. 1995). Similarly, cleavage of SPARC can produce a potent pro-angiogenic peptide containing the (K)GHK motif which stimulates endothelial cord formation in vitro and angiogenesis in vivo (Lane and Sage 1994). Despite a focus on intracellular and cell associated SPARC in the current study, we did identify secreted SPARC in the cell culture supernatant and
associated with the ECM. It is beyond the scope of this study, but remains to be
determined what role different concentrations or different compositions of the SPARC
protein (whole or fragments) may play in regulating these processes.

Several opportunities appear to exist for physiological and regulatory interactions
between SPARC and VEGF, the prototypical regulator of angiogenesis. Exposure of
human dermal microvascular endothelial (HMEC-1) cells to VEGF increased SPARC
expression and SPARC subsequently regulates VEGF signalling (Kato, Lewalle et al.
2001). In proliferative endothelia, secreted SPARC gates VEGF signal transduction
through the pro-angiogenic VEGFR2 by blocking binding to and signalling via the anti-
angiogenic VEGFR1 (Nozaki, Sakurai et al. 2006; Uehara, Luo et al. 2010). In this
regard, SPARC transduces proliferative effects through modulation of VEGF signalling.

In this study, VEGF was included as a positive control intended to drive SPARC
expression in hCMEC/D3. This decision was based on work by Kato et al. (2002) in
which VEGF treatment in the absence of sera increased SPARC expression at the
protein and mRNA level in HMEC-1 microvascular endothelia. In the present study,
VEGF treatment had no significant effect on SPARC levels compared to untreated
controls, a difference which may be explained by our continued inclusion of serum in
the culture media. However, Ki-67 expression and cell counts were reduced in VEGF
treated hCMEC/D3 cultures compared to untreated controls. In these experiments,
VEGF may have out-competed SPARC for VEGFR1 with anti-proliferative effect
(Nozaki et al. 2006). Immunocytochemistry analysis revealed levels of SPARC and Ki-
67, previously positively correlated in untreated hCMEC/D3 cultures, instead exposure
to VEGF diminished incidence of Ki-67 positive nuclei with no apparent effect on the
distribution of SPARC expression in subconfluent hCMEC/D3 cultures. This observation
suggests that SPARC expression is not definitively tied to the proliferative status or abilities of the endothelial cells, and that differential regulation of SPARC expression and proliferation may indeed exist.

This thesis offers novel \textit{in vitro} evidence that human cerebral endothelia constitutively express SPARC during expansion/proliferation and downregulate SPARC as cells establish a BBB phenotype. \textit{In vivo} SPARC expression coincides with blood vessels during embryogenesis and postnatal development, but not in mature blood vessels of the normal adult CNS. To that effect, \textit{in situ} hybridization showed SPARC mRNA levels are enriched in migrating endothelia and pia-derived blood vessels in embryonic and postnatal tissue, and progressively downregulated with maturity in blood vessel of the adult CNS (Mendis and Brown 1994; Mendis, Ivy et al. 1998; Mothe and Brown 2001; Vincent, Lau et al. 2008). In the normal adult CNS, SPARC at the protein level is predominantly restricted to neurogenic subventricular zones, choroid plexus, activated microglia, specialized radial glia (bergman and müller glia), astrocytes (Au, Richter et al. 2007; Vincent, Lau et al. 2008). These data suggest that SPARC may be expressed by cerebral endothelia in a spatiotemporal manner consistent with a role in CNS vascularisation.

4.3 Regulation of SPARC expression during infection and inflammation

Earlier \textit{in vivo} studies found that blood vessels express high levels of SPARC at sites of injury, infection, and neoplasia (Porter, Sage et al. 1995; Mendis, Ivy et al. 1998; Chlenski and Cohn 2010; Chlenski, Guerrero et al. 2010). In adult tissue, SPARC expression increases in response to various environmental stressors, including heat shock, heavy metal, and endotoxin (Liu, Mosher et al. 2009), during angiogenesis.
(Iruela-Arispe, Diglio et al. 1991; Iruela-Arispe, Lane et al. 1995) and wound repair (Bradshaw and Sage 2001) associated with a variety of pathological conditions.

