Chemokine and chemikine receptor expression by human brain endothelium: an in vitro and in situ study

Thesis

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Chemokine and chemokine receptor expression by human brain endothelium: An *in vitro* and *in situ* study

A thesis submitted for the degree of doctor of philosophy

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Submitted in August 2005
Acknowledgements

I would first like to thank my supervisors without whom this thesis could not have been possible. Thanks to Nacho for giving me the opportunity to make a PhD, for his availability and advice during the four years. Thanks to David for his enthusiasm and much valued ideas and knowledge. On a more personal level, I am very grateful for the hospitality I have received when I came to London (thanks as well to Margot). Finally, thank you to John for his useful overview of my work and his input.

This work would not have been possible without a certain number of people. Amongst them, special thanks to the members of the immunology group, Jane and Payam for their help and enthusiasm, Karen, Monica, Sandeep, Hadassah, Suzanne, Sarah and Jackie for being such a brilliant and easy people to work with as well as good friends. I would like to thank Filipe and Seema for the good time in the N0076 office, and all the passionate discussions about chemokines. Thanks to Marc for his help with the statistics.

Thanks as well to the group of the Institute Cochin in Paris in particular Babette Weksler who carried out the viral work for the immortalisation of brain endothelial cells and Pierre-Olivier Couraud who made it possible to develop the immortalised human brain endothelial cells. Heather and Frances, who did the ultrathin sections, of the Electron Microscopy group for sharing their expertise and for giving me invaluable help with all the electron microscopy experiments as did Julio, Chris and Howard.

Finally, the frustration that arise with the uncertainty of sciences would have been much harder if it were not for the presence and care of my favourite housemate Yoseph, for all my sport friends who kept me healthy among who Dnyanesh, Dinar, Vanessa, Vee, and co., and finally my friends and family back in France and around Europe.
Abstract

The infiltration of leukocytes across the BBB is thought to be mediated by chemokines released in the CNS. This study investigated the production of chemokines and expression of chemokine receptors by human brain endothelial cells (HBEC) as well as the consequences of CXCL10 stimulation on brain endothelial function in terms of leukocyte migration and endothelial permeability.

Purified primary HBEC as well as a novel human brain endothelial cell line, called hCEMC/D3 and generated in collaboration with the Institute Cochin, were used in the present study. Characterisation of hCMEC/D3 cells was effected by determining the expression and localisation of HBEC markers such as tight junction and adherens junction proteins. Both primary and immortalized HBEC were tested for the production of four chemokines (CCL2, CCL5, CXCL8 and CXCL10) considered to play a role in multiple sclerosis. CXCL8 and CCL2 were constitutively released, whereas CXCL10 and CCL5 could not be detected at basal levels. By contrast, all these chemokines were up-regulated in response to either TNF-α or IFN-γ or a combination of both. TNF-α had the most striking effect, up-regulating the production of CCL2, CCL5 and CXCL8, while IFN-γ up-regulated CXCL10 exclusively. The chemokine receptors CXCR1 and CXCR3, whose ligands include CXCL8 and CXCL10, were expressed constitutively by HBEC both in vitro and in vivo, and only CXCR3 was up-regulated in response to cytokine stimulation in vitro. Furthermore, CXCR3 on endothelial cells is functional as CXCL10 induces phosphorylation of p42/44 MAP kinase, p38 MAP kinase and JNK/SAPK. However, activation of HBEC by CXCL10 did not induce any permeability change nor did it modulate leukocyte adhesion.

These results demonstrate that endothelial cells could play an important role in chemokine production and hence leukocyte infiltration during inflammatory pathologies such as MS. The role of chemokine receptors requires further investigation.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>aa</td>
<td>Amino acids</td>
</tr>
<tr>
<td>ACM</td>
<td>Astrocyte condition medium</td>
</tr>
<tr>
<td>AF-6</td>
<td>Afadin 6</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>APS</td>
<td>Amonium per sulfate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BMEC</td>
<td>bone marrow endothelial cells</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate sodium salt</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DARC</td>
<td>Duffy antigen/receptors for chemokines</td>
</tr>
<tr>
<td>dNTPs</td>
<td>deoxyribonucleoside triphosphates</td>
</tr>
<tr>
<td>DMEC</td>
<td>Dermal microvascular endothelial cells</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorter</td>
</tr>
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<td>FCS</td>
<td>Foetal calf serum</td>
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<td>FITC-Dextran 70kDa</td>
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<td>FGF</td>
<td>Fibroblast Growth Factor</td>
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<td>HBEC</td>
<td>Human brain endothelial cells</td>
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<tr>
<td>HBSS</td>
<td>Hank's balanced salts solution</td>
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<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<td>Human leukocyte antigen</td>
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<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>ICAM</td>
<td>InterCellular Adhesion Molecule</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor I</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JAM</td>
<td>Junctional adhesion molecule</td>
</tr>
<tr>
<td>JNK/SAPK</td>
<td>Jun N-terminal kinase/stress-activated protein kinase</td>
</tr>
<tr>
<td>LMEC</td>
<td>Lung microvascular endothelial cells</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen-activated protein</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>Min</td>
<td>Minute</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>NAWM</td>
<td>Normal appearing white matter</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa B</td>
</tr>
<tr>
<td>ORO</td>
<td>Oil-Red O</td>
</tr>
<tr>
<td>PB</td>
<td>Phosphate Buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Platelet endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PP-MS</td>
<td>Primary progressive multiple sclerosis</td>
</tr>
<tr>
<td>RR-MS</td>
<td>Relapsing-remitting multiple sclerosis</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>S.D.</td>
<td>Standart deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Sec</td>
<td>Second</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>Standart error of the mean</td>
</tr>
<tr>
<td>SP-MS</td>
<td>Secondary progressive multiple sclerosis</td>
</tr>
<tr>
<td>SVEC</td>
<td>Saphenous vein endothelial cells</td>
</tr>
<tr>
<td>STAT1</td>
<td>Signal transducer and activator of transcription 1</td>
</tr>
<tr>
<td>SV40</td>
<td>Siaman virus 40</td>
</tr>
<tr>
<td>TEER</td>
<td>Transendothelial electrical resistance</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N,N-tetramethyl-ethylene dramine</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight junctions</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>vWF</td>
<td>Von Willebrand factor</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zonula occludens protein 1</td>
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1. Chapter 1: General Introduction
1.1 **Multiple sclerosis**

Multiple sclerosis (MS) is considered to be an autoimmune disorder affecting the central nervous system (CNS). It is characterized by the infiltration of leukocytes from the systemic compartment into multifocal areas in the CNS where active demyelination of neuronal axons occurs. The regions of inflammation and demyelination are named plaques or lesions. MS affects 2,500,000 people worldwide, and represents the most frequent cause of neurological disability in young adults, with an average age of onset between 20 and 40 years old, predominantly affecting women over men at a ratio of 2 to 1 (Confavreux *et al.*, 1980). This female: male ratio in morbidity is commonly observed in many autoimmune disorders, including Graves’ disease, rheumatoid arthritis, systemic lupus erythematosus and Hashimoto's disease.

MS was first identified and characterised in 1865 by the French clinician Jean-Martin Charcot and his pupil Alfred Vulvian (Charcot, 1865). He named it *la sclerose en plaques disseminées*, *la sclerose multiloculaire* or *la sclerose generalisée*. Despite extensive research over the last 140 years, the aetiology of the disease is still unknown and much debated.

**1.1.1. Aetiology**

MS is a disease with uneven geographic and ethnic distribution. The overall risk of developing MS is higher in northern (the prevalence, meaning the number of individuals in whom the diagnosis has been made in a given population, is around 100/105 in Scandinavian countries) and southern (a prevalence of around 60/105 in New-Zealand) hemispheres, than in equatorial regions (occasional cases) (Rosati, 2001). The aetiology of MS is extremely complex but it appears to be triggered by environmental factors in...
genetically-susceptible individuals. Table 1.1 lists some of the major factors that have been currently proposed to contribute to MS.

<table>
<thead>
<tr>
<th>Genetic</th>
<th>major histocompatibility complex (MHC) class I</th>
<th>HLA-A3</th>
<th>(Fogdell-Hahn et al., 2000)</th>
</tr>
</thead>
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<tr>
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<td>MHC class I</td>
<td>HLA-B7</td>
<td>(Bertrams and Kuwert, 1976)</td>
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<td>Genetic</td>
<td>MHC class II</td>
<td>DRB1<em>1501-DQB1</em>0602</td>
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<td>Virus</td>
<td>Human herpes virus 6</td>
<td>(Clark, 2004)</td>
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<tr>
<td>Environmental</td>
<td>Virus</td>
<td>Epstein-Barr virus</td>
<td>(Wagner et al., 2004)</td>
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<tr>
<td>Environmental</td>
<td>Virus</td>
<td>Varicella zoster virus</td>
<td>(Ross, 1998)</td>
</tr>
<tr>
<td>Environmental</td>
<td>Bacteria</td>
<td>Chlamydia pneumonia</td>
<td>(Contini et al., 2004)</td>
</tr>
<tr>
<td>Environmental</td>
<td>Solar radiation UV</td>
<td>Biosynthesis of vitamin D</td>
<td>(Hayes, 2000)</td>
</tr>
<tr>
<td>Other</td>
<td>Sex hormones</td>
<td>Female hormones</td>
<td>(Coo and Aronson, 2004)</td>
</tr>
</tbody>
</table>

Table 1.1: List of some proposed factors contributing to MS as reviewed by Sospedra and Martin (2005).

Several studies have indicated that MS may be causally linked to one or several genetic components. Indeed, a small but significantly higher incidence rate of MS has been observed within families (approximately 20% higher than in the general population) (Pratt et al., 1951). The prevalence in monozygotic twins ranges from 21% to 30% and in dizygotic twins is around 5% (Ebers et al., 1986; James, 1982; Mumford et al., 1994). Adopted siblings of MS patients do not appear to have a higher tendency to develop MS (Ebers et al., 1995). However, the inheritance of the genes involved does not follow classical Mendelian patterns. The first candidate gene suspected to confer susceptibility to MS is the MHC encoding region on chromosome 6q21. Other genes that may be implicated are on chromosome 14 in the area encoding the variable region of the immunoglobulin heavy chain, involving several genetic regions, as well as on chromosomes 1p, 17q, Xq,10p, 11p, 19p and 20p as reviewed by (Hensiek et al., 2003). At
present, the MHC region on chromosome 6p has given the most reproducible data for establishing a relationship of MS with a genetic component. The human leukocyte antigen (HLA) complex consists of 4000kb DNA mapped to chromosome 6q21.3. It consists of 3 classes of genes, MHC class I, MHC class II and MHC class III. A strong association with MS has been reported for HLA DR2 (Olerup et al., 1987; Heard et al., 1989), mainly among Caucasians in northern and central Europe, northern America and Australia, but this association was weak or lacking in other ethnic groups (Spurkland et al., 1991; Clerici and Fernandez, 1992).

Studies on the prevalence of MS carried out on migrants from Europe underline the importance of environmental factors in the aetiology of this disease. In some cases, those migrants show lower prevalence rates than in their country of origin, and the residential place during childhood appears to be of critical importance. For example, observations made in Israel (Alter et al., 1962) suggested that the place of residence in early life affects the probability of developing MS as migrants from northern Europe had lower MS prevalence rates when they had migrated before the onset of puberty therefore emphasizing the importance of environmental factors. While this indirect evidence suggests that a pathogen, whose infection was acquired most certainly during childhood, may represent an environmental factor that determines the onset of MS, no one as yet has identified a specific agent. Instead, a variety of common viral disorders developing in late childhood (measles, mumps, rubella, and mainly Epstein-Barr virus) have been proposed over the years as significant factors predisposing to MS (Operskalski et al., 1989) (some of these are reported in table 1.1).

Another suggested environmental factor, reported in table 1.1, that may protect individuals from developing MS is sunlight and its effect through vitamin D production on the immune system. In a study involving American women, vitamin D supplementation in the diet has
been shown to be inversely correlated with the risk of developing MS (Munger et al., 2004). Furthermore, the finding of seasonal variation of immune function in serially magnetic resonance imaging (MRI)-monitored MS patients suggests an environmental role in T-cell activation (Killestein et al., 2002).

1.1.2. The symptoms of multiple sclerosis

The clinical course of MS can follow different patterns, and this observation has led to the classification of distinct types of MS. In the majority of cases, the disease follows a relapsing-remitting (RR-MS) course, where the initial attack is followed by partial or complete recovery, with relapses occurring months or years later. Around 50% of RR-MS patients will convert to a secondary progressive (SP-MS) form, in which the progression towards incapacitating manifestations of the disease will be gradual with or without relapses. Another form of MS is called primary progressive (PP-MS), where the disease follows a progressive course from the onset. The PP-MS form is usually diagnosed at an older age and with a greater proportion of male to female patients than in the RR-MS form. Finally, in fewer than 5% of the cases, the disease is manifested as a progressive relapsing (PR-MS) form, which is similar to the PP-MS but displays one or more exacerbations (Noseworthy et al, 2000).

The symptoms displayed by MS patients are related to the distribution of their CNS lesions which varies from patient to patient. There is a higher probability that lesions affect the white matter of the cerebrum, cerebellum, brain stem, spinal cord, the optic nerves and chiasm (Poser et al., 1979). Because areas in the CNS where lesions can develop are different amongst individuals, the resulting symptoms are also extremely varied from patient to patient. Table 1.2 describes symptoms associated with the location of lesions in the CNS (adapted from Compston and Coles, 2002).
Site | Symptoms
--- | ---
Cerebrum | Cognitive impairment; Hemi-sensory and motor disturbances; Affective disorder (mainly depression); Epilepsy (rare) Focal cortical deficits (rare)
Optic nerve | Unitateral painful loss of vision
Cerebellum and cerebellar pathways | Tremor, Clumsiness and poor balance
Brainstem | Diplopia, Oscillopsia; Vertigo; Impaired speech and swallowing; Paroxysmal symptoms
Spinal cord | Erectile impotence; Bladder dysfunction; Weakness; Stiffness and painful spasms
Other | Pain; Temperature sensitivity and exercise intolerance; Fatigue

Table 1.2: Lesion sites and corresponding syndromes developing in MS. Adapted from Compston and Coles (2002).

Many of these symptoms are also related to the neuropathology of the lesion (see section 1.1.3. below), as a result of either demyelinated axons, or partially demyelinated axons. Demyelinated axons can discharge spontaneously and show increased mechanical sensitivity. This accounts for the sensation of seeing light flashes triggered by eye movement (phosphenes) and for the electric shock-like sensation running down the spine or limbs on neck flexion (Lhermitte’s symptom and sign). Symptoms and signs characteristically appear after exercise or a hot bath (Uhthoff’s phenomenon) and are related to the sensitivity of partially demyelinated axons which cannot sustain the fall in membrane capacitance induced by a rise in temperature leading to electrical conduction failure (Compston and Coles, 2002).

1.1.3. The pathology of multiple sclerosis

The predominant pathological features of MS lesions are inflammation, axonal demyelination and gliosis (see sections 1.1.3.1 to 3 below). The pathology of MS lesions varies due to a number of factors, related to the temporal state of activity of the lesions. Histological studies of MS lesions have attempted to provide a system of classification. The following paragraph will describe some lesion classification systems.
MS lesions may be divided into active, chronic active and chronic silent according to Bo et al. (1994). This classification reflects the pathological characteristics of the lesions. Active lesions are composed of infiltrating leukocytes positive for MHC class II, and are hypercellular (Gay et al., 1997). Chronic active lesions are characterised by a central hypocellular gliosis area surrounded by an active MHC class II positive hypercellular border, where both demyelination and inflammation occur concomitantly. In the centre of a chronic active lesion, demyelinated axons and reactive astrocytes are found. In chronic silent lesions, there is apparent demyelination of the axon, but the inflammation is not visible and the staining for MHC class II is negative. Mainly microglia and macrophages are present, as indicated by CD68 positive staining (Bo et al., 1994).