Using an *in vitro* model of the BBB, this thesis offers the first description of proinflammatory regulation of SPARC in cerebral endothelia. TNF-\(\alpha\) and LPS endotoxin treatment increased SPARC protein levels, however TNF-\(\alpha\) and IFN-\(\gamma\) cotreatment of cerebral endothelia abrogated SPARC induction observed after TNF-\(\alpha\) alone, suggesting neuroimmune cross-talk between TNF-\(\alpha\) and IFN-\(\gamma\) that may regulate levels of SPARC expression in cerebral endothelia. Although the present study is the first mention of proinflammatory regulation of SPARC expression in cerebral endothelia, other CNS injury models and neoplastic syndromes highlight the association between inflammation and SPARC enriched blood vessels. In the normal adult CNS, SPARC levels are low and expression is primarily restricted to astrocytes and microglia of synaptic rich regions but typically not blood vessels. During the peak phase of EAE, CD31 positive blood vessels become intensely SPARC positive by immunohistochemistry (Roskams lab, unpublished data). Injury to the CNS during EAE appears to increase the amount of SPARC detected in association with cerebrovascular endothelial cells. In a study of incisional wound injury of the cerebral cortex, SPARC mRNA was increased in blood vessels proximal to the wound (Mendis, Ivy et al. 1998). Following dermal wound injury, SPARC reactivity is evident not only in endothelia but also in macrophages, megakaryocytes, platelets, and fibroblastic cells as illustrated by immunohistochemistry and *in situ* hybridization (Reed, Puolakkainen et al. 1993). Repair after injury of neural tissue occurs through the inflammatory activation of resident microglia and astrocytes followed by the recruitment of peripheral leukocytes required for the removal of cellular debris, formation of a glial scar, production of trophic
factors, and the development of new blood vessels (Mendis et al. 1997). The induction of SPARC in newly sprouted blood vessels suggests a role for SPARC in angiogenesis, but may also implicate SPARC in immune cell trafficking, and other processes involved in the CNS inflammatory response (Fancy, Kotter et al. 2010). Indeed, increased bioavailability of SPARC at the neurovascular niche may promote CNS regeneration by supporting neurite outgrowth after neuronal injury (Au, Richter et al. 2007).

SPARC is undetectable in normal quiescent tissue but abundant in reactive endothelia of damaged tissue undergoing rapid remodelling and repair (Porter, Sage et al. 1995; Brekken and Sage 2001; Capper, Mittelbronn et al. 2010). SPARC is highly expressed in human neoplastic syndromes associated with certain inflammatory components (i.e. VEGF, PGE2), histological damage and increased cellular turnover (Yoshimura 2006). Chelenski et al. (2010) found xenograft neuroblastoma tumours had structurally abnormal, tortuous blood vessels, while tumours treated with SPARC had thin walled, structurally more normal blood vessels in lower density. Normalizing blood vessel cytoarchitecture is but one of numerous tissue protective features ascribed to SPARC.

In the present study, TNF-α treatment of hCMEC/D3s increased SPARC levels relative to controls. In a recent study using the same hCMEC/D3 cell line, the same dose of TNF-α significantly decreased BrdU incorporation, without an effect on cell number or migration (Chen and Easton 2010). Thus, inflammatory induction of SPARC expression is likely governed by signalling distinct from that which upregulates SPARC during angiogenesis. Moreover, an examination of human astrocytic tumours with high inflammatory activity did not find a clear association between SPARC levels and proliferating endothelial cells (Porter, Sage et al. 1995). Inflammatory regulation of
SPARC has not previously been studied in an in vitro BBB system. Novel data here show specific inflammatory molecules influence SPARC expression supporting a role for SPARC in CNS inflammatory responses.

Neuropathological investigations show a higher than normal density of blood vessel MS brain. Further analysis revealed endoglin (CD105) – a specific marker of endothelial proliferation- was elevated in all lesion types, the highest of which in normal appearing white matter (NAMW) (Holley, Newcombe et al. 2010). Proliferating endothelia in NAWM either indicate an early pathogenic or protective activation of endothelia. High microvessel density in MS brain is not completely novel, as an earlier study reported endothelial reactivation and proliferation in chronic MS lesions (Lassmann 2003).

Angiogenesis promotes inflammation in the CNS by increasing the sum total of inflammatory factors secreted by cerebral endothelia cells (Chen and Easton 2010). However, angiogenesis provides a means to protect and repair CNS tissue. A classical proinflammatory molecule, TNF-α not only contributes to apoptotic clearance of immune cells in the CNS, but also promotes cerebral endothelia expression of BDNF, TGF-β, IL-6, and pleiotrophin, which collectively promote neuronal survival and growth (Bayas, Hummel et al. 2002; Kallmann, Wagner et al. 2002; Rieckmann and Maurer 2002). Leukocytes that infiltrate the CNS through extant and new vessels also carry neurotrophic factors that conserve nervous tissue during a CNS inflammatory response (Martino, Adorini et al. 2002; Rieckmann and Maurer 2002).

Only a few in vitro studies have investigated inflammatory cytokine and growth factor regulation of SPARC expression, none of which were endothelial or CNS related.
TGF-β treatment of human fibroblasts increased SPARC expression 3.5 fold after 24 hours (Reed, Vernon et al. 1994). In line with this *in vitro* data, TGF-β and SPARC *in vivo* are abundant in connective tissue undergoing rapid remodelling during wound healing (Pavasant and Yongchaitrakul 2008). Interestingly, SPARC has been associated with the normalization and negative regulation of inflammatory signalling in ovarian cancer. Said et al. (2007) found that SPARC ameliorated peritoneal ovarian carcinomatosis by downstream suppression expression of macrophage chemoattractant protein-1 (MCP-1), IL-6 and PGE2 production and activity. Also, SPARC overexpression in ovarian cancer cells down regulated NFkB mediated gene expression and ameliorated ovarian cancer inflammation (Said, Elmarakby et al. 2008). These anti-inflammatory properties suggest SPARC expression may be upregulated under inflammatory conditions to negatively regulate inflammatory gene expression (Said, Socha et al. 2007). In the present study, cerebral endothelia express more SPARC in the presence of inflammatory molecules. In the context of MS, an inflammatory neurodegenerative disease, increased SPARC bioavailability at the BBB under inflammatory conditions may then help attenuate neuroinflammation and serve to normalize lesioned tissue exhibiting robust inflammatory gene and protein expression.

### 4.4 Tight junction regulation at the BBB

A role for SPARC in development, maintenance, and breakdown of blood-neural barriers is suggested by its localization at the BBB and BCSFB during development and inflammation. Ependymal cells of the BCSFB line brain ventricles and the choroid plexus and express SPARC during embryogenesis and postnatal development, and at barrier sites in the lateral ventricle of adult mice (Vincent, Lau et al. 2008). Analogously, cerebral endothelia strongly express SPARC during embryogenesis, but down regulate
SPARC in early postnatal development as they establish a BBB (Mendis and Brown 1994). In adult mice, SPARC is concentrated in mature astrocytic endfeet of the BBB (Vincent, Lau et al. 2008). SPARC colocalization with mature S100 positive astrocytic endfeet at the BBB was confirmed by immunofluorescence deconvolving 50µm Z-stack of adult spinal cord tissue (Vincent et al. 2008; Ash Anwar, Roskams Lab, unpublished data). Astrocytes and soluble mediators they produce can upregulate tight junction (TJ) and transporter expression on brain endothelia and are thought to be a major regulator of BBB development (Gaillard, van der Sandt et al. 2000; Abbott, Ronnback et al. 2006). The anatomical association of SPARC in astrocytic endfeet and cerebral endothelia place SPARC in an ideal niche to induce, maintain, or disrupt blood-neural barrier characteristics (Kim, Park et al. 2006). SPARC in end feet could reflect an uptake of endothelial-derived SPARC or endogenous astrocyte-derived SPARC concentrated at its end feet, or both. In addition, BBB dysfunction is evident in SPARC null mice brains, where FITC-Na tracer typically absent in wild type mice is observed around the lateral ventricles of SPARC null brains, similar to when the BBB is perturbed using mannitol (Roskams lab, unpublished data).