Sanders et al. (1993) has proposed classifying the lesions into four different sub-types, type I, type II, type III and type IV, based upon cellularity criteria, oil-red O (ORO) staining (ORO stains lipids, and when it is present in leukocytes, it is thought to reflect phagocytosed degraded myelin), and expression of HLA-DR, which is one of the MHC class II molecules and a marker for leukocyte activation. As a result, active lesions from the classification above are subdivided into two sub-types: type I lesions, corresponding to the earliest lesion, where there are HLA-DR positive cells which are negative for ORO, hence no evidence of myelin degradation; and type II lesions, where HLA-DR/ORO doubly positive cells correspond to activated macrophages and microglia. The ORO staining of type I lesions has proven to be controversial. Li et al. (1993) described the earlier stage of the lesion as being ORO positive, implying myelin degradation as an early event whereas Gay et al. (1997) described the early lesions as being ORO negative, but with signs of myelin phagocytosis and digestion. Type III lesions correspond to the chronic active lesions with a hypocellular centre and a hypercellular edge constituted by HLA-DR/ORO positive cells. Type IV lesions are equivalent to chronic silent lesions, with
demyelinated axons, amoeboid microglia, and macrophages which are positive for CD68 (Bo et al., 1994), but little HLA-DR/ORO positive staining. Interestingly, the majority of the lesions present are different in type depending on the duration of the disease. Around 80% of lesions are type I in patients with disease duration of less than 5 years, whereas very few are present in patients that have been diagnosed over 30 years earlier (2%). The relation is inversely true for type IV lesions (around 20% of all lesions in patients with less than 5 years of clinical history compared to 85% in those with a 30 year clinical history) (Sanders et al., 1993).

Even more detailed characterisation of MS lesions has been carried out, using a wider range of histological markers for macrophage activation such as MRP14, 27E10 or 25F9 and CD3, a marker for lymphocytes. Two other types of lesions have thus been added, the early remyelinating stage and the late remyelinating stage, the latter also named shadow plaque and recognisable by their pale appearance (Bruck et al., 1996). These shadow plaques were described very early on by Marburg (1906) and were first thought to reflect the degree of demyelination. It has since become clear that they represent remyelination of complete plaques (Lassmann et al., 1983). The early remyelinated lesion is characterised by the presence of many cells, both lymphocytes and macrophages, and by clusters of thinly myelinated axons, while in the late remyelinated lesion, only a few macrophages are present among the thinly myelinated axons. The abundance of remyelinated lesions is apparent from studies of acute or early multiple sclerosis (Prineas et al., 1993).

Inter-individual differences in MS lesions have also been reported, suggesting that the pathology of this disease may be heterogeneous (Lassmann et al, 2001). The authors investigated active and inactive MS lesions in different patients and reported four different patterns of inflammation. These are macrophage-mediated demyelination (pattern I),
antibody-mediated demyelination (pattern II), oligodendriopathy (pattern III) and primary oligodendrocyte damage (pattern IV) (Lassmann et al., 2001).

1.1.3.1. Demyelination

The loss of myelin sheaths around axons is the earliest pathological feature of MS plaques. The myelin sheath is formed by gyring of several layers of the plasma membrane of an oligodendrocyte around a portion of an axon. An oligodendrocyte may be responsible for the myelination of several axons. In MS, disruption of the myelin sheath may lead to the death of the oligodendrocyte. Oligodendrocytes are found in reduced numbers in lesions especially in chronic silent and/or active lesions (Mews et al., 1998; Prineas et al., 1984), whereas in earlier lesions, their numbers may appear unchanged and there is even some active remyelination (Ozawa et al., 1994). It has been suggested that in the early stage of the lesion, the destruction of oligodendrocytes is followed by recruitment of progenitor cells from surrounding areas. This may explain the remyelination observed by MRI and the shadow plaque (Li et al., 2003b).

The extent of axonal damage varies greatly between individuals and lesion types. It is characterised by transacted axons and axonal swelling with the formation of spheroids and regeneration sprouts (Dahl et al., 1989). These phenomena are most evident in active lesions (Trapp et al., 1998). In chronic silent lesions, axonal damage may lead to Wallerian degeneration (corresponding to the degeneration of an axon and its myelin sheath distal to the site of an injury), which is manifested by brain atrophy. As neurons are not replaceable and do not regenerate, neuronal loss is most certainly responsible for the neurological deficits observed in patients as demonstrated by magnetic resonance spectroscopy (Davie et al., 1997).
1.1.3.2. Gliosis

Glial scar formation, also known as gliosis, is one of the features of demyelinated MS lesions. Gliosis generally consists of highly reactive astrocytes, microglia and oligodendrocyte precursors but in demyelinated plaques in MS, it consists mostly of scar-type astrocytes and naked axons (Fawcett and Asher, 1999). The two most commonly used markers for gliosis in MS are the intermediate filament glial fibrillary acidic protein (GFAP) and vimentin (Holley et al., 2003), whose up-regulation is associated with hypertrophy of astrocyte processes. Early on, in active lesions, astrocytes show signs of reactivity with increased expression of lysosomal enzyme (Allen et al., 1981). In chronic silent lesions, the demyelinated axons are embedded in an important fibrillary network of astrocyte processes. It has been suggested the astrocyte reactivity is a secondary response to the immunological factors present in the lesion, such as tumor necrosis factor alpha (TNF-α) which has a proliferative effect on astrocytes (Selmaj et al., 1990).

1.1.3.3. Inflammation

Originally, the CNS was considered to be an immune-privileged organ since it was suggested that the immunosurveillant lymphocytes which are patrolling elsewhere in the organism were excluded from the brain (Barker and Billingham, 1977). It is now clear that this is not the case, and that leukocyte can infiltrate the brain under pathophysiological circumstances. In MS lesions, macrophages and lymphocytes have been identified in the CNS parenchyma, whereas plasma cells and B cells are present in the perivascular space (Prineas and Wright, 1978). Most lymphocytes within the lesion are T cells, both CD4+ and CD8+ predominantly at the early stage of the lesions. CD4+ lymphocytes, also called Helper T-cells, facilitate antibody production by B-cells, and help the expansion of CD8+ T lymphocytes, also called cytotoxic T-cells, which are involved in the cytolytic destruction of antigen-presenting cells. The distribution of leukocytes within the lesion varies according to the type of lesion (Lucchinetti et al., 2000). Although most studies
indicate that both CD4 and CD8 positive cells are present within lesions, there is still much discussion as to which of these T-lymphocyte sub-types prevails during the early stages of lesion formation. Transfer of reactivated myelin specific CD4+ T cells induces Experimental Autoimmune Encephalomyelitis (EAE) in animal models for MS, strengthening the hypothesis that it is the CD4+ subpopulation that initiates the autoimmune reaction (Pettinelli and McFarlin, 1981). However, recent data shows an important role for CD8+ T cells in the development of neuropathology in MS (Bitsch et al., 1999; Babbe et al., 2000). Not only is clonal expansion in MS lesions more important in the CD8+ population than in CD4+ cells (Babbe et al., 2000), but also the number of CD8+ cells within the lesion correlates better with the extent of acute axonal injury than the number of CD4+ cells (Bitsch et al., 1999).

Macrophages constitute another type of immune cell which are found in abundance, together with T lymphocytes, in MS lesions. They are predominantly present during the active phase of the lesion, and participate in myelin degradation and breakdown, as indicated by the presence of immunoreactive myelin peptide fragments in their cytoplasm (Bruck et al., 1995). Not only do early and late chronic lesions contain lower numbers of macrophages, but also the differentiation of those macrophages changes with the lesion type, as indicated by a series of markers such as MRP14, 27E10, 25F9, MRp8, Ki-M1P and Ki-M4P (Bruck et al., 1996); MRP14 and 27E10 are expressed in early and late active lesions, whereas 25F9 is detected throughout all the different lesion type. It has been suggested the macrophages are the key cells responsible for myelin degradation through the production of macrophage soluble mediators. In vitro, macrophage conditioned medium triggered apoptosis in cultured neurons (Flavin et al., 1997). Among the soluble factors identified are pro-inflammatory cytokines (such as TNF-α and IFN-γ), free radicals, glutamate and some proteases such as the matrix metalloproteinases (MMP); all potential neurotoxic mediators, as reviewed by Hendriks et al. (2005).
1.1.3.4. Blood-brain barrier dysfunction

The BBB is certainly compromised in MS and blood vessel damage concomitant with leakage of serum proteins into the CNS parenchyma appears to be one of the earliest events during the formation of active MS lesions (Gay and Esiri, 1991). The BBB as an anatomical structure and biochemical barrier in both physiological conditions and, particularly, its malfunction and relevance to MS pathology is described in the following section.

1.2 The blood-brain barrier

Goldmann (1913) first introduced the concept of a BBB when he demonstrated that after intravenous injection of the trypan blue dye into rats, all organs with the exception of the brain stained blue. The cerebro-spinal fluid (CSF) remained free of dye, whereas the choroid plexus and the meninges appeared stained. By contrast if the dye was injected directly into the CSF, the brain would stain blue.

The BBB is thought to be important in maintaining the homeostasis of the CNS parenchyma, as neuronal function is dependent on small fluctuations in the concentration of ions and/or molecules such as neurotransmitters. The BBB protects the brain from exogenous substances such as drugs, toxins and infectious agents and also from endogenous substances such as elevated ion concentrations, blood-borne toxic constituents and immunological components. The BBB also eliminates by-products of CNS metabolism, such as neurotransmitters, whose high concentrations would be potentially deleterious for neuronal function. In addition, the entry of many substances necessary for the normal functioning of the CNS is actively regulated at the level of the BBB and these include amino acids, glucose, and sodium, potassium and chloride ions (Pardridge, 1984; Hawkins and Davis, 2005)
The three cellular elements of the brain microvasculature are endothelial cells, astrocyte end-feet, and pericytes (fig 1.1). Brain endothelial cells, in contrast to endothelial cells from the peripheral microvasculature, form a continuous layer of cells, with no detectable fenestrations. Brain endothelial cells are also unique in that they have a reduced pinocytic activity (Broadwell, 1989) and form tight molecular junctions (Rubin and Staddon, 1999). Astrocytes extend foot-like projections surrounding the capillaries, and play an important role in inducing and maintaining the barrier (see section 1.2.2). Less frequent than the astrocytes, pericytes and perivascular cells can also be found scattered along the brain capillary endothelial cells. Pericytes are thought to be a second defence barrier due to their phagocytic properties although their exact role and function remain to be determined.

**Fig 1.1:** The three main cell types of the brain microvasculature: interactions between the brain capillary endothelium, the pericytes, and the astrocyte foot processes are crucial for maintaining the BBB. From Miller (1999)

### 1.2.1. Brain endothelial properties

Microvascular brain endothelial cells are unique in that they present a barrier phenotype. These observations were initially made in electron microscopy studies which demonstrated that tracers administrated intravenously, such as ionic lanthanum, do not cross the BBB
and are retained at the level of the vascular endothelium (Donahue, 1964). CNS endothelial cells present a physical barrier to the passage of blood-borne substances due to the presence of tight junctions between them (Bazzoni and Dejana, 2004). In addition, they show functional polarity due to the different distribution of transporters and receptors between the luminal and abluminal membranes, enabling regulation of transport. Certain transporters and intracellular enzymes function as biochemical barriers to many lipophilic substances (table 1.3).

At the electron microscopy level, brain endothelial cells show electron dense contact points between them, which represent tight junctions that can physically restrict flux between the blood and the brain (Reese and Karnovsky, 1967). The presence of tight junctions restricts passage of molecules with a diameter greater than 8-18 Å. There are common features between endothelial cells and epithelial cells. In epithelial cells, junctions are better organized, with tight junctions and adherens junctions following a well-defined spatial distribution along the intercellular cleft. Tight junctions are concentrated at the luminal side of the rim, while AJ are located below the tight junctions. In contrast, in endothelial cells, the junctional architecture is less defined and, along the cleft, adherens junctions are intermingled with tight junctions (Simonini et al., 2000). Moreover, epithelial cells also form desmosomes, which are absent in the endothelium. The organization of endothelial junctions varies along the vascular tree in function of organ-specific requirements (Simonini et al., 2000). For instance, in the brain, where a strict control of permeability between blood and the nervous system is required, junctions are well developed and rich in tight junctions (Wolburg and Lippodt, 2002). In the brain, they are formed by a complex of transmembrane proteins, the extracellular part of the protein being in direct interaction with its homologous protein on the adjacent cell and are located on the most apical side. Some of these proteins have been identified, such as claudin-3 and claudin-5, forming the primary seal of the tight junctions (Furuse et al., 1999). Other transmembrane tight junctional proteins include occludin, which has been suggested to be a regulatory protein in
terms of the permeability of the BBB (Hirase et al., 1997), and junctional adhesion molecules (JAM) which participate in regulating the migration of leukocytes (Martin-Padura et al., 1998). In association with transmembrane proteins are cytosolic proteins which participate in the maintenance and regulation of the tight junctions, such as zonula-occludens (ZO) proteins-1, -2, -3 (Huber et al., 2001) (fig 1.2). Adherens junctions are located on the abluminal side in relation to tight junctions. They are found in the endothelium of most vascular beds, and are not specific to the BBB. However, they participate in the formation of a barrier, as tight junctions cannot develop in the absence of adherens junctions. Adherens junctions contain cadherins, specifically VE-cadherin, and a submembranous complex of proteins belonging to the catenin family which bind cadherins to actin microfilaments.

Fig 1.2: Proposed interactions of the major proteins associated with tight junctions at the blood–brain barrier (BBB). From Huber et al. (2001). (AF6 stands for Afadin 6)
As tight junctions prevent the paracellular passage of hydrophilic substances, the endothelial cells are responsible for regulating the transcellular movement of substances. Transcellular diffusion is possible for small lipophilic substances (<600 Da) and small molecules such as O$_2$ and CO$_2$ (Grieb et al., 1985) by penetrating the lipid membrane of the endothelial cells. Many lipid soluble substances are substrates of efflux transporters and actively pumped out of the brain (Scherrmann, 2002). The function of efflux pumps is to protect the brain from various substances. An example of such a transporter is P-glycoprotein (P-gp), a membrane-bound ATP-dependent efflux transporter which protects the brain against xenobiotics (Fairchild et al., 1987). Another limiting factor of the endothelial cells to drug permeability is the presence of several enzyme implicated in drug oxidation (Ghersi-Egea et al., 1994). Hydrophilic substances can traverse the BBB via specific carrier mediated transport mechanisms, such as for amino acids, glucose and nucleosides. For example, glucose, one of the main sources of energy for the brain, is transported across the endothelial cells in its D-form via a transporter termed GLUT-1 (Dobrogowska and Vorbrodt, 1999). Vesicular transport may be receptor-mediated or, alternatively, cationic proteins can be taken up via electrostatic interactions with anionic sites on the endothelial surface triggering adsorptive endocytosis (absorptive mediated transcytosis). Most of these transmembrane proteins are in the luminal or abluminal membranes of the endothelial cells and control the uptake of numerous drugs (Scherrmann, 2002). Table 1.3 summarizes some of the transporters found on the luminal and abluminal sides of endothelial cells at the BBB.
<table>
<thead>
<tr>
<th>Transporters</th>
<th>Substrates</th>
<th>Luminal</th>
<th>Abluminal</th>
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<tr>
<td>GLUT-1</td>
<td>Glucose</td>
<td>+</td>
<td>++++</td>
<td>(Mann et al., 2003)</td>
</tr>
<tr>
<td>System L1</td>
<td>Branched chain and aromatic neutral amino acid</td>
<td>+</td>
<td>+</td>
<td>(Mann et al., 2003)</td>
</tr>
<tr>
<td>System B&lt;sup&gt;(0)&lt;/sup&gt;</td>
<td>Neutral and dibasic amino acid</td>
<td>-</td>
<td>+</td>
<td>(Mann et al., 2003)</td>
</tr>
<tr>
<td>Organic anion transporters-1</td>
<td>Anionic compounds</td>
<td>+</td>
<td>-</td>
<td>(Mann et al., 2003)</td>
</tr>
<tr>
<td>Organic cation transporters</td>
<td>Cationic compounds</td>
<td>+</td>
<td>-</td>
<td>(Mann et al., 2003)</td>
</tr>
<tr>
<td><strong>EFFLUX TRANSPORTERS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-gp</td>
<td>Cationic or highly lipophilic compounds</td>
<td>+</td>
<td>-</td>
<td>(Sun et al., 2003)</td>
</tr>
<tr>
<td>Multidrug resistance</td>
<td>Organic anion transporters</td>
<td>+</td>
<td>+</td>
<td>(Sun et al., 2003)</td>
</tr>
<tr>
<td>Breast cancer resistance</td>
<td>molecules of either negative or positive charge, organic anions and sulphate conjugates</td>
<td>+</td>
<td>-</td>
<td>(Cooray et al., 2002)</td>
</tr>
<tr>
<td><strong>RECEPTOR MEDIATED TRANSCELLATION</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Transferrin receptor</td>
<td>transferrin</td>
<td>+</td>
<td>-</td>
<td>(Roberts et al., 1993)</td>
</tr>
<tr>
<td><strong>ADSORPTIVE TRANSCYTOSIS</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Acidic glycoproteins</td>
<td>Cationic peptides</td>
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<td></td>
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<td></td>
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<tr>
<td>γ-Glutamyl Transpeptidase</td>
<td>Large and neutral amino acids</td>
<td>+</td>
<td>+</td>
<td>(Cancilla et al, 1993)</td>
</tr>
</tbody>
</table>

Table 1.3: Transporters found on the luminal and abluminal sides of endothelial cells at the BBB.