The present study examined the effect of SPARC on cerebral endothelia tight junction protein expression by immunoblotting using the hCMEC/D3 model of the BBB. The TJ adaptor protein, ZO-1 (~220kDa), as well as two integral transmembrane junctional molecules, occludin (~65kDa) and claudin-5 (~22kDa), were candidate tight junction proteins analyzed in these experiments. These TJ proteins share a structural connection as domains of claudins and occludin bind ZO-1 which in turn anchors the actin cytoskeleton and other peripheral membrane associated signalling components (Matter and Balda 2003; Katsuno, Umeda et al. 2008). Many signalling pathways
regulate tight junction expression, including G-proteins, serine, threonine and tyrosine kinases, extra- and intracellular calcium levels, cAMP levels, proteases, and TNF-α (Kniesel and Wolburg 2000). TNF-α mediated a down regulation of tight junction protein expression and therefore served as a control treatment throughout the experiments in this thesis to substantiate the responsive nature of the hCMEC/D3 in vitro BBB model.

The present study quantified TJ protein levels in lysates from confluent hCMEC/D3 cultures treated for 24 hours with recombinant human (rh)SPARC or rhTNF-α. The results provide the first documentation of SPARC concentration dependent regulation of TJ expression in endothelial cells regardless of tissue origin. Compared to SPARC-null conditions, addition of a physiological SPARC dose as measured in serum or plasma from healthy individuals consistently demonstrated increased ZO-1 and occludin expression. These results are tempting to interpret as a role for low levels of SPARC in maintaining the integrity of the BBB vasculature. Conversely, SPARC at physiological concentrations associated with neoplasia or other inflammatory or infectious conditions lowered both ZO-1 and occludin expression.

Notably, the trends observed for ZO-1 and occludin were reversed for claudin-5. Physiological low doses of SPARC lowered levels of claudin-5 compared to untreated controls but increased claudin-5 expression when added at concentrations greater than 1 μg/ml consistent with values obtained from patients with defined pathological processes. This claudin-5 paradox is evident in the distinction between BBB opening and pathological barrier loss in cerebral endothelia (Gavard and Gutkind 2008) and may explain the differential response to SPARC in regulation of claudin-5 expression. TJ and adherens complexes are polarized in epithelia but intermingled in endothelia (Crosby, Fleming et al. 2005; Stamatovic, Keep et al. 2008). Close proximity suggests adherens
such as VE-cadherins influence TJ assembly and disassembly. VE-cadherin maintains claudin-5 expression, whereas the absence and inactivation of VE-cadherin represses claudin-5 expression (Dejana 2004; Gavard and Gutkind 2008; Taddei, Giampietro et al. 2008). For example, TNF-α, mediates the removal of VE-cadherin which lowers claudin-5 expression and drives BBB dysfunction (McKenzie and Ridley 2007).

SPARC alters endothelial barrier function, at least partly by its abrogation of focal adhesions. Exposure of bovine pulmonary endothelial cells with exogenously applied SPARC abrogates focal adhesions (Young, Wang et al. 1998) involving VE-cadherin disassembly which, as described above, subsequently suppresses claudin-5 expression. Concomitant to this effect, SPARC has been shown to enhance β-catenin, ILK and PI3k/AKT signalling which promotes claudin-5 expression (Barker, Baneyx et al. 2005; Shi, Bao et al. 2007; Nie and Sage 2009; Nie and Sage 2009). Claudin-5 is protected by the way ILK prevents β-catenin degradation and PI3K/AKT sequesters FOXO1 in the cytoplasm and both these processes enhance claudin-5 expression (Young, Wang et al. 1998; Gavard and Gutkind 2008; Taddei, Giampietro et al. 2008). Therefore it is feasible to see how SPARC may paradoxically reduce claudin-5 expression by VE-cadherin abrogation on a background of feedback signalling that supports claudin-5 expression. The presented results report such a biphasic concentration dependent trend where low dose SPARC lowered claudin-5 expression, and subsequently higher doses reconstituted claudin-5 protein content in cerebral endothelia.