1.2.2. Role of astrocytes in barrier phenotype induction

As shown in fig 1.1, astrocytic end feet are in close contact with the basement membrane surrounding the endothelium. Astrocytes are important for the induction and maintenance of a barrier phenotype in brain endothelium. Indeed, astrocytes are thought to produce brain endothelial trophic factors. This was demonstrated by a classical *in vivo* experiment in which a piece of CNS tissue was grafted into a non-brain tissue in rat (Svendgaard *et al.*, 1975). The non-CNS capillaries growing into the grafted CNS tissue developed some
properties of the BBB, tested by its barrier properties toward L-dopa (Svendgaard et al., 1975). Astrocytes were confirmed to be the main candidate cell type for this effect since cultured rat astrocytes, implanted into the anterior eye chamber, normally with leaky vessels, were able to induce tightening of the endothelium (Janzer and Raff, 1987). These observations were further reinforced by in vitro studies. Isolated endothelial cells from brain tissue lose some of their phenotype, but either co-culture with astrocytes or addition of astrocyte conditioned medium (ACM) are able to reinduce some BBB properties. Indeed, in the presence of astrocytes, brain endothelial monolayers form tighter junctions with higher transendothelial electrical resistance (TEER) and lower rates of paracellular permeability (Rubin et al., 1991; Hayashi et al., 1997). Some specific transport systems are also up-regulated under these conditions such as GLUT-1 and P-gp (El Hafny et al., 1997; Sobue et al., 1999). However, the ability to acquire a barrier phenotype is not specific to brain endothelial cells. In human umbilical vein endothelial cells (HUVEC), the immunostaining for tight junction proteins is inherently discontinuous in the absence of inducing factors contrary to what is observed with brain endothelial cells but expression of these proteins increases dramatically in the presence of ACM (Burns et al., 1997).

1.2.3. Diapedesis across the Blood-brain barrier

Leukocyte migration from the blood, across the vascular endothelium and into a tissue area of inflammation has been shown to be highly directed and follows a specific sequence of events. The transmigration of the leukocytes themselves has been described as a process involving three interdependent steps. The first step involves the transient adhesion of leukocytes to the vascular endothelium. In this step, the blood flow causes the leukocytes to roll over the endothelial cells and is known as ‘rolling’. The second step consists of the activation of leukocytes by chemokines presented on the endothelial surface and their firm adhesion to the endothelium. Finally, the last step involves the spreading of the immune cell and the migration through the endothelial barrier, a phenomenon also known as
diapedesis (Ley, 1996; Millan and Ridley, 2005). These three steps are further explained in the following sub-sections.

1.2.3.1. Capture and rolling

The capture and rolling of leukocytes on endothelial cells is a process in which free circulating leukocytes in the blood start to interact with the endothelial cells and tether towards the site of inflammation. Supported by the blood flow, leukocytes roll along the endothelium (Gonzalez-Amaro and Sanchez-Madrid, 1999). Selectins are the proteins responsible for this initial weak bond between endothelial cells and leukocytes. Three selectins have been identified: P-, E- and L-selectin.

P-selectin is a highly glycosylated 140 kDa protein constitutively expressed by endothelial cells. P-selectin is stored in Weibel–Palade bodies, endothelial-specific vesicles that also store von Willebrand factor (vWF). Upon stimulation by inflammatory mediators, Weibel–Palade bodies fuse with the plasma membrane, which results in the surface expression of P-selectin within minutes (Dole et al., 2005). Like P-selectin, E-selectin is expressed by endothelial cells (Hooft van Huijsduijnen et al., 1992). The relative molecular weight of unglycosylated E-selectin is about 64 kDa but ranges between 107 and 115 kDa depending on the level of glycosylation. E-selectin is not constitutively expressed by endothelial cells, instead, E-selectin expression is transcriptionally regulated by mediators such as TNF-α and IL-1 and its peak expression occurs at 6 h following cytokine stimulation (Wyble et al., 1997). L-selectin is expressed in most peripheral blood leukocytes, and has been suggested to be involved in leukocyte trafficking (Lewinsohn et al., 1987). The ligands for P- and E-selectins include CD24, P-selectin glycoprotein ligand-1 and E-selectin ligand-1 on the leukocytes, and the ligands for L-selectin include CD34, mucosal addressin cell adhesion molecule-1 and glycosylation-dependent cell adhesion molecule-1 on the endothelial cells (Millan and Ridley, 2005).
Fig 1.3: The different stages of leukocyte migration, showing capture and rolling, firm adhesion of leukocytes to endothelial cells, and finally transmigration by either paracellular or transcellular processes. Adapted from Millan and Ridley (2005).

The duration of selectin-mediated rolling is about 50-500ms and is followed by rapid integrin avidity changes in leukocytes induced by chemokines (Alon et al., 1997) known as firm adhesion.

1.2.3.2. Firm adhesion

The proteins responsible for firm adhesion and subsequent arrest of leukocytes on the endothelial cells are called integrins. Leukocyte firm adhesion is mediated by activation of leukocyte integrins which involve integrin clustering and higher affinity/avidity. These changes are initially triggered by the binding of chemokines present on the endothelial cells bound to glycosaminoglycans onto chemokine receptors expressed by leukocytes (Tanaka et al., 1993; Kitayama et al., 1998) activating heterotrimeric G-protein-dependent
signalling. Chemokine induced signal transduction results in changes in integrin avidity/affinity which then facilitates integrin interactions with their counterpart Immunoglobulin-like adhesion molecules on endothelial cells. Among the different integrins present on leukocytes, α4β1 integrin, which binds Vascular Cell Adhesion Molecule (VCAM) present on the endothelial cells, is thought to be important in the early stages of the firm adhesion. VCAM-α4β1 integrin interactions can up-regulate the avidity of α1β2 integrin for its ligand InterCellular Adhesion Molecule (ICAM) present on the endothelial cells (Chan et al., 2000). The expression of cell adhesion molecules of the immunoglobulin superfamily on the endothelial cell surface can be up-regulated by cytokines (dos Santos et al., 1996). Effectively, the binding of integrins to adhesion molecules triggers downstream signals in both leukocytes and endothelial cells. These intracellular signals will subsequently lead to morphological changes which allow the final step of transmigration. Leukocytes spread out and consequently are able to squeeze (in amoeboid fashion) between the endothelial cells. ICAM-1 cross-linking using antibodies mimicking interactions resulting from leukocyte integrin clustering, induces an increase in Ca2+ flux in endothelial cells (Etienne-Manneville et al., 2000). An increase in intracellular Ca2+ leads to phosphorylation of myosin light chains and unfolding of myosin II, causing the endothelial cells to retract facilitating the passage of leukocytes (Hixenbaugh et al., 1997). Inhibition of an increase in intracellular Ca2+ in endothelial cells blocks neutrophil transmigration (Huang et al., 1993).

1.2.3.3. Transendothelial migration

Transmigration is a rapid process by which leukocytes crawl between tightly apposed endothelial cells. The initial stage involves a leukocyte extending a pseudopod along the endothelial cell border. While in non-CNS endothelial cells, most investigators are in agreement that the majority of leukocytes enter the sites of inflammation by passing between endothelial cells, the route of transmigration of leukocytes across brain
endothelium is much less clear due to the presence of tight junctions. Two likely transmigration routes have been proposed: a paracellular route that involves crawling through transiently opened tight junctions, and a transcellular route that involves a leukocyte being engulfed by the endothelial cell and being released on the abluminal side near the endothelial cell junctional area (fig 1.3) (Millan and Ridley, 2005). Whatever the route, leukocyte transmigration requires a rapid disassembly of the cytoskeleton of leukocytes on the apical side and a reassembly on the abluminal side of the endothelium (Brown, 2001). Similarly to the steps of capture and firm adhesion, transendothelial migration requires the interaction of transmembrane proteins between leukocytes and endothelial cells, although in this case it involves homophilic interactions. These include Platelet endothelial cell adhesion molecule-1 (PECAM-1), CD99, and proteins of the JAM family, all of which are located at the cell-cell junction on endothelial cells and are strongly expressed by activated leukocytes (Liu et al., 2004). PECAM-1 and JAM are members of the immunoglobulin gene superfamily whereas CD99 is a unique heavily glycosylated type I transmembrane protein. The role of PECAM-1 in leukocyte transmigration is well established as blocking PECAM-1 with a neutralizing antibody inhibits diapedesis in vivo (Bogen et al., 1994). CD99 appears to be involved at a later stage than PECAM-1 since blocking CD99 arrested monocytes at a point where they had partially migrated through the junction of HUVEC (Schenkel et al., 2002). The role of JAM is still unclear as they have only recently been identified (Martin-Padura et al., 1998). Nevertheless, using neutralizing antibodies, it has been possible to demonstrate the importance of JAM in transmigration. Indeed, an antibody against JAM-c blocked migration of human polymorphonuclear leucocytes across a HUVEC monolayer in response to a CXCL12 chemokine gradient (Martin-Padura et al., 1998).
1.2.4. The blood-brain barrier in multiple sclerosis

1.2.4.1. Alterations in adhesion molecule expression

As described in section 1.2.3, endothelial cells play an active role in leukocyte recruitment. In addition, endothelial cell function appears to be altered in MS. Isolated brain microvessels from MS patients exhibit a higher adhesion capacity together with an increase in ICAM-1 expression (Lou et al., 1997). This up-regulation of ICAM-1 has been confirmed in MS brains and was much greater in the MS lesions (Bo et al., 1996). A soluble form of ICAM-1, VCAM-1 and L-selectin can also be detected in the blood and in the intrathecal fluid of MS patients (Duran et al., 1999). This allows a non-invasive follow up of ICAM-1 expression in MS and its correlation with symptoms during MS. The concentration of soluble ICAM-1 is higher in both the serum and the CSF in patients with acute relapse compared to patients with stable disease (Alves-Leon et al., 2001). Furthermore, soluble ICAM-1 production in the CSF correlated with appearance of lesions on gadolinium-MRI and immunoglobulin G (IgG) level in the CSF (Acar et al., 2005).

In the CSF, VCAM-1 and E-selectin soluble form are also increased during the duration of the disease (Correale and Bassani Molinas Mde, 2003). Soluble PECAM-1 was reported to increase in the serum in MS only during the formation of active lesions (Losy et al., 1999). No studies on JAM-1 and CD99 in MS have been reported to date. The involvement of VLA-4/VCAM-1 interactions in leukocyte entry into the CNS and subsequent disease has been demonstrated using a monoclonal antibody against VLA-4 which diminished the infiltration of VLA-4-positive cells into the brain of an animal model of MS, EAE, and suppressed the clinical and histopathological signs (Soilu-Hanninen et al., 1997). The increase in adhesion molecule expression may be the result of activation of endothelial cells by cytokines. Indeed, it has been shown that cytokines such as TNF-α and IFN-γ
induce an up-regulation of adhesion molecules by endothelial cells in vitro, and cytokines are largely up-regulated in MS, as described in section 1.3.5.1.

1.2.4.2. Changes in blood-brain barrier permeability

In MS, there is also an increase in the permeability of the BBB which can be visualised by MRI gadolinium enhancement, showing the opening of the BBB at the tight junctions level (Werring et al., 2000). The integrity of the BBB is an important factor in the normal function of the brain since it forms a barrier that protects the CNS cells from blood-born substances. The integrity of the tight junctions in MS is compromised and discontinuous staining of tight junction proteins, such as ZO-1, in MS lesions has been demonstrated, most importantly in active lesions, but there is also abnormal ZO-1 staining in inactive lesions (Kirk et al., 2003). Interestingly there is also a change in ZO-1 expression in normal appearing white matter (NAWM) tissue when compared to tissue from healthy controls (Plumb et al., 2002) where a small (around 4%) blood vessel opening can be observed (Kirk et al., 2003). Another tight junction protein which has been reported to be altered in inflammatory conditions is claudin-3 which is lost in EAE lesions (Wolburg et al., 2003).

A variety of factors that can affect the permeability of the BBB are present in MS lesions or the serum. Indeed, the serum from MS patients down regulates occludin and VE-cadherin expression in cultured brain endothelial cells (Minagar et al., 2003). This effect could be the result of cytokine activation, since cytokines are also detected in MS lesions. Among some of these cytokines are TNF-α and IFN-γ, which have been shown to reduce the expression of occludin in vitro in epithelial cells (Mankertz et al., 2000). Other factors that can disrupt the organization of tight junctions is vascular endothelial growth factor (VEGF), present in chronic and active lesions (Proescholdt et al., 2002), or the MMP, such as MMP-9, which is increased in the serum of MS patients (Correale and Bassani Molinas
Mde, 2003) and display a strong immunoreactivity in blood vessel walls in active lesions (Lindberg et al., 2001).

Other changes that can contribute to the increased permeability of the BBB in MS patients have been reported. These may involve vesicular exchange, as pinocytotic vesicles have been shown to be markedly increased in endothelial cells in MS (Brown, 1978).

The pathology of vessels has been proposed previously as possible pathogenic mechanism for this disease (Courville, 1968). In this model, vascular damage would result in myelin destruction as a result of loss of CNS homeostasis, neurons being particularly sensitive to these variations. This hypothesis was reinforced by the detection of antibodies directed against proteins of human brain vessels (Souberbielle et al., 1992) but the presence of these antibodies could not be correlated with the clinical course of the disease or with abnormal MRI activity (Tintore et al., 1996). The time course the alteration of the BBB is still not clear, some studies showing an early disruption (Werring et al., 2000) prior to the inflammation while others showed that the change of permeability of the BBB occurs in parallel with leukocyte infiltration. In addition, it has been demonstrated that monocytes use a transcellular migration mechanism without disrupting BBB integrity suggesting that leukocyte diapedesis is not always associated with a change in tight junction organisation and BBB permeability (Wolburg et al., 2005).

1.3 Chemokines

1.3.1. Structure and function

Chemokines are small molecular weight peptides responsible for adhesion, activation, and chemotaxis of leukocytes. Based on genomic studies, it has been estimated that there are as many as forty to fifty human chemokines. Many of the genes encoding chemokines have
been found to cluster at specific loci, mainly grouped at 17q11.2-12 and 4q13 (Oppenheim et al., 1991). Chemokines are structurally related, possessing a pattern of conserved cysteine residues near the amino-terminal domain (Murphy et al., 2000). It is the position of the first and second conserved cysteine residues that determines the basis for the classification of chemokines into four families (CC, CXC, CX3C and XC or the α, β, δ and γ subfamilies in the old nomenclature, respectively). CC chemokines have no amino acids between the two cysteine residues whereas CXC and CX3C chemokines have one and three, respectively. XC chemokines only have one cysteine residue (fig 1.4).

**Fig 1.4:** Classification of chemokine receptors based on the structural characteristics of their relative ligands (stylized beside). The list of chemokines binding each receptor is indicated. From Bajetto et al. (2002).

CXC chemokines can be further subdivided into ELR (glutamic acid/leucine/arginine)-containing and non-ELR-containing chemokines depending on the presence of an ELR
motif just prior to the CXC signature. The ELR-containing C-X-C chemokines attract neutrophils whereas the non-ELR-containing C-X-C chemokines attract lymphocytes (Baggiolini, 1998). It has also been shown that CXC chemokines with an ELR+ motif are angiogenic, while chemokines with an ELR- motif are angiostatic with the exception of CXCL12 and CXCL2 (Bernardini et al., 2003). The requirement of the ELR motif for the angiogenic effect has been demonstrated by site-directed mutagenesis substitution of the ELR motif in the sequence of an angiogenic chemokine (i.e. IL-8/CXCL8) with a non-ELR motif of the angiostatic molecule (Mig/CXCL9) and vice versa. The shift in angiogenic properties of the mutated chemokines to angiostatic in both in vitro and in vivo assays strongly supports the importance of the ELR motif as a structural domain for angiogenic activity (Strieter et al., 1995).

The second major branch of the CC chemokines are chemoattractants for a wide range of cells including monocytes, granulocytes other than neutrophils, various subpopulations of lymphocytes including thymocytes, and dendritic cells. The CC subfamily contains the largest number of chemokines (Murphy, 2002). The only XC chemokine cloned to date, XCL1, has been reported to selectively attract CD8+ T lymphocytes. This chemokine has only two cysteines in its primary amino acid sequence (Kelner et al., 1994). Finally, CX3CL1 also called fraktokine, the only member of the CX3C subfamily, has a unique primary amino acid sequence with three intervening amino acids between its first two cysteines (Pan et al., 1997). It is an unusual chemokine in that it is a larger protein, with a chemokine module positioned at the N-terminus, and contains a transmembrane domain and a cytoplasmic tail (Bazan et al., 1997).

1.3.2. Chemokine receptors

Chemokines exert their biological effects on target cells via binding to cell surface receptors. Chemokine receptors are classified into CXC, CC, XC and CX3C receptors
according to the family of chemokines their ligands belong to (fig. 1.4). A remarkable feature of the chemokine receptor superfamily, however, is their promiscuity as far as ligand binding is concerned (fig 1.4), although binding to a receptor only occurs for chemokines from the same sub-group. For example, CCR5 binds to CCL3, CCL4, CCL5 and CCL8 whereas CXCR3 binds to CXCL9, CXCL10 and CXCL11 (fig 1.4). To date, nineteen receptors have been identified among which six receptors bind CXC chemokines (CXCR1-CXCR6), eleven receptors bind CC chemokines (CCR1-CCR11), one receptor for CX3CL1 (CX3CR1) and one receptor for XCL1 (XCR1). D6 and Duffy antigen/receptors for chemokines (DARC) are two other chemokine receptors which are able to bind promiscuously to both CC and CXC chemokines. These two chemokine receptors do not signal, and are thought to be decoy receptors having the role of maintaining chemokine gradients (Comerford and Nibbs, 2005).

Chemokine receptors are typically 340-370 amino acids in length with 25-80% amino acids identical. All chemokine receptors identified to date are G protein-coupled seven transmembrane domain receptors (GPCR). A unique feature of these GPCRs is the presence of an amino acid sequence (DRYLAIV) in the second intracellular loop domain (Murphy, 1994). They all have an extracellular domain which includes the N-terminus and three extracellular loops which constitute the binding sites for the chemokine ligands. In addition, the N-terminus stalk plays a role in the selectivity and affinity of the ligand (Rajagopalan and Rajarathnam, 2004). The intracellular region is composed of three loops and the C-terminus domain. The C-terminus is the site where the G protein binds upon stimulation by chemokines, leading to G protein phosphorylation and binding of a molecule of guanine triphosphate to the \( \alpha \) subunit of the heterotrimeric protein. Indeed, the G protein, composed of three sub-units (the \( \alpha \), \( \beta \) and \( \gamma \) sub-units), separates into the \( \alpha \) and the \( \beta \gamma \) sub-unit, each of them activating other signalling proteins such as adenyl and...
guanylyl cyclases, phosphodiesterases, phospholipase A2 and phospholipase C, leading to an increase in second messengers, such as intracellular calcium, important for cytoskeletal changes during transmigration. Functional responses induced by most chemokines are blocked by *Bordatella pertuxin* toxin, indicating that intracellular signalling is mediated by the Go1 sub-unit (Bokoch, 1995). The increase in second messengers and also activation of kinases such as phosphoinositide-3-kinase, can in turn, depending on the cell type, chemokine ligand and its receptor, activate other downstream proteins such as protein kinase A, protein kinase C, the p38 mitogen-activated protein (MAP) kinase, SAPK/JNK and p42/44 MAP (also known as erk) kinase pathways. In some cases, some transcription factors are activated, such as CREB, c-Jun and c-Fos (Bokoch, 1995; Marinissen and Gutkind, 2001).

1.3.3. Physiological functions of chemokines

Chemokines play an important role in orchestrating the immune response, as they activate the recruitment of specific subsets of leukocytes (Baggiolini, 1998; Thelen, 2001), especially during the step of firm adhesion to the endothelium, already described in section 1.2.3.2. The mechanism by which chemokines control leukocyte function will be further described in section 1.3.4.

In addition, chemokines play a role in the formation of blood vessels i.e. angiogenesis, especially members of the CXC chemokine family, whose CXCR family receptors are expressed by endothelial cells (Bernardini *et al.*, 2003). Within this context, CXCL12 (the only CXCR4 ligand) expression has been shown to have a fundamental role during vascular development. CXCR4 or CXCL12 gene-deficient mice die prenatally and exhibit defects in the formation of gastrointestinal tract arteries, as well as defects in vessel development, hematopoiesis, cardiogenesis, and cerebellar development (Nagasawa *et al.*, 1996; Tachibana *et al.*, 1998). Subsequently, a study on CXCR2 gene-deficient mice has
shown that this receptor represents the common mediator for the angiogenic activity of several chemokines, including CXCL1, CXCL2, CXCL3, CXCL5, CXCL6 and CXCL7, and angiogenesis in response to these factors is impaired in the cornea of CXCR2 gene-deficient mice (Addison et al., 2000). Conversely, CXCL4, CXCL9, CXCL11, CXCL13 and CXCL10 inhibit angiogenesis and as such are called angiostatic chemokines (Bernardini et al., 2003).

In addition to their role in leukocyte migration and angiogenesis, it has been suggested that chemokines are implicated in many autoimmune disorders due to their importance in controlling the immune system (Baggiolini, 2001). In addition to MS, other diseases where different chemokines have been reported to play an important role in their pathogenesis include arteriosclerosis, where CCL2 has been suggested to attract monocytes to the atherosclerotic plaque, rheumatoid arthritis, asthma (CCL3) and inflammatory bowel disease (Santamaria, 2003; Christopherson and Hromas, 2004).

Finally, certain chemokine receptors have been identified as co-receptors for viral entry into cells, in particular that of the human immunodeficiency virus (HIV). Chemokines, as natural ligands for these receptors have been shown to block HIV infection. (Cocchi et al., 1995) showed that CCL3, CCL4 and CCL5 could inhibit infection of macrophages by HIV. These three chemokines are ligands for CCR5, which, together with CD4, is the major co-receptor for HIV R5 subtypes (Deng et al., 1996).

1.3.4. Coordination of the immune system

Chemokines may be involved in homeostastic leukocyte trafficking into tissues, as is the case for CXCL12, and in this role they are expressed constitutively by many cell types in a tissue-specific manner. Other chemokines are termed inducible and have been shown to be expressed only in response to specific inflammatory stimuli. For example, IFN-\(\gamma\), produced
by T helper cell type 1 (Th1) lymphocytes, triggers the production of CXCL9 and
CXCL10 (both ligands of CXCR3) which will in turn attract more Th1 lymphocytes. This
is the case in MS lesions where an increase of CXCL10 is correlated with the occurrence
of CXCR3 positive T cells (Balashov et al., 1999). Interleukin (IL)-4 and IL-13 are
produced by activated Th2 lymphocytes. These two cytokines induce the production of
chemokines such as CCL2, CCL11, CCL27, CCL17 and CCL22 which lead to a Th2
lymphocyte pattern of recruitment (Syrbe et al., 1999).

The specific response of cells from the immune system in inflammation is mediated by the
distinct expression of chemokine receptors by different subsets of leukocytes (Table 1.3).
Furthermore, the level of expression of chemokine receptors is dependent on the activation
state of the cell. Indeed, only activated effector lymphocytes respond to inflammatory
chemokines, because naïve cells do not express the corresponding receptors (Syrbe et al.,
1999).

<table>
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<th>Chemokine receptor</th>
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</tr>
<tr>
<td>Th1 T cells</td>
<td>CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR10, CCR3, CCR4, CCR5, CCR6, CX3CR1, XCR1</td>
</tr>
<tr>
<td>Th2 T cells</td>
<td>CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR10, CXCR3, CXCR4, CXCR5, CXCR6, CX3CR1, XCR1</td>
</tr>
<tr>
<td>α4β7+ T cells</td>
<td>CCR9</td>
</tr>
<tr>
<td>CLA+ T cells</td>
<td>CCR10</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>CCR1, CCR3</td>
</tr>
<tr>
<td>Basophils</td>
<td>CCR1, CCR2, CCR3</td>
</tr>
<tr>
<td>Neutrophils</td>
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<td>Monocytes</td>
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</tr>
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<td>Macrophages</td>
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</tr>
<tr>
<td>NK cells</td>
<td>CXCR3, CCR2, CX3CR1</td>
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</tr>
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<td>Mast cells</td>
<td>CXCR1, CXCR2, CCR3</td>
</tr>
</tbody>
</table>

Table 1.4: Functional expression of chemokine receptors in different leukocyte populations. Adapted from Olson and Ley (2002)
1.3.5. Cytokines, chemokines and chemokine receptors in multiple sclerosis

1.3.5.1. Implication of cytokines in multiple sclerosis

MS is thought to be a Th1 lymphocyte-mediated disease. CD4+ T-cells have been classified into three sub-types, Th1, Th2 and Th0 cells, characterised by their ability to produce specific subsets of cytokines. Th1 cells produce mainly TNF-α and IFN-γ and the role of these cytokines has been extensively investigated.

IFN-γ has not been shown to be up-regulated in CSF and serum of MS patients (Gallo et al., 1991), whereas the elevation of TNF-α is more controversial. A study showed no significant increase of TNF-α in the serum and CSF in PP-MS or RR-MS (Rovaris et al., 1996; Kleine et al., 2003), while others have demonstrated periodic increases of TNF-α in CSF and serum of patients with RR-MS (Spuler et al., 1996). However, clinical trials with a recombinant TNF receptor p55 immunoglobulin fusion protein which is protective in EAE, showed an increased number of exacerbations occurring earlier in MS patients (The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group, 1999). In another study, a different TNF-α-based therapeutic regime induced the appearance of new lesions, as indicated by MRI studies when MS patients were treated with the monoclonal anti-TNF-α antibody cA2 (van Oosten et al., 1996). Intravenous injections of recombinant IFN-γ worsens MS symptoms (Panitch et al., 1987a; Panitch et al., 1987b), whereas treatment with anti-IFN-γ antibody in SP-MS results in significant improvement of symptoms (Skurkovich et al., 2001). However, although promising, the latter study by (Skurkovich et al., 2001) was carried out on a small group of individuals and within a short period of time, and not all patients responded to anti-IFN-γ antibody treatment. Overall, the results from clinical MS studies are very different to those obtained from EAE studies (section 1.3.6.1).
In addition to TNF-α and IFN-γ, other cytokines may be implicated in MS pathogenesis. For example, transforming growth factor (TGF)-β1 mRNA levels in peripheral blood mononuclear cells have been shown to correlate with MRI disease activity in MS (Bertolotto et al., 1999), and serum levels of TGF-β1 are elevated in both RR- and SP-MS patients (Nicoletti et al., 1998). In active MS lesions, the three forms of TGF-β were detected mainly on foamy macrophages and hypertrophic astrocytes (De Groot et al., 1999). TGF-β2 reduces demyelination, virus antigen expression, and macrophage recruitment in a viral model of multiple sclerosis (Drescher et al., 2000). Other cytokines, regarded as important in the regulation of T-cell mediated inflammation, such as IL-2 (Gallo et al., 1991), IL-1 (Rovaris et al., 1996) or IL-6 (Rovaris et al., 1996) have been shown to increase in serum or in CSF of MS patients.

1.3.5.2. Chemokine plasma and cerebrospinal fluid levels in multiple sclerosis

Alterations in the concentration of chemokines in plasma may reflect the level of activity of the immune system during the development of MS. An increase in CXCL8 plasma levels during the relapse phase of RR-MS has been reported (Saruhan-Direskeneli et al., 2003; Lund et al., 2004; Bartosik-Psujek and Stelmasiak, 2005). However, whether the plasma levels of other chemokines such as CXCL10 and CCL2 increase in MS remains unclear, with some studies showing similar plasma levels in MS and control patients (Saruhan-Direskeneli et al., 2003; Bartosik-Psujek and Stelmasiak, 2005) whereas others showed an increase in plasma levels in MS patients (Scarpini et al., 2002).

A more reliable method that should more closely reflect changes in chemokine concentrations within the CNS parenchyma is to determine their levels in the CSF obtained by lumbar puncture. Another advantage of this method is that levels of different chemokines can be correlated with the presence of distinct populations of leukocytes within the CSF, which are usually absent in healthy individuals. The concentration of a
number of chemokines has been shown to be altered in the CSF of MS patients. Among them, CSF CXCL10 levels have been shown to increase in MS patients (Mahad et al., 2002; Scarpini et al., 2002) and this increase has been associated with relapses (Mahad et al., 2002). As MS is considered to be a Th1-driven autoimmune disorder, the increase of CXCL10 in the CSF (Mahad et al., 2002) correlates with the presence of CXCR3+ T lymphocytes in the CFS and with the appearance of lesions on the MRI scans (Sindem et al., 2002). In addition, other immune cells such as macrophages and B-cells have been detected in the CSF of MS patients (Cepok et al., 2001). Most interestingly the level of CCL2, known to attract macrophages, decreases in association with relapses (Sindem et al., 2001; Mahad et al., 2002; Scarpini et al., 2002; Saruhan-Direskeneli et al., 2003; Bartosik-Psujek and Stelmasiak, 2005). Other candidate chemokines, whose elevated CSF levels may reflect infiltration of macrophages into the CNS are CCL3 and CCL5. Initially, CCL3 was shown to moderately increase in the CSF of MS patients (Miyagishi et al., 1995), although levels were too low to be accurately determined and this effect was later disputed (Mahad et al., 2002). By contrast, increases in CCL5 CSF levels (Bartosik-Psujek et al., 2004; Bartosik-Psujek and Stelmasiak, 2005) correlate with active Gadolinium-enhancing lesions on MRI revealing BBB breakdown (Sindem et al., 2001), as gadolinium does not cross the BBB under normal circumstances. Neutrophils are not reported to play an important role in MS, as they are not present in the leukocyte infiltrates in MS lesions. Nevertheless, CXCL8 which is known to be an important chemoattractant for neutrophils, is increased in the CSF of MS patients (Bartosik-Psujek et al., 2004; Bartosik-Psujek and Stelmasiak, 2005).

1.3.5.3. Chemokine and chemokine receptor expression in multiple sclerosis lesions

Because of the poor availability of human tissue material, only a small number of studies have compared the expression of chemokines and chemokine receptors between MS lesions and NAWM. Interestingly, the changes in the expression of chemokines and
chemokine receptors observed in MS lesions correlate well with the alterations in chemokine levels detected in the CSF, as described in section 1.5.5.2

CXCL10 is not detected in NAWM, but becomes strongly expressed in all MS lesion types, in particular in association with reactive astrocytes and T lymphocytes as determined by immunohistochemistry (Sorensen et al., 1999; Simpson et al., 2000b; Sorensen et al., 2002). CXCL10 expression has also been detected in perivascular cuffs of infiltrating leukocytes (Sorensen et al., 2002) and in macrophages (Simpson et al., 2000b). Its only receptor, CXCR3, is expressed by CD3+ cells, corresponding to T lymphocytes, predominantly appearing in early lesions (Sorensen et al., 1999; Simpson et al., 2000b; Sorensen et al., 2002; Trebst et al., 2003) and by astrocytes (Simpson et al., 2000b). Simpson et al. (2000b) also demonstrated the expression of CXCL9, another ligand for CXCR3, with a similar pattern of expression to that observed for CXCL10.

Similarly to CXCL10, CCL2 has been shown to co-localise with astrocytes and macrophages in MS lesions (McManus et al., 1998; Simpson et al., 1998). CCL2 staining on these cells was more intense when the lesion was active and tended to decrease in parallel with the level of active demyelination. Within the active lesion, some staining was located on the blood vessels in the centre of the lesion, although the exact cellular origin of CCL2 could not be ascertained. According to the authors, perivascular CCL2 expression may be the result of CCL2 released by activated astrocytes which would then form a gradient resulting in chemotactic leukocyte migration (McManus et al., 1998; Simpson et al., 1998). CCR2 expression was detected on macrophages and astrocytes and in the perivascular cuff but not in NAWM (Simpson et al., 2000a).

Finally, CCL5 was detected exclusively in MS lesions associated with blood vessels, both in the perivascular cuff and on the endothelial cells while there was a diffuse staining of
endothelial cells in the control tissue (Simpson et al., 1998). Expression of the receptors for CCL5, CCR1 and CCR5, in MS lesions is also cell type-specific. CCR1 is only expressed on leukocytes, either lymphocytes or macrophages (Sorensen et al., 1999; Trebst et al., 2001; Trebst et al., 2003), while CCR5 is detected on the microglia within the lesion only (Sorensen et al., 1999).