TNF-α applied exogenously decreased total expression of all TJ proteins studied, consistent with previous reports in the literature (Forster, Burek et al. 2008; Aveleira, Lin et al. 2010; Nagyoszi, Wilhelm et al. 2010). Hydrocortisone offered some protection against the reduction of ZO-1 and claudin-5 but not occludin. This is explained by a
greater ability of hydrocortisone to drive both claudin-5 and ZO-1 expression to a greater degree than occludin in hCMEC/D3 cultures (Forster, Burek et al. 2008).

The relative contributions of occludin, claudin-5, and ZO-1 towards barrier function have been studied using transgenic mice (Saitou, Furuse et al. 2000; Nitta, Hata et al. 2003) and in vitro cell culture models (Romero, Radewicz et al. 2003; Weksler, Subileau et al. 2005; Forster, Burek et al. 2008; Van Itallie, Fanning et al. 2010). Occludin knockout (KO) mice revealed no gross phenotype; however, histological assessments revealed perivascular calcification in the brain (Saitou, Furuse et al. 2000). Peptide fragments of the first external loop of occludin impairs resealing paracellular junctions opened by Ca^{2+} removal imploring that occludin differentially regulates rather than structurally seals barriers (Wolburg and Lippoldt 2002). Localization and barrier function of occludin appear to be dependent on phosphorylation of its serine and threonine residues (Hawkins and Davis 2005; Morgan, Shah et al. 2007). Occludin is also required for TNF-α and IFN-γ cytokine induced disruption of tight junctions in MDCK cells (Van Itallie, Fanning et al. 2010). Claudin-5, but not occludin, comprises the structural back bone of TJ strands (Umeda, Ikenouchi et al. 2006). VEGF knocks down claudin-5 expression contributing to BBB breakdown (Agraw et al. 2009). Claudin-5 KO mice develop normally, except transgenic cerebral blood vessels were selectively more permeable to small molecules (<800Da) than wildtypes (Nitta et al. 2003). ZO-1 KO—a devastating transgenic mutation—causes aberrant yolk sac angiogenesis and embryonic cell apoptosis resulting in embryonic lethality (Katsuno et al. 2008). ZO-1 and ZO-2 directs the correct localization of claudin-5 and promotes TJ assembly in cells (Umeda et al. 2006). The potent anti-inflammatory steroid dexamethasone has been shown to upregulate ZO-1 and occludin protein expression.
and decrease transendothelial permeability of immortalized rat brain endothelial monolayers (Romero, Radewicz et al. 2003; Forster, Burek et al. 2008).

SPARC has been studied using *in vitro* endothelial cell culture models and is considered a key modulator of endothelial barrier function. Exogenously applied RhSPARC (15µg/ml) induced rounding and increased transendothelial albumin flux 1.3 to 3.6 fold through confluent bovine pulmonary artery endothelial monolayers. SPARC affected barrier permeability in a dose dependent manner that could be blocked by neutralizing SPARC antibody (Goldblum et al. 1994). SPARC treated endothelial cells exhibit a rounded morphology and intercellular gaps through F-actin remodelling in a manner prevented by prior phallicidin F-actin stabilization (Goldblum et al. 1994). BBB permeability was investigated *in vitro* by TEER measurement in postconfluent porcine brain capillary endothelial cells (PBCECs) grown on transwell inserts coated with a 1:1 mixture of type IV collagen and SPARC (Tilling et al. 1998). Combinatorial SPARC and type IV collagen coating of inserts reduced TEER measurements compared to collagen alone (Tilling et al. 1998). The prevailing understanding from these experiments was that SPARC increases transendothelial permeability and disrupts endothelial barrier function of *in vitro* endothelial monolayers. This reduction in barrier protein expression and function is consistent with our data demonstrating increased TEER and permeability to FITC-dextran (3 and 10 kDa) diffusion in hCMEC/D3 monolayers cultured with SPARC (Quandt lab, unpublished data). Clearly, SPARC has the potential to influence the integrity of the BBB, but the nature and outcome of this influence, reparative or pathological, is yet to be determined.