1.3.6. Cytokines and chemokines and chemokine receptors in experimental autoimmune encephalomyelitis

EAE is not MS, but a model for neuroinflammation and delayed-type hypersensitivity. It has nevertheless permitted the study of the cascade of immunological and molecular mechanisms of neuroinflammation. In relapsing EAE models, T-cells are activated in the peripheral lymphoid tissue by injection of antigenic immunopeptides (Voskuhl, 1998). They traffic into the bloodstream, and once in the bloodstream they get into the CNS where they may or may not be restimulated. If they are stimulated, T-cells produce inflammatory cytokines, chemokines and IFN-γ, which induce the accumulation of other mononuclear cells. This induces a new wave of CNS inflammation, and T cell accumulation results in clinical disease progression (Fife et al, 2001).

1.3.6.1. Cytokines in experimental autoimmune encephalomyelitis

TNF-α appears to play an important role in the pathogenesis of EAE, the animal model for MS, although whether its action is beneficial or detrimental is still debated. In the mouse model of transfer EAE, where myelin basic protein-specific T cells are injected into naïve mice, blocking antibodies directed towards lymphotoxin or towards TNF-α reduced the severity of the disease (Ruddle et al., 1990). In addition, treatment with soluble TNF-α receptor, in a relapsing-remitting model of EAE, using mice which were injected with encephalitogenic PLP peptide, also reduced clinical symptoms, and inhibited elevated CNS chemokine levels (Glabinski et al., 2004). By contrast, TNF-α knock-out mice showed a
much more severe form of EAE than wild type mice, when they were immunized with myelin oligodendrocyte glycoprotein (Liu et al., 1998). This exacerbation of symptoms could be reversed by TNF-α administration. IFN-γ is not crucial for the induction or the clinical course of EAE, as IFN-γ knockout mice immunized with myelin basic protein, still developed the disease (Ferber et al., 1996). Furthermore IFN-γ is thought to be important for the recovery phase of EAE, as intrathecal injection shortened the recovery phase in mice which were EAE induced by injection of myelin oligodendrocyte glycoprotein (Furlan et al., 2001).

1.3.6.2. Variations in chemokines in experimental autoimmune encephalomyelitis

CCL2, CCL3, CCL4, CCL5, CXCL10 and other chemokines are rapidly expressed one or two days before onset of clinical symptoms in EAE (Godiska et al., 1995). As the disease progresses there is an increase in the expression of chemokines (Glabinski et al., 2003). Chemokine expression varies, with CCL3 expression, greater than CCL5 expression which is higher than CCL4. CCL2 does not become fully expressed until the relapsing phase of disease where its expression in CNS correlates with clinical disease score in EAE (Kennedy et al., 1998). CNS infiltrated T-cells express CCL3, CCL4, CCL5, and CXCL10 (Glabinski et al., 1997). Monocytes and macrophages that infiltrate the CNS from the periphery express CCL3, CCL5 and CCL2 (Miyagishi et al., 1997), whereas resident astrocytes express CXCL10, CCL2, CCL5 and CXCL10 in the lesions (Glabinski et al., 1997; Miyagishi et al., 1997; Galbinski et al., 1999; Nygardas et al., 2000). Anti-CCL3 antibody inhibits acute EAE development (Kennedy et al., 1998). However the usage of knockout mice reveals the complex regulation of chemokines in EAE, as CCL3 knockout mice developed equally severe symptoms as normal mice (Tran et al., 2000a). Absence of CCL2 in mice leads to decreased local macrophage recruitment and antigen-specific Th1 immune response in EAE (Izikson et al., 2000; Huang et al., 2001). Results concerning the blocking of CXCL10 or CCL3 in EAE gave contradicting results. Administration of
plasmid DNA encoding self CXCL10, leading to production of self-specific antibodies, could suppress EAE (Wildbaum et al., 2002). Others demonstrated that neutralization of CXCL10 exacerbates EAE (Narumi et al., 2002) or showed that CXCL10 deficient mice had a greater number of CNS lesions and a lower threshold for induction of the disease (Klein et al., 2004).

1.4 Aims

The general aim of the present work was to investigate the effects of specific chemokines on BBB function in terms of both immunological responses (expression of adhesion molecules and leukocyte adhesion) and permeability. The specific objectives were:

1) To develop and characterise a method to isolate primary HBEC that are suitable as an in vitro human BBB model.

2) To develop an immortalised HBEC line in collaboration with Cochin Institute in Paris, to facilitate the investigation of chemokine actions at the BBB.

3) To characterise the basal and cytokine-induced expression of chemokines and chemokine receptors by HBEC both in vitro and in situ.

4) To investigate the functional consequences at the in vitro human BBB of specific chemokines for which elevated levels of receptors were found to be expressed by HBEC.
2. Chapter 2: Chemokine expression by primary human brain endothelial cells *in vitro*
2.1 Introduction

Whether it is activated circulating leukocytes or a local inflammatory reaction that triggers
the subsequent leukocyte infiltration is still a much debated subject, but a direct correlation
between chemokine production and demyelination in the CNS of MS patients has been
established. Chemokines have been suggested to be produced by resident cells such as
astrocytes and microglia in response to the up-regulation of cytokines in the brain
parenchyma (Croitoru-Lamoury et al., 2003; Kremlev et al., 2004). MS is mainly
associated with infiltration of activated Th1 T-lymphocytes which express CXCR3 and
CCR5 receptors (Balashov et al., 1999). The Th1 T-lymphocyte subset of T cells is a
source of IFN-γ and IL-2 whereas activated Th2 lymphocytes release IL-4 and IL-10. In
addition, an increase in IFN-γ and TNF-α has been shown to precede relapses in MS
patients, whereas in chronic stages of the disease, both cytokines are present (Beck et al.,
1988). IFN-γ induces the production of CXCL9, CXCL10 and CXCL11, and could further
lead to the recruitment of activated CXCR3+ lymphocytes. By contrast, TGF-β appears to
have a suppressive role in MS. Elevated TGF-β levels have been found in the CSF of MS
patients during remissions (Carrieri et al., 1997) and in the stable phase of MS (Carrieri et
al., 1998).

HBEC are the first cells to be in contact with leukocytes when they enter the brain.
Chemokines bound to the proteoglycans of the luminal surface of endothelial cells are
presented to circulating leukocytes and trigger their firm adhesion to the endothelium by
activation of the corresponding chemokine receptor expressed on the surface of the
leukocytes. CNS resident cells are able to produce chemokines following cytokine
stimulation and this has been demonstrated both in vivo and in vitro for astrocytes (Oh et
al., 1999; Croitoru-Lamoury et al., 2003; Carpentier et al., 2005), and microglia (Lee et al.,
The release of chemokines by these cells, transported across the endothelial cells before being presented at its luminal surface, create a chemical gradient guiding leukocyte entry into the CNS (Brown, 2001; Biernacki et al., 2004). Another potential source of chemokines is the HBEC constituting the brain microvessels. Although a number of studies have shown that HBEC are able to produce chemokines in response to a wide range of stimuli (see table 2.1), whether particular cytokines important in MS and/or combinations of them act synergistically to induce the expression of several chemokines by HBEC remains to be established.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Endothelium Cell type</th>
<th>Chemokines</th>
<th>Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Zidovetzki et al., 1999)</td>
<td>HBEC</td>
<td>CXCL8 (mRNA)</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>(Vadeboncoeur et al., 2003)</td>
<td>HUVEC HBEC</td>
<td>CXCL8 CCL2</td>
<td>Streptococcus suis</td>
</tr>
<tr>
<td>(Lee et al., 2003b)</td>
<td>HBEC</td>
<td>CXCL8</td>
<td>VEGF</td>
</tr>
<tr>
<td>(Omari et al., 2004)</td>
<td>HBEC</td>
<td>CCL2 CCL5</td>
<td>CD40/CD40L interactions</td>
</tr>
<tr>
<td>(Salmaggi et al., 2002)</td>
<td>HBEC HUVEC</td>
<td>CXCL10</td>
<td></td>
</tr>
<tr>
<td>(Weiss et al., 1998)</td>
<td>HUVEC HBEC</td>
<td>CCL2</td>
<td>TNF-α, IFN-γ, IL1-β</td>
</tr>
<tr>
<td>(Frigerio et al., 1998)</td>
<td>HBEC HUVEC</td>
<td>CCL2</td>
<td>TNF-α , IFN-γ</td>
</tr>
<tr>
<td>(Chen et al., 2001)</td>
<td>HBEC</td>
<td>CCL2</td>
<td>Endothelin-1, TNF-α, IL1-β</td>
</tr>
<tr>
<td>(Biernacki et al., 2004)</td>
<td>HBEC</td>
<td>CXCL8 CXCL10 CCL2</td>
<td>Supernatant of activated Th1</td>
</tr>
<tr>
<td>(Hofman et al., 1999)</td>
<td>HBEC</td>
<td>CXCL8</td>
<td>TNF-α, TGF-β, Tat</td>
</tr>
<tr>
<td>(Shukaliak and Dorovini-Zis, 2000)</td>
<td>HBEC HUVEC</td>
<td>CCL5</td>
<td>IFN-γ, TNF-α, IL1-β, LPS</td>
</tr>
</tbody>
</table>

Table 2.1: Chemokine production by HBEC and HUVEC under different stimuli.
In this study, the release of chemokines (CCL2, CCL5, CXCL8, and CXCL10) into the supernatants of HBEC monolayers in vitro was investigated at the constitutive level, but also when stimulated individually by IFN-γ, TNF-α and TGF-β, and in combination since they have been described as playing a crucial role in the pathogenesis of MS. The intracellular distribution of these chemokines and possible location within the storage vesicles of HBEC was investigated in vitro.

In order to work on HBEC in vitro, a method to purify HBEC had first to be established. The method first used to grow endothelial cells from isolated rat brain capillaries was initially developed by Hughes and Lantos (1986) and later adapted by Abbott et al. (1992). This method had the advantage that it allowed a good yield of brain endothelial cells. However, the presence of other cell types restricted the use of brain endothelial cells in vitro to passage 0 or even passage 1. Other methods used to isolate endothelial cells have been developed to increase the purity of the endothelial cultures by taking advantage of endothelial specific markers. These include the selective binding of the lectin *Ulex europaeus I* to the surface of endothelial cells (Jackson et al., 1990), or more commonly anti-PECAM-1 (Demeule et al., 2001), both used in combination with magnetic beads for isolation of endothelium. Other endothelial-specific isolation methods have employed the use of flow cyometric cell sorting (Craig et al., 1998). Both magnetic beads and the FACS are cumbersome, require specific equipment, are rather expensive, and result in low yield of endothelial cells. Furthermore, its application to isolation of HBEC from human brain has the additional disadvantage of further damaging already fragile cells exposed to the stressful conditions of the post-mortem environment such as hypoxia and low pH. Since the methods described above commonly result in either a low yield of cells or low purity of culture, a method recently published by Perriere et al. (2005) designed to generate high purity cultures of rat brain endothelium derived from fresh tissue samples was adapted for
isolation of HBEC from post-mortem tissue. This method relies on the expression of P-gp by HBEC (Demeule et al., 2001) and the use of puromycin, which is a substrate of the P-gp efflux transporter, toxic to cultured cells. This method was here adapted for use on HBEC.
2.2 Material and methods

2.2.1. Culture medium

For HBEC the endothelial medium was composed of an endothelial basal medium (EBM-2, Cambrex, Berkshire, UK) with supplements of: 5% foetal bovine serum, hydrocortisone, VEGF, epidermal growth factor (EGF), insulin-like growth factor I (IGF-1), human fibroblastic Growth Factor (FGF), ascorbic acid and Gentamicin Sulfate and Amphotericin-B (the concentrations of the supplements included by the supplier were not made available). The medium was optimised by cambrex in order to induce endothelial cell proliferation.

For astrocytes the culture medium was composed of 45% MEM (minimum essential medium)-alpha, 45% F-10, 10% FCS, 1% human serum (for astrocyte proliferation) and 50U/ml penicillin, 50μg/ml streptomycin, and 50μg/ml fungizone to prevent contaminations (Invitrogen, Renfrewshire, UK). The culture medium was made up under sterile conditions and stored at 4°C.

For the differentiation medium, ACM was obtained by adding 45% MEM-alpha, 45% F-10, 50U/ml penicillin, 50μg/ml streptomycin, and 50μg/ml fungizone to confluent astrocytes for 24 h. 150mM of 8-(4-chlorophenylthio)adenosine 3'5'cyclic monophosphate sodium salt (cAMP) and 17.5μM of RO 20-1724 also named 4-(3-Butoxy-4-methoxybenzyl)imidazolidin-2-1 was added to ACM and the differentiation medium was frozen at -80°C (I. Romero, personnal communication).
2.2.2. Preparation of growth surface

HBEC were grown on 25cm² plastic flasks (Greiner, Gloucestershire, UK) which has been previously coated with collagen type 1 from calf skin at the initial concentration of 1mg/ml (Sigma-Aldrich, Dorset, UK). The collagen was diluted by a factor of 1/20 into 5ml of Hank's balanced salts solution (HBSS) and left for 1 hour (h) on the surface of the flask. The flask was then rinsed with three successive washes of normal HBSS.

The same procedure was followed for the astrocytes but using poly-L-lysine (Sigma-Aldridge, Dorset, UK) to coat the plastic flask. Astrocytes were generally grown in 75cm² or 175cm² flasks (Greiner, Gloucestershire, UK). The poly-L-lysine at 0.01% was diluted by a factor of 1/100 in HBSS, and left on the flask for 15 minute (min) to 1 h. Excess of poly-L-lysine was removed by washing 3 times with HBSS.

2.2.3. Isolation of primary HBEC

Solution A: HBSS containing 10mM HEPES, 50U/ml penicillin, 50μg/ml streptomycin, 2.5μg/ml fungizone (Invitrogen, Renfrewshire, UK) and 0.4 % bovine serum albumin (BSA) (Sigma-Aldrich, Dorset, UK)

Collagenase/disnase solution: 1mg/ml collagenase/disnase (Roche Diagnostics Ltd, East Sussex, UK) containing 10μg/ml DNase I (Roche Diagnostics Ltd, East Sussex, UK) and 0.147μg/ml N-p-tosyl-L-lysine chloromethyl ketone (Sigma-Aldrich, Dorset, UK)

Primary HBEC were isolated according to the method initially developed by Hughes and Lantos (1986), with some changes. Human brain tissue was obtained post-mortem from MS patients (table 2.2) fom the MS Tissue Bank, London, UK or from cerebral cortical tissue obtained during lobectomies conducted to relieve medically intractable seizures carried out at Kings College Hospital, London. The investigation conformed to the
principles outline in the declaration of Helsinki and following the Open University guidelines of the Ethics Committee. Details of patients were kept confidential and the brain tissue was in these cases processed 24h after the operation. Human brain tissue was cleared of meninges and cut into small pieces with a scalpel blade. Tissue fragments were then transferred into 10ml solution A and centrifuged for 5 min at 300g (ALC PK121R from Labcare). The pellet was resuspended in 15ml of the collagenase/dispase solution and incubated for 1 h at 37 °C. After digestion, microvessels were isolated by density-dependent centrifugation on 25% BSA. The myelin floating layer was removed, by gently turning the tube at an angle to detach it from the tube, and placed in 25 ml of HBSS. Cells within the myelin layer were isolated by centrifuging the myelin for 5 min at 300g. Single cells in the myelin consisted mainly of astrocytes and were grown on poly-L-lysine, 75cm² coated flasks in 10 ml astrocyte culture medium. The pellet containing microvessel fragments underwent a further 2 h enzymatic digestion with 5ml collagenase/dispase solution to separate HBEC from other cells associated with capillaries. A percoll gradient (50% percoll in HBSS centrifuged for 1 h at 25,000g) was then used to separate microvessel fragments from erythrocytes and single cells surrounding vessels. The microvessel suspension was placed on 7ml of percoll gradient and centrifuged for 10 min at 1000g. The resulting middle fraction, containing the HBEC, was harvested using a Pasteur pipette and after a final centrifugation in 10ml of solution A at 100g for 5 min, was plated on collagen-coated (1/20) 25 cm² flasks in 5ml EGM-2. They were placed in an incubator at a constant temperature of 37°C with an atmosphere of 5%CO₂ and 95% water saturated air. For most cultures, the growth medium after 48 h was changed and 0.5μg/ml of puromycin was added to the medium for 5 days with one medium change in between. The purity of the culture at passage 2 was confirmed by staining for vWF as an endothelial cell marker.

66
In order to assess the effect of puromycin on HBEC on morphology and cell purity, different concentrations of puromycin were added to cells grown in 24 well plates coated with collagen in normal medium. The cells survived and grew in a puromycin concentration range of between 0.1µg/ml to 1µg/ml as well as in control wells in the absence of puromycin. The medium was changed every 2-3 days. After 7 days of treatment, the cells were passaged on glass coverslip coated with collagen. The cells were left to grow without puromycin for 7 further days.

<table>
<thead>
<tr>
<th>Post-mortem tissue from MS patient</th>
<th>Time post-mortem (h)</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Cause of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>12</td>
<td>92</td>
<td>Male</td>
<td>&quot;Old age&quot;</td>
</tr>
<tr>
<td>Patient 2</td>
<td>24</td>
<td>70</td>
<td>Female</td>
<td>Aspiration pneumonia, sepsis, multiple sclerosis</td>
</tr>
<tr>
<td>Patient 3</td>
<td>36</td>
<td>59</td>
<td>Female</td>
<td>Respiratory failure, cardiac arrest</td>
</tr>
<tr>
<td>Patient 4</td>
<td>36</td>
<td>53</td>
<td>Male</td>
<td>Septicaemia, multiple sclerosis</td>
</tr>
<tr>
<td>Patient 5</td>
<td>36</td>
<td>59</td>
<td>Female</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>Patient 6</td>
<td>24</td>
<td>50</td>
<td>Female</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>Patient 7</td>
<td>24</td>
<td>34</td>
<td>Female</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>Patient 8</td>
<td>36</td>
<td>44</td>
<td>Female</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>Patient 9</td>
<td>36</td>
<td>71</td>
<td>Female</td>
<td>bronchopneumonia, multiple sclerosis</td>
</tr>
<tr>
<td>Patient 10</td>
<td>24</td>
<td>82</td>
<td>Female</td>
<td>Small bowel obstruction, Multiple sclerosis</td>
</tr>
<tr>
<td>Patient 11</td>
<td>36</td>
<td>63</td>
<td>Male</td>
<td>Metastatic carcinoma of prostate, Multiple sclerosis</td>
</tr>
</tbody>
</table>

Table 2.2: MS Donor details.

2.2.4. Maintenance of brain cell cultures

HBEC were passaged onto collagen coated flasks and grown in EGM-2 medium at 37°C (Cambrex, Berkshire, UK) until they reached confluence. The medium was changed every 2-3 days. Confluent cell monolayers were detached from the extracellular matrix by washing with HBSS without Ca$^{2+}$ and Mg$^{2+}$ for 1 min and incubated with 0.25% w/v porcine trypsin-ethylene diamine tetraacetic acid (EDTA) (Invitrogen, Renfrewshire, UK).
Once the cells were detached, the remaining active trypsin was neutralized by diluting in culture medium and the cells were divided 1/3 into another collagen coated flask. The cells were passaged as long as they represented a high percentage of HBEC, based on morphological appearance. In the present study, cells at passage 2 were routinely used.

To evaluate whether there were any preferential side for chemokines release by HBEC, cells at passage 2 were seeded on an insert in a Boyden chamber (0.4 μm diameter pore used, this size allow the passage of the chemokines in the bottom chamber) (fig 2.1) at a concentration of $10^5$ cells/ insert (surface area of 1 cm$^2$). 0.5ml of normal medium was added in the upper part of the chamber and 1.5 ml was added to the outer well of the 12 well plate.

The same procedure was followed to detach astrocytes and to passage the cells. Astrocytes in culture could be used until passage 8, but were in general used at passage between 2 and passage 5. The purity of the astrocyte culture was confirmed by fluorescence-activated cell sorter (FACS) using GFAP (dilution used at 1/100, DakoCytomation Ltd, Cambridgeshire, UK) as a marker (data not shown). The protocol used is as described in section 4.2.7.
Upper (inner) chamber

Bottom (outer) chamber

0.4 μm pore-size tissue culture insert

**Fig 2.1: Illustration of a Boyden chamber.**

### 2.2.5. Capture ELISA

**Wash buffer:** 0.05% Tween-20 in phosphate buffered saline (PBS)

**Elisa Diluent:** 0.1% BSA (sigma-Aldrich, Dorset, UK), 0.05% Tween-20 in tris buffered saline, pH 7.3

**TMB solution:** 0.1mg/ml of tetramethylbenzidine (TMB) (Sigma-Aldrich, Dorset, UK) 0.03% hydrogen peroxide and 0.0375M citric acid (BDH Chemicals, Dorset, UK) in 0.1 M citrate acetate buffer (Sigma-Aldrich, Dorset, UK)

**Phosphate Buffered Saline (PBS):** 13mM sodium phosphate dibasic, 2mM potassium phosphate dibasic, 3mM potassium chloride, 134mM Sodium chloride, pH 7.4.

HBEC (passage 2) were cultured on collagen coated 24 well plates until confluence and treated for 48 h with 2 μl cytokines at the following final concentrations: TGF-β at
25ng/ml, TNF-α at 25ng/ml and INF-γ at 100ng/ml (R&D Systems, Oxfordshire, UK) in 1ml medium. The concentrations of cytokines selected for this experiment were of the same order as those used for other experiments in Hillyer et al. (2003). Culture supernatants were then collected and frozen at -20°C until further analysis. For chemokine determination in culture medium, capture antibodies (R&D systems, Oxfordshire, UK, table 2.3) were diluted in the appropriate concentration (reported in table 2.3) in 100μl PBS and left overnight to allow binding to the surface of 96 well ELISA plates (Nunc Maxisorb, scientific laboratory supplies, Nottingham, UK). The plates were washed three times with 200μl of wash buffer. Non-specific binding sites were blocked using 1% BSA and 5% sucrose for 1 h at room temperature (RT). To ensure chemokine detection was within the assay limits, culture supernatants were first diluted at 1/50 (to determine basal level) to 1/100 (for the supernatant from stimulated cells) in Elisa Diluent and 100 μl of the diluted supernatant was added to each well and incubated for 2 h at RT in duplicate, as were the standards, ranging from 0 to 8000pg/ml. After three washes in the wash buffer, a biotinylated goat anti-human chemokine detection antibody (R&D systems, table 2.3) was added for 2 h in ELISA Diluent. Following three washes in the wash buffer, signal amplification was achieved by incubation with 100μl streptavidin-horseradish peroxidase (Amersham Biosciences, Buckinghamshire, UK) diluted at 1:500 in Elisa Diluent solution for 1 h. After the final 3 washes, the peroxidase reaction was visualised by incubation with 100μl of TMB solution containing substrate of the peroxidase. The reaction was stopped with 10% sulphuric acid (BDH Chemicals, Dorset, UK). The optical density of the plate was read on a spectrophotometer (Lab systems Mulitskan, Meadow rose Ltd, UK) at a wavelength of 450 nm.

The concentration of chemokines in the solution was determined by plotting the absorbance value against the values given by the standards. The standard curve, when the concentrations were transformed to logarithmic numbers, gave a sigmoid type of curve (fig
2.2). The central part of the curve was linear, and the dilutions of the supernatant samples were carefully assessed and verified to be located within the linear part of the curve for each experiment.

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Monoclonal capture antibody concentration (µg/ml)</th>
<th>Standard range (ng/ml)</th>
<th>Polyclonal detection antibody concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>1</td>
<td>0.03-8</td>
<td>50</td>
</tr>
<tr>
<td>CCL5</td>
<td>4</td>
<td>0.03-8</td>
<td>5</td>
</tr>
<tr>
<td>CXCL8</td>
<td>4</td>
<td>0.03-8</td>
<td>20</td>
</tr>
<tr>
<td>CXCL10</td>
<td>2</td>
<td>0.03-8</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2.3: Optimal concentrations of antibodies for capture and detection of chemokines in culture supernatants by capture ELISA, and concentration of standards.

Fig 2.2: Example of Standard curve
Graphs display typical standard curve for absorbance of CXCL8 at different concentration (from 0 to 3600 pg/ml) made up using recombinant CXCL8.

2.2.6. Immunocytochemistry

2.2.6.1. Immunofluorescence
HBEC were grown on collagen coated Labtek chambered slides (Nunc, Scientific laboratory supplies, Nottingham, UK). Cells were fixed with 4% para-formaldehyde (PAF) dissolved in PBS on ice for 10 min, and permeabilised for 5 min with 0.1% Triton X-100
in PBS. For those experiments concerning the effect of puromycin on HBEC culture purity and vWF staining, cells were fixed with methanol at -20°C for 10 min.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Antigen specificity</th>
<th>Host species</th>
<th>Concentration</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWF</td>
<td>Rabbit IgG</td>
<td>1/200</td>
<td>Sigma-Aldrich</td>
<td></td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Mouse</td>
<td>1/50</td>
<td>Serotec</td>
<td></td>
</tr>
<tr>
<td>JAM-1</td>
<td>Mouse</td>
<td>1/400</td>
<td>provided by Dr E. Dei ana</td>
<td></td>
</tr>
<tr>
<td>TRITC-Phalloidin conjugated</td>
<td>Actin</td>
<td>1/2000</td>
<td>Sigma-Aldrich</td>
<td></td>
</tr>
<tr>
<td>Beta-catenin</td>
<td>Mouse IgG1</td>
<td>2µg/ml</td>
<td>Santa Cruz Biotechnology</td>
<td></td>
</tr>
<tr>
<td>Gamma-catenin</td>
<td>Mouse IgG1</td>
<td>5µg/ml</td>
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<td>Alpha-catenin</td>
<td>Rabbit</td>
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<td>Sigma-Aldrich</td>
<td></td>
</tr>
<tr>
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<td>Zymed</td>
<td></td>
</tr>
<tr>
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<td>Zymed</td>
<td></td>
</tr>
<tr>
<td>Claudin-5</td>
<td>Mouse</td>
<td>10µg/ml</td>
<td>Zymed</td>
<td></td>
</tr>
<tr>
<td>CXCL10</td>
<td>Rabbit</td>
<td>0.4µg/ml</td>
<td>Peprotech</td>
<td></td>
</tr>
<tr>
<td>Biotinylated-CXCL10</td>
<td>Goat</td>
<td>1µg/ml</td>
<td>R&amp;D</td>
<td></td>
</tr>
<tr>
<td>CXCL10</td>
<td>Mouse</td>
<td>5µg/ml</td>
<td>R&amp;D</td>
<td></td>
</tr>
<tr>
<td>CXCL8</td>
<td>Rabbit</td>
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<td>Peprotech</td>
<td></td>
</tr>
<tr>
<td>CCL2</td>
<td>Mouse</td>
<td>10µg/ml</td>
<td>Peprotech</td>
<td></td>
</tr>
<tr>
<td>CCL5</td>
<td>Rabbit</td>
<td>0.4µg/ml</td>
<td>Peprotech</td>
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</tr>
<tr>
<td>Rabbit-IgG</td>
<td>Goat</td>
<td>10µg/ml</td>
<td>Chemicon</td>
<td></td>
</tr>
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<td>mouse-IgG</td>
<td>Goat</td>
<td>10µg/ml</td>
<td>Chemicon</td>
<td></td>
</tr>
<tr>
<td>Peroxidase conjugated Rabbit-IgG</td>
<td>Swine</td>
<td>1/100</td>
<td>Dako Corporation</td>
<td></td>
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<tr>
<td>Peroxidase conjugated mouse-IgG</td>
<td>Rabbit</td>
<td>1/100</td>
<td>Dako Corporation</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4: List of antibodies used in immunocytochemical analysis. The final concentration of the antibody used is given or else the dilution factor is reported for those antibodies where no original concentration was available.
The cells were then blocked in 10% normal goat serum diluted in PBS for 1 h. The primary antibodies (see table 2.4) were applied for 1 h at RT. After 3 washes with PBS, the corresponding fluorescence-conjugated secondary antibody (table 2.4) was added to the cells and incubated for 1 h. A drop of solution containing DAPI (DakoCytomation Ltd, Cambridgeshire, UK) was added for 5 mins at RT. After 3 final washes, the slides were mounted with a glass coverslip with Dako Fluorescent Mounting Medium (DakoCytomation Ltd, Cambridgeshire, UK) and viewed with a fluorescence microscope (Olympus BX61).

2.2.6.2. Intracellular staining for chemokines

Cells were grown on Labtek Chambered slides (Nunc, UK) coated with collagen for one week. They were then fixed using 4% PAF for 10 min on ice. In order to block endogenous peroxidase as well as to permeabilised the cells, absolute methanol was applied for 2 min with 3.5% hydrogen peroxide. After having intensively washed the cells with PBS, a blocking bluffer was applied for 1 h (10% rabbit serum or 10% swine serum in PBS for the secondary rabbit anti-mouse or the Swine anti-rabbit antibody, respectively). The primary antibodies were left overnight on the cells at RT. For negative control, mouse IgG or rabbit IgG (10 μg/ml, DakoCytomation Ltd, Cambridgeshire, UK) were used instead of primary antibodies. The secondary biotinylated antibody (Dako Corporation, Cambridgeshire, UK), rabbit anti-mouse or swine anti-rabbit relative to the species of which the primary antibody originated (table 2.4) was used at the dilution of 1/100 and incubated for 1 h. To enhance the signal, a further incubation was performed with the VECTASTAIN Elite ABC Kit (Vector Laboratories, Peterborough, UK) which contains avidin-biotin complex to which the peroxidase associates. After 40 min, the presence of peroxidase was revealed with the VIP dye (Vector Laboratories, Peterborough, UK) which is a dye reacting with the peroxidase and enables visualization of the immunoreaction product, indicating the
presence of the chemokines in a pink-purple colour. The VIP agent was applied for 3 min, and the reaction was stopped with 2 rinses of distilled water. The slides were then mounted with DPX after dehydratation through a series of ascending concentrates of alcohol for 2 min in each. The staining was viewed with a light microscope (Nikon microphot-FX), and the images were acquired using the software Image Pro-plus Image Analysis.

2.2.7. Statistical analysis

Significance was determined by a one way or two way ANOVA, followed by a post hoc Tukeys test. P<0.05 was considered significant. For the results concerning CXCL8 production by HBEC, the data were not normally distributed and a Kruskal-Wallis and Mann-Whitney test was used.
2.3 Results

2.3.1. Morphology

Primary HBEC growing out of isolated capillary fragments mainly consisted of elongated fusiform-shaped cells (fig 2.3). These cells represented the majority cell type present in the culture for the first 10 days. After that time, and depending on the quality of the preparation, other cells with different morphology started to grow at a faster rate. Contaminating cells based on their fibroblast-like morphological appearance were mainly astrocytes although other cell types such as pericytes could not be excluded.

2.3.2. Effect of puromycin treatment on the purity of HBEC cultures

The antibiotic puromycin is a substrate of P-gp, an efflux transporter strongly expressed by brain endothelium of the BBB. Therefore it has been proposed recently as a selective agent to improve the purity of the rat brain endothelial cell culture (Perriere et al., 2005), as these cells should be more resistant to the cytotoxic actions of puromycin than the non-P-gp expressing contaminating cells. The effect of different concentrations of puromycin on the proliferation of both human astrocytes, which is a possible source of contaminating cells in the HBEC culture, and on HBEC was here investigated as well as the purity of the HBEC culture (Greenwood, 1992).

Using a pure endothelial batch, the effect of puromycin on HBEC was investigated. The growth rate of HBEC incubated in puromycin was unaffected at a concentration of 0.5μg/ml and lower during the first seven days while the cells grown in 1μg/ml did not proliferate (fig 2.3). After 16 days, the cells at 0.1μg/ml also showed signs of slower division rate compared to the control, while the cells grown in 0.5μg/ml were starting to detach (fig 2.3).
From a batch of cells containing contaminant cells in the HBEC culture, the use of puromycin to obtain a pure endothelial cells culture was investigated. In 0.5μg/ml of puromycin, treatment of the culture did not prevent the growth of other contaminant cells (fig 2.4) while based on their morphological appearance, 0.5μg/ml and above prevented the growth of cells other other HBEC (fig 2.4). The type of contaminant cell was not determined.

The cytotoxic effects of 0.75 and 1μg/ml of puromycin on cultured astrocytes were confirmed on a culture of astrocytes only. Cells grown in 0.1μg/ml proliferate similarly to the control cells, whereas the astrocytes grown in 0.5μg/ml and above died after seven days (fig 2.5).