SPARC modulates transendothelial permeability through protein tyrosine kinase (PTK) phosphorylation signalling as Herbimycin A, a potent PTK inhibitor, diminished
SPARC-induced changes in permeability. A marked 12 fold increase in phosphotyrosine containing proteins was immunolocalized to interendothelial borders within 1 hour of SPARC treatment concomitant to barrier opening as evidenced by immunocytochemistry and BSA diffusion permeability assay, respectively. β-catenin and paxillin were two candidate substrates for tyrosine phosphorylation (Young, Wang et al. 1998). Phosphorylated β-catenin signalling in endothelia confers a apoptosis-resistant phenotype and mediates angiogenesis, barriergenesis, and barrierstasis in vivo and in vitro (Lammert 2008; Polakis 2008; Nie and Sage 2009; Nie and Sage 2009; Chang, Wei et al. 2010).

In the present study, compared to media lacking exogenously supplied SPARC, addition of a baseline physiological dose of SPARC always increased levels of ZO-1 and occludin, and higher SPARC levels supported claudin-5 consistent with a role for SPARC in maintenance of BBB integrity. Conversely, SPARC concentrations expected during angiogenesis, neuroinflammation, and wound repair lowered ZO-1 and occludin expression. TJ depletion is one mechanism conventionally associated with BBB breakdown; although, the distinction between transient BBB opening and BBB breakdown is not always clear. VEGF-induced BBB breakdown is associated with claudin-5 and occludin downregulation and severe disability in EAE, a model of MS (Argaw, Gurfein et al. 2009). Remarkably, transduced recombinant claudin-5 expression protected brain microvascular endothelial cells from VEGF-induced barrier loss; yet, occludin expressed at the same promoter was not protective (Argaw, Gurfein et al. 2009). This thesis reports that SPARC above normal levels opens the BBB while supporting claudin-5 expression consistent with temporary barrier opening rather than BBB breakdown (Gavard and Gutkind 2008). By extension, it may be inferred that loss
of claudin-5 may be the most stringent marker of pathological barrier dysfunction, whereas loss of ZO-1 and occludin expression may occur during both temporary BBB opening and pathological BBB breakdown.

BBB disruption and leukocyte CNS trafficking are among the earliest preclinical cerebrovascular events in MS pathogenesis (Minagar and Alexander 2003). BBB dysfunction in CNS disease, such as MS may arise secondary to inflammatory, vasoconstrictive, or hypoxic/ischemic conditions because of downregulation, proteolytical degradation, and internalization of TJs (Minagar and Alexander 2003; Hawkins and Davis 2005; Stamatovic, Keep et al. 2008; Stamatovic, Keep et al. 2009). Markers of hypoxia-like damage and repair are present in pattern III MS lesions with evident distal oligodendrogliopathy and apoptosis (Lassmann, Bruck et al. 2007). Expression of HSP70 and HIF are elevated in pattern III MS lesions and implicate ischemia, anoxia, hypoxic stress, or excess excitatory stimulation (Aboul-Enein, Rauschka et al. 2003; Lassmann 2003). Levels of Endothelin-1 (ET-1), a potent vasoconstrictor, are elevated in CSF and sera of MS patients compared to controls. ET-1 overexpression foreseeably exacerbates MS by restricting endoneural flow to nervous tissue with already high metabolic demand (Timoshin, Sazonova et al. 2000). Sheer stress from blood flow is necessary for endothelial barrier tightness. hCMEC/D3 cultures, a model of the BBB, establish a tighter monolayer with lower transendothelial permeability under flow conditions compared to static conditions (Cucullo, Couraud et al. 2008). Therefore, intraluminal flow is important for BBB integrity.

Transient global cerebral ischemia diminished SPARC mRNA expression in laser-captured microdissected (LCM) blood vessels (Bauman et al. 2009). Parallel to the diminished SPARC immunoreactivity in this same study, functional assays showed
that post-ischemic cerebral microvessels were more permeable for small and large molecular weight tracers (sucrose and fibrinogen) compared to control vessels. Mild post-ischemic hypothermia (~33°C) attenuated loss of SPARC content in the basement membrane and protected against BBB breakdown (Bauman et al. 2009). Mild hypothermia protects against depletion/rapid-release of SPARC from the basement membrane after ischemic injury and maintains BBB integrity in doing so.