These observations were confirmed by staining the cells for vWF (fig 2.6) and DAPI to help identify the endothelial cells. In cultures grown in 0.1 and 0.2μg/ml, as many cells had grown as in the control culture, but only few were HBEC as shown by vWF staining (fig 2.6). The HBEC appeared to grow in colonies in those cultures. On the contrary, cells that grew in concentrations of 0.5μg/ml and above were composed mainly of vWF positive cells, with little difference between the three different concentrations used (fig 2.6). In conclusion, 0.5μg/ml of puromycin treatment for seven days appeared to be the most appropriate to obtain a pure HBEC culture without affecting the growth rate of the cells.

2.3.3. Phenotype of primary HBEC: morphology and expression of endothelial markers

The expression of junctional molecules and specific HBEC markers was determined by immunocytochemistry. HBEC were largely positive for vWF. However there were some differences in staining pattern between different cells within the same culture, as vWF in
some cells were detected in rod-shape vesicles, corresponding to Weibel-Palade bodies, whereas in other cells, the staining was more diffuse throughout the cell cytoplasm (fig 2.7).

Figure 2.8 shows the expression of vWF, and actin organisation as well as junctional PECAM-1 and JAM-1 which are thought to be important for the transmigration of leucocytes (Muller et al., 1993; Johnson-Leger et al., 2002). PECAM-1 and JAM-1 were found at the point of cell-cell contact, with a light staining within the cytoplasm around the nucleus (fig 2.8). Actin organisation is important for maintaining the tight junction structure, as tight junction proteins such as ZO-1 are connected to actin filaments and dynamic regulation of perijunctional actin may function as a controlling factor in regulating paracellular permeability (Char-Huei et al., 2005). Actin distribution at the edge of the cells is regarded as a sign of low permeability and high electrical resistance, whereas appearance of stress fibers is associated with increase of permeability in cultured brain endothelium (Lu et al., 2004). In HBEC, actin was located mostly at the junctions of the cells although the presence of stress fibres could also be observed unequally distributed among different cells. In some cells, many stress fibres were present within the cytoplasm, whereas in others, actin was restricted to the junction (fig 2.8).

Figure 2.9 shows the expression by the HBEC of VE-cadherin, α-, γ- and β-catenin. The three members of the catenin family, α-, γ- and β-catenin as well as VE-cadherin, all constituent proteins of adherens junctions, are all highly expressed by cultured HBEC (fig 2.9). All four were mainly detected in a linear distribution at the junction between cells. There was also a slight positive staining for β- and γ-catenin within the cytoplasm (fig 2.9). Every cell expressed α-, β-, γ-catenin and VE-cadherin, although some differences were observed in the intensity of the staining. Mostly, it appeared that there were colonies of
cells within the monolayer with higher intensity labelling for the adherens junction proteins, as is illustrated with the staining of α-catenin (fig 2.9).

Figure 2.10 shows the distribution of the tight junctional proteins, claudin-3, claudin-5, occluding and ZO-1, which are expressed by HBEC but not by endothelial cells of other vascular beds. A faint expression of claudin-3, claudin-5 and occludin at the junctional areas was observed, some diffuse expression was detected within the cytoplasm. The expression of claudin-5 and occludin was mostly patchy, with areas of closely apposed cells showing strong staining at the cell-cell junctions. All 3 junctional proteins showed a punctuate expression along the cell edges, indicative of immature tight junctions. By contrast, claudin-1 was only detected in the cytoplasm as previously reported for HBEC (Ishizaki et al., 2003). ZO-1 was expressed by all cells in the monolayer, and the staining appeared continuous at the junction. The figures show staining of cells at passage 2. Nevertheless, there was no or little variation between passages 1 and 2 for the expression of any junctional protein by HBEC in culture (not shown).

2.3.4. Basal and cytokine-induced chemokine release by HBEC

2.3.4.1. Constitutive release of CCL2, CCL5 and CXCL10

The level of chemokines constitutively produced by HBEC was measured by harvesting culture supernatants from confluent HBEC after 48 h (fig 2.11). No significant difference in the release of chemokines was reported between the HBEC that originate from the MS donors and from the epileptic donors. They were hence treated as one group. CCL2 was produced at basal level (11ng/ml ± 3.8ng/ml) whereas CXCL10 and CCL5 were absent in the supernatant of unstimulated HBEC, suggesting that there is no release of these two chemokines at basal levels in vitro.
2.3.4.2. Constitutive release of CXCL8 by HBEC

CXCL8 released at constitutive level was found to be highly variable among different donors ranging from low (3ng/ml) to elevated constitutive release (207ng/ml) (fig 2.11). Out of the eight donors, the cells from two of the donors were above 100ng/ml/48h and cells from three different donors release around 10ng/ml in the supernatant within 48 h. The cells from the three other donors were in between (45, 56 and 96ng/ml). Details from the MS patients concerning the gender, age and cause of death were available (table 2.5) and no correlations between those factors and the release of CXCL8 at basal level could be detected. Similarly, there was also a variation in the production of CXCL8 in response to TNFα (table 2.5). Also the sample number was too small to draw any conclusions. Details from the three epileptic donors were not available, but since the HBEC from the three epileptic donors were from the same brain region (the temporal lobe), this indicated that the differences could be donor dependent. Indeed, the cells from the epileptic patients also display a range of constitutive levels for CXCL8 (45, 56, 142ng/ml).

<table>
<thead>
<tr>
<th>Basal (ng/ml)</th>
<th>TNF-α (ng/ml) (fold increase)</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Cause of death</th>
</tr>
</thead>
<tbody>
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<td>Patient 1</td>
<td>96</td>
<td>262 (x2.7)</td>
<td>92</td>
<td>Male &quot;Old age&quot;</td>
</tr>
<tr>
<td>Patient 2</td>
<td>207</td>
<td>364 (x1.8)</td>
<td>70</td>
<td>Female Aspiration pneumonia, sepsis, multiple sclerosis</td>
</tr>
<tr>
<td>Patient 3</td>
<td>3</td>
<td>83 (x25.7)</td>
<td>59</td>
<td>Female Respiratory failure, cardiac arrest</td>
</tr>
<tr>
<td>Patient 4</td>
<td>9</td>
<td>411 (x44.4)</td>
<td>53</td>
<td>Male Septicaemia due to UTI, multiple sclerosis</td>
</tr>
<tr>
<td>Patient 5</td>
<td>17</td>
<td>357 (x20.7)</td>
<td>59</td>
<td>Female Pneumonia</td>
</tr>
</tbody>
</table>

Table 2.5: MS Donor details: cells used in CXCL8 assays.

2.3.4.3. Constitutive release of CXCL8 and CCL2 by HBEC grown on filters

Chemokines are presented to circulating lymphocytes on the endothelial surface and, after rolling, trigger firm adhesion. When endothelial cell cultures are polarized, a greater release of chemokines should be observed on the apical side than on the basal side. HBEC
were grown on filters to investigate whether there was any preferential side for chemokine release. The experiment was conducted with HBEC from two different donors. For both CCL2 and CXCL8, a preferential release of the chemokines on the apical side could be observed (fig 2.12). For CCL2, 66% of the total was released on the apical side (22±0.55ng in the upper chamber, and 12±6.8ng in the lower chamber, n=2 different donors). Similarly, three times more CXCL8 was found in the upper chamber at basal level compared to the lower chamber of the culture system (31±10.4ng in the upper chamber and 9±6.8ng in the lower chamber, n=2). These results suggest that HBEC are polarised on filter, but also that there is a low permeability of the HBEC monolayer to small molecular weight proteins such as chemokines (table 2.6), hence that tight junctions were formed. There could also be an active transport of the chemokines from the basolateral side to the luminal side which has already been proposed for CCL2 (Andjelkovic and Pachter, 2000).

<table>
<thead>
<tr>
<th>Chemokines</th>
<th>Molecular weight (kDa)</th>
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</thead>
<tbody>
<tr>
<td>CXCL8</td>
<td>8.9</td>
</tr>
<tr>
<td>CXCL10</td>
<td>8.5</td>
</tr>
<tr>
<td>CCL2</td>
<td>8.6</td>
</tr>
<tr>
<td>CCL5</td>
<td>7.8</td>
</tr>
</tbody>
</table>

Table 2.6: Molecular weight of CXCL8, CXCL10, CCL2 and CCL5.

2.3.4.4. Effect of IFN-γ on chemokine production by HBEC

When used alone, 100 ng/ml of IFN-γ strongly induced the release of CXCL10 by HBEC (from 0ng/ml to 99±16.5ng/ml, P=0.02, n=8 different donors). By contrast, IFN-γ tended to reduce the mean secretion for CXCL8 compared to basal levels although this was not significant. IFN-γ had no significant effect on the basal level of CCL2 secretion (10±2.7ng/ml), and induced a small but non-significant increase in CCL5 secretion (2±0.8ng/ml) (fig 2.11).

When used in combination with TNF-α, IFN-γ-induced release of CXCL10 in the supernatant was greater than with IFN-γ alone (200±50.9ng/ml versus 99±16.5ng/ml,
$P=0.02$, n=8 different donors). IFN-γ alone failed to induce any significant changes in the production of CCL5, but when added in combination with TNF-α CCL5 production was greatly increased compared to TNF-α alone (43±2.8ng/ml versus 5±3ng/ml, respectively, $P=0.0001$, n=5 different donors). IFN-γ had an inhibitory effect on the production of CXCL8 induced by TNF-α, leading to a significant reduction (72±27.2ng/ml versus 162±31.1ng/ml, $P=0.046$, n=8 different donors) whereas no significant difference was observed between control and IFN-γ-treated HBEC. Finally, IFN-γ had no effect on CCL2 production by HBEC when added to TNF-α (fig 2.11).

2.3.4.5. Effect of TNF-α on chemokine production by HBEC

TNF-α (25ng/ml) increased significantly the production of CCL2 and CXCL8 (34±8ng/ml versus 12±3.8ng/ml, $P=0.04$, n=6 different donors; 308±60.5ng/ml versus 72±27.2ng/ml, $P=0.029$, n=8 different donors, respectively) and there was a small but non-significant increase in CCL5 production (5±3ng/ml versus 0ng/ml). TNF-α alone had no significant effect on CXCL10 production, but displayed a synergistic effect when added with IFN-γ (200±50.9ng/ml versus 99±16.5ng/ml, $P=0.023$, n=8 different donors) (fig 2.11).

2.3.4.6. Effect of TGF-β on chemokine production by HBEC

TGF-β (25ng/ml) had no effect on the production of any the chemokines tested when used at the concentration of 25ng/ml. When used in combination with other cytokines, the only significant effects on HBEC observed were an inhibitory effect on the TNF-α–induced stimulation of CXCL8 production (134±23.6ng/ml versus 308±60.5ng/ml, $P=0.04$, n=8) and an increase in CCL5 production when used in combination with TNF-α (5±3ng/ml versus 22±4.7ng/ml, $P=0.00014$, n=5) (fig 2.11).
2.3.4.7. Effect of differentiation medium on chemokine production by HBEC

Increased intracellular cAMP levels and culture medium conditioned by astrocytes has been shown to induce a differentiated phenotype in cultured brain endothelium, including those of human origin (Rubin et al., 1991). When the cells were grown in differentiation medium, CXCL10 release induced by TNF-α and IFN-γ was decreased compared to basal level (48±0.9ng/ml versus 127±0.6ng/ml, n=1), whereas CXCL8 was increased (67±10.9ng/ml versus 29±3ng/ml, n=1). The significance of these results could not be assessed as the experiment was only conducted in duplicate on cells coming from one donor (fig 2.13).

2.3.5. Intracellular expression and compartmentalisation of chemokines in HBEC

For CCL2 and CCL5, only a faint cytoplasmic staining could be detected in unstimulated cells, suggesting the absence of intracellular stores of these chemokines at basal level. Stimulation of HBEC with cytokines did not change the pattern of the staining in any of the three donors tested (fig 2.14).

CXCL10 labelling in endothelial cells in vitro was conducted with three different antibodies from different animal recipients (fig 2.15). A mouse anti-human CXCL10, a rabbit anti-human CXCL10 and a goat anti-human CXCL10 were used. The pattern of staining in unstimulated cells differed between the three antibodies. The goat anti-human CXCL10 antibody did not label unstimulated HBEC whereas the mouse anti-human CXCL10 labelled faintly the cytoplasm of HBEC. When the rabbit anti-human CXCL10 was used, a clear labelling within small vesicles and part of the cytoplasm was observed within unstimulated HBEC. When HBEC were stimulated with IFN-γ and TNF-α, strong positive staining within vesicular structures was observed with all CXCL10 antibodies (fig 2.15). These vesicles were mainly located around the nucleus, in agreement with a positive
staining of the Golgi apparatus. A stronger and more diffuse cytoplasm staining was also observed with the mouse anti-human CXCL10 antibodies.

Intracellular staining for CXCL8 in HBEC in vitro exhibited a different pattern for each of the three donors although one antibody was used (fig 2.16). In unstimulated HBEC, only cells from one donor stained positive for CXCL8. In this case, there were some small vesicles located around the nucleus positive for CXCL8. Stimulation with IFN-γ and TNF-α induced the intracellular expression of CXCL8 in cells from two out of the three donors. In one case the cytoplasm was diffusely stained, as well as some vesicles resembling the vWF positive Weibel-Palade bodies. In the other case small vesicles around the nucleus were stained (fig 2.16).

2.3.5.1. Effect of differentiation medium on CXCL10 intracellular expression in HBEC

In one experiment HEBC grown in differentiation medium for a week, and left unstimulated or further stimulated with TNF-α and IFN-γ for 48 h, were stained for CXCL10. As shown in figure 2.17 differentiation medium decreased the staining of CXCL10 in unstimulated cells, but most importantly decreased the number of vesicles induced by IFN-γ and TNF-α. It should be noted that there was no effect of differentiation medium on the change of morphology induced by the cytokine treatment (fig 2.17).
**Fig 2.3:** Phase contrast micrographs of cultured HBEC incubated in different concentrations of puromycin.

HBEC were seeded on collagen-coated 24 well plates and were treated for one week with different concentrations of puromycin (0.1µg/ml, 0.5µg/ml, 1µg/ml) or were left to grow in normal medium. Phase contrast images were taken after two days (left column), after nine days (middle column) and after sixteen days (right column). Data shown were obtained from one experiment, with duplicate wells per treatment. Scale bar = 100µm.
Fig 2.4: Phase contrast micrographs of cultured HBEC incubated in different concentrations of puromycin.
HBEC were seeded on collagen-coated 24 well plates and were treated for one week with different concentration of puromycin (0.1μg/ml, 0.5μg/ml, 1μg/ml) or were left to grow in normal medium. Phase contrast images were taken with a light microscope after 2 days (left column), and after 7 days (right column). Results are representative of four different experiments with a single well per treatment. Scale bar = 100 μm.
Fig 2.5: Phase contrast micrographs of cultured astrocytes incubated in different concentrations of puromycin.

Cells were seeded on collagen-coated 24 well plates and were treated for one week with different concentrations of puromycin (0.1µg/ml, 0.5µg/ml, 1µg/ml) or were left to grow in normal medium. Phase contrast images were taken with a light microscope after two days (left column) and after seven days (right column). Results are representative of four different experiments with a single well per treatment. Scale bar = 100 µm.
Fig 2.6: Immunocytochemical detection of vWF and the cell nucleus using DAPI staining of cells derived from primary HBEC isolation grown in different concentrations of puromycin.