4.5 SPARC: friend or foe in CNS development and response to injury or repair

MS pathology is characterized by BBB dysfunction, immune infiltrate, and perivascular nervous tissue damage. Based on current data, elevated levels of SPARC expected during growth and inflammation open the BBB and likely neuroimmune mediated damage and repair in the CNS (Rieckmann and Smith 2001; Rieckmann and Maurer 2002). Opening the BBB appears a useful regenerative strategy for immune-mediated and passive removal of cell debris, complement, antibodies, and cytotoxic agents that otherwise accumulate and perpetuate inflammation (Hohlfeld, Kerschensteiner et al. 2000; Hohlfeld, Kerschensteiner et al. 2007; Harrer, von Bodingen et al. 2009; Kerschensteiner, Meinl et al. 2009). Active lesions and NAWM of MS brain contain evidence of inflammation. This thesis reports two canonical proinflammatory molecules (TNF-α and LPS) increased SPARC expression in cerebral endothelia. Indeed, enriched SPARC bioavailability at the neurovascular niche could conceivably enhance neural regeneration by promoting neurite outgrowth at sites of neuroinflammation (Au, Richter et al. 2007).

A recent study using HUVEC, and primary lung and cardiac endothelial cell cultures demonstrated SPARC is a VCAM-1 (vascular cell adhesion molecule) ligand
that induces cytoarchitectural rearrangement and intercellular gap formation, two processes critical for leukocyte trafficking (Kelly, Allport et al. 2007). VCAM-1 is upregulated on inflammatory transformed cerebral endothelia to facilitate leukocyte firm adhesion (Kelly, Allport et al. 2007). Inflammatory recruitment of neutrophils, eosinophils, and monocytes/macrophages to an inflamed peritoneum was compromised in SPARC-null mice compared to wildtype in vivo (Kelly, Allport et al. 2007). SPARC engagement of VCAM-1 may function analogous to VLA-4 mediated leukocyte trafficking, as they both bind to and signal through VCAM-1 to increase endothelial monolayer permeability. Paradoxically, data presented in this thesis suggest SPARC not only disrupts but also maintains endothelial barrier stasis. These data support the notions that SPARC maintains BBB stasis under normal conditions but opens the BBB after injury. Therefore, SPARC deficiency or excess may ultimately exacerbate BBB breakdown in MS.

4.6 Future Directions

As a followup to the results and explanations offered in this thesis, certain questions remain unresolved and should be considered for further study. Firstly, we could ask the question: Is SPARC an anti-inflammatory negative regulator of NFκB activation in cerebral endothelia? Said et al. (2008) found SPARC ameliorated peritoneal ovarian carcinomatosis by suppression of macrophage chemoattractant protein-1 (MCP-1), IL-6, PGE2 production associated with negative regulation of NFκB activation. In the current study, TNF-α treatment of cerebral endothelia increased endogenous SPARC levels possibly as a negative feedback regulator of inflammatory gene expression. If SPARC is a negative regulator of NFκB activation, nuclear fractions of hCMEC/D3 cotreated with TNF-α and SPARC should contain less NFκB than TNF-α
treatment alone. This research would further implicate SPARC as a regenerative, anti-inflammatory, neurotrophic factor in the CNS.

Secondly, the present study analysed TJ protein levels by immunoblotting not immunocytochemistry. Further immunocytochemistry studies are needed to investigate SPARC’s effects on regulation of TJ gene expression as well as TJ protein localization. Above and beyond any potential influence on gene expression which may occur rapidly at the post transcriptional level, it is possible that SPARC changes hCMEC/D3 monolayer permeability through internalization, dephosphorylation, and degradation of TJ proteins (Stamatovic, Keep et al. 2008; Stamatovic, Keep et al. 2009). Each of these possibilities warrant further investigation.
References


Aveleira, C. A., C. M. Lin, et al. (2010). "TNF-\{alpha\} signals through PKC{zeta}/NF-{kappa}B to alter the tight junction complex and increase retinal endothelial cell permeability." Diabetes.


Furlan, R., E. Brambilla, et al. (2001). "Intrathecal delivery of IFN-gamma protects C57BL/6 mice from chronic-progressive experimental autoimmune