HBEC were seeded on collagen-coated 24 wells plate and were treated for one week with different concentrations of puromycin (0.1μg/ml, 0.2μg/ml, 0.5μg/ml, 0.75μg/ml, 1μg/ml) or were left to grow in normal medium. The cells were then fixed and permeabilised with Triton X-100 and processed for detection of vWF by immunocytochemistry and for detection of the nucleus using DAPI staining. Data shown were obtained from one experiment, with duplicate wells per treatment. Scale bar=100μm.
Fig 2.7: Immunocytochemical detection of vWF on cultured primary HBEC.
HBEC were grown on collagen-coated labtek chamber slides, fixed, permeabilised with Triton X-100, and then processed for detection of vWF. In some cells vWF is located in Weibel-Palade body like structures (arrows), whereas in some other cells the staining appears diffuse within the cytoplasm (star). The bottom images show, cells with different pattern of vWF vesicles within the same culture. Data shown were obtained from one experiment. Scale bar=50μm.
Fig 2.8: Immunocytochemical detection of JAM-1, actin, PECAM-1 and vWF on primary HBEC.
HBEC were grown on collagen-coated Labtek chamber slides, fixed and permeabilised with Triton X-100 then processed for detection of JAM-1, actin, PECAM-1 and vWF by immunocytochemistry. Results are representative of three different experiments for PECAM-1, actin and vWF and show one single experiment for JAM-1 with a single well per treatment. Scale bar=100 μm.
Fig 2.9: Immunocytochemical detection of β, γ, α-catenin and VE-cadherin on primary HBEC.
HBEC were grown on collagen-coated Labtek chamber slides, fixed and permeabilised with Triton X-100 then processed for detection of β, γ, α-catenin and VE-cadherin by immunocytochemistry. Results are representative of three different experiments for β-catenin and show one single experiment for γ, α-catenin and VE-cadherin with a single well per treatment. Scale bar=100 μm.
Fig 2.10: Immunocytochemical detection of claudin-1, -3, -5, occludin and ZO-1 in primary HBEC.
HBEC were grown on collagen-coated Labtek chamber slides, fixed, permeabilised with Triton X-100 then processed for detection of claudin-1, -3, -5, occludin and ZO-1 by immunocytochemistry. Results are representative of three different experiments with a single well per treatment. Scale bar=50 μm.
Fig 2.11: Chemokine release into culture medium by HBEC following stimulation by cytokines for 48 h.
Supernatants from confluent monolayers of HBEC seeded at same density were collected after 48 h stimulation with TNF-α (50ng/ml), IFN-γ (100ng/ml) and TGF-β (25ng/ml) or a combination of them and assayed by sandwich ELISA for different chemokines. Results presented are means ± S.E.M. of eight different experiments for CXCL8 and CXCL10, six for CCL2 and five for CCL5 (one well per treatment). Significant differences were determined by ANOVA followed by a post hoc t-test for CCL2, CCL5 and CXCL10 and by a Kruskal-Wallis and Mann-Whitney test for CXCL8. * significant difference (P <0.05) versus the control; Δ significant difference (P<0.05) of sample treated with a combination of cytokines versus TNF-α treated sample; • significant difference (P<0.05) of sample treated with a combination of cytokines versus IFN-γ treated sample.
Fig 2.12: Total CXCL8 and CCL2 release into the apical and baso-lateral culture medium by HBEC grown on filters after 3 h and 48 h.

Supernatants from confluent monolayers of HBEC grown on filters were collected after 3 h and 48 h from the apical (white column) and the baso-lateral sides (striped column) and assayed by sandwich ELISA for chemokine production. Results presented are means from two different experiments with two replicate filters each.
Fig 2.13: CXCL8 and CXCL10 release by HBEC following stimulation by TNF-α and IFN-γ for 48 h in normal medium or in differentiation medium (DF).
Supernatant from confluent monolayers of HBEC were collected after 48 h stimulation with TNF-α (50ng/ml) and IFN-γ (100ng/ml) and assayed by sandwich ELISA for CXCL8 and CXCL10 production. Results presented are means of duplicate wells from one experiment per treatment.
Fig 2.14: Immunocytochemical detection of CCL2 and CCL5 in HBEC.
HBEC were grown on collagen-coated Labtek chamber slides, fixed, permeabilised with Triton X-100, and then processed for detection of CCL2 and CCL5 by immunocytochemistry in unstimulated cells (left column) or TNF-α (50ng/ml) and IFN-γ (100ng/ml) stimulated cells (right column). Cells labelled with either mouse IgG (Ms IgG) or Rabbit IgG (Rb IgG) as negative controls. Results are representative of three different experiments with a single well per treatment. Scale bar = 50µm.
Fig 2.15: Immunocytochemical detection of CXCL10 in HBEC.
HBEC were grown on collagen-coated Labtek chamber slides, fixed and permeabilised with Triton X-100 and then processed for detection of CXCL10 by immunocytochemistry using three different antibodies, a mouse anti-human CXCL10, and rabbit anti-human CXCL10 and a goat anti-human CXCL10 in unstimulated cells (left column) or TNF-α (50ng/ml) and IFN-γ (100ng/ml) stimulated cells (right column). Results are representative of three different experiments with a single well per treatment. Scale bar = 50μm.
Fig 2.16: Immunocytochemical detection of CXCL8 in HBEC originating from three different donors.

HBEC from three different donors were grown on collagen-coated Labtek chamber slides, fixed, permeabilised with Triton X-100 and then processed for detection of CXCL8 by immunocytochemistry in unstimulated cells (left column) or TNF-α (50ng/ml) and IFN-γ (100ng/ml) stimulated cells (right column). Results show one experiment with a single well per treatment. Scale bar=50 μm.
Fig 2.17: Immunocytochemical detection of CXCL10 in HBEC after stimulation with cytokines of cells grown in normal and differentiation medium (DF).
HBEC were grown on collagen-coated Labtek chamber slides, fixed, permeabilised with Triton X-100 and then processed for detection of CXCL10 by immunocytochemistry in unstimulated cells or TNF-α (50ng/ml) and IFN-γ (100ng/ml) stimulated cells grown in normal or differentiation medium. Results show one experiment with one well per treatment. Scale bar=50μm.
2.4 Discussion

Many studies have underlined the importance of a variety of chemokines in MS. In this study the contribution that HBEC may make to the overall production of chemokines was investigated. The aim was to test the hypothesis that endothelial cells are not only a physical barrier that protects the brain from blood-borne substances and/or cells, but also that they may play an active role in brain inflammation and in particular, in directing leukocyte diapedesis. This was tested by investigating the production of a number of chemokines by cultured HBEC both constitutively and following stimulation by cytokines known to be up-regulated in MS brains. Here, we have shown that human brain capillary endothelial cells are able to produce some of the chemokines which are up-regulated in MS brains and/or CSF such as CXCL10, CCL2, CXCL8 and CCL5.

2.4.1. Isolation and purification of primary HBEC.

Isolation and long term culture of brain endothelial cells has often proved a difficult task, as the endothelial cells of microvessels are in close contact with fibroblasts, astrocytes, smooth muscle cells and pericytes within the vascular unit (Greenwood, 1992). Contaminating cells usually overgrow the endothelial cells as they have better proliferative capacities under culture conditions. A method recently published by Perriere et al. (2005), based on P-gp expression by HBEC (Demeule et al., 2001) and the use of puromycin, one of its substrates, was designed to generate high purity cultures of rat brain endothelium derived from fresh tissue samples was adapted for isolation of HBEC from post-mortem tissue. This method relies and the use of puromycin, which is a substrate of the P-gp efflux transporter and is toxic to cultured cells.
P-gp is an efflux pump belonging to the ATP-binding cassette family of transporters with wide specificity for high molecular weight lipophilic substances. Tumour cells have been shown to express high levels of this transporter and it has been suggested that its overexpression confers resistance of tumour cells to therapeutic levels of cytotoxic drugs (Hendrikse et al., 1999). Under physiological conditions, HBEC express high levels of P-gp (Jette et al., 1993) and in culture, brain endothelial cells retain the capacity to exclude drugs such as the antibiotic puromycin attaining low intracellular levels whereas the concentration of puromycin in the cytoplasm of non-P-gp expressing cells is sufficient to block the de novo production of proteins and induce cell death. Puromycin causes the degradation of polyribosomes and triggers the release of nascent peptides from ribosomes (Cundliffe et al., 1974).

Perriere et al. (2005) used puromycin at a concentration of 3µg/ml for three days from the time when the isolated rat brain capillaries are seeded on a collagen matrix. In our system, this concentration proved to be extremely cytotoxic for HBEC, and fewer capillaries would adhere to the collagen extracellular matrix. The higher sensitivity of HBEC to puromycin compared to rat brain endothelial cells is further reinforced by the fact that in their study, Perriere et al. (2005) showed that rat brain endothelial cells were treated for 5 weeks with 0.5µg/ml without any apparent cytotoxic effects. However, the same concentration of puromycin induced signs of degradation and apoptosis in HBEC after only 2 weeks. Why HBEC are more sensitive to puromycin than rat brain endothelial cells remains to be established. It is possible that human brain endothelium expresses lower amounts of P-gp (or other unidentified efflux pumps for which puromycin may be a substrate) than rat brain endothelium in vivo rendering human cells more sensitive to the cytotoxic actions of drugs. Indeed, it has been shown that there are interspecies differences in P-gp activities in several marine and freshwater invertebrates which account for the sensitivity of particular species to pollution (Smital et al., 2000). It is possible that rat brain endothelial cells...
express P-gp, or an unidentified efflux pump, for which puromycin may be a substrate, at higher level than HBEC. Alternatively, HCBE may be more sensitive to culture conditions and lose their barrier phenotype more rapidly than rat brain endothelial cells. Nevertheless, short treatments with 0.5μg/ml puromycin did not induce any apparent signs of distress in HBEC. Hence, when the cells were treated with the lower concentration of 0.5μg/ml and for a period of 5 days after the 2 initial days when the cells were allowed to adhere to the collagen, the yield of endothelial cells was improved and purity maintained. These conditions proved to be the most efficient and the least toxic, based on the cell morphology and vWF staining and amount of cells present under these conditions. Astrocytes also express P-gp in vitro (Pardridge et al., 1997). Indeed, this could explain why the astrocytes did not die immediately following puromycin treatment. The expression of P-gp in vivo by astrocytes is more controversial. It has been suggested that expression of P-gp by astrocytes is an artefact due to the culture conditions, as no P-gp could be detected by immunohistochemistry on brain tissue of normal human brain (Tatsuta et al., 1992). Others have showed that P-gp is weakly expressed by astrocytes in vivo and is up-regulated in drug refractory human epileptic tissue (Marchi et al., 2004).

2.4.2. Expression of endothelial junctional proteins by primary HBEC

Primary HBEC displayed many characteristic features of brain endothelium in vivo. These include the expression of not only the adherens junction molecules but also the presence of a number of tight junction protein types which are specific to brain endothelial cells such as claudin-5 (Craig et al., 1998). Some of the junction proteins studied were not uniformly expressed in the culture. In some patches of cells, the protein expression was either strongly expressed or more linearly distributed whereas in other patches, as with occludin and claudin-5, the cells were negative for these proteins at the junctions. This may be explained by a difference in the confluence state. The same phenomenon has already been reported previously for a BBB associated enzyme, gamma-glutamyl transpeptidase enzyme.
When a rat brain endothelial cell line was grown in the presence of bovine FGF and on collagen, the confluent culture was reported to develop sprouts that extended above the monolayer and expressed this enzyme (Roux et al., 1994). The patches of cells that express strongly the tight junction proteins were similar in that they were slightly above the other cells monolayer.

2.4.3. Constitutive production of chemokines by primary HBEC

Chemokines, known for their chemotactic activities, are mostly functional at concentrations between 2 and 200ng/ml. In vitro, the concentration-dependent response of leukocytes to chemokines usually follows a “bell shape” curve, that is, a maximal effect is achieved at a certain chemokine concentration (Bryan et al., 2002).

Some chemokines are known to be expressed constitutively by certain cell types, whereas others are released only upon stimulation with cytokines. Out of the chemokines tested, only CCL2 and CXCL8 were found to expressed by HBEC constitutively at concentrations above 2ng/ml (CCL2 ~ 10ng/ml and CXCL8 ~ 70ng/ml). Our results on CXCL8 and CCL2 are similar to those previously described by others showing CXCL8 and CCL2 to be secreted into the supernatant of HBEC (Hofman et al., 1999; Biemacki et al., 2004).

Constitutive expression of CCL2 and CXCL8 by cultured HBEC might not reflect physiological conditions in vivo. In healthy humans, the concentration of CXCL8 and CCL2 in plasma is approximately 20pg/ml and 300pg/ml respectively (Bartosik-Psujek and Stelmasiak, 2005), much lower than that found in our cultures. Although it is possible that plasma proteases keep circulating levels of chemokines low and that the local concentrations within the brain capillaries may be high, it is likely that some factors present in the growth medium are stimulatory for CCL2 and CXCL8. HBEC in culture express CCL2 abundantly, whereas no CCL2 transcript was present in freshly isolated
capillaries, or whole brain homogenate (Zach et al., 1997). Furthermore, there is no detectable CCL2 expression in the normal rat brain, but CCL2 expression can be induced rapidly, within 3 h following ischemia, where there is a predominant staining of CCL2 on blood vessels (Chen et al., 2001). It is possible, therefore, that endothelial cells of capillaries in post-mortem tissue that have been exposed to ischemia over-express chemokines such as CCL2. Alternatively, species differences may determine whether endothelial cells express certain chemokines. By contrast with rat brain, CCL2 appears to be localised to the endothelial cells in normal human brain sections (Sanders et al., 1998) and this may account for the basal expression found in the endothelial cell culture in this study. Similarly, CXCL8 expression is also up-regulated by the presence of growth factors such as VEGF (Lee et al., 2003b). However, by contrast with CCL2, there is no detectable CXCL8 in normal human brain, although CXCL8 has been detected on HBEC of inflamed brains such as in HIV encephalitis (Sanders et al., 1998). The culture medium used in this study for maintaining endothelial cell cultures contains several growth factors (EGF, FGF, VEGF, IGF-1) which may account for the basal in vitro expression not only of CCL2 but also CXCL8 which contrasts with the in vivo lack of CXCL8 expression.

2.4.4. Effect of cytokine stimulation on chemokine expression by HBEC.

Following cytokine stimulation, the up-regulation of CCL2 secretion by HBEC, and of other chemokines by endothelial cells of peripheral vascular beds, has been well documented (Weiss et al., 1998; Frigerio et al., 1998; Beck et al., 1999; Hofman et al., 1999; Crane et al., 2000; Chen et al., 2001; Sundstrom et al., 2001; Hillyer et al., 2003). However, the regulation of CXCL8, CXCL10 and CCL5 by cytokines in HBEC is less clear. In this study, we attempted to make a systematic investigation of the effects of three cytokines, TGF-β, TNF-α and IFN-γ, alone or in combination, on the expression of these four chemokines (CCL2, CCL5, CXCL8 and CXCL10). No cinetique or dose/response studies were made due to material restriction, but it would have been of interest.
Previous studies have shown that TNF-\(\alpha\) and IFN-\(\gamma\) induce HBEC to secrete CCL2 (Frigerio et al., 1998; Weiss et al., 1998; Chen et al., 2001). According to Frigerio et al. (1998), an up-regulation of CCL2 mRNA induced by TNF-\(\alpha\) and INF-\(\gamma\) in HBEC only occurs in cells originally from MS donor tissue. By contrast, HBEC from non-MS patients showed an up-regulation of CCL2 mRNA only following treatment with IFN-\(\gamma\) but not with TNF-\(\alpha\). In our HBEC culture system, an up-regulation of CCL2 protein to similar levels in the supernatant was observed in response to TNF-\(\alpha\) in both HBEC from MS and non-MS patients (temporal lobe epileptic individuals). Altogether, this data would suggest that at least TNF-\(\alpha\) may regulate CCL2 production by a post-transcriptional mechanism. In addition, CCL2 up-regulation was observed following TNF-\(\alpha\) treatment but not IFN-\(\gamma\) which is in contrast to the observations reported by Weiss et al. (1998) and Chen et al. (2001) where both cytokines induced an effect. Furthermore, no synergistic effect of IFN-\(\gamma\) on the action of TFN-\(\alpha\) was observed in the present study. These different results may be due to differences in the initial concentration of IFN-\(\gamma\) used to stimulate the cells and not to individual donor variation as cells from all 6 donors tested responded in a similar manner in our study. It would be difficult, however, to relate the concentrations of IFN-\(\gamma\) between different studies since the concentration of IFN-\(\gamma\) previously reported was given in U/ml, whereas in this study the solutions were in ng/ml. In another study on endothelial cells from other vascular beds, Hillyer et al. (2003) used the same concentration of cytokines and observed a marked variability in the response to IFN-\(\gamma\) among the different endothelia. IFN-\(\gamma\) failed to up-regulated CCL2 in HUVEC and liver endothelial cells whereas it stimulated CCL2 production in saphenous vein endothelial cells (SVEC), bone marrow endothelial cells (BMEC), lung microvascular endothelial cells (LMEC) and dermal microvascular endothelial cells (DMEC). A lack of increase of CCL2 by IFN-\(\gamma\) was also reported in HUVEC and LMEC by Beck et al. (1999), who used concentrations of IFN-\(\gamma\)
as high as 250ng/ml. There was no synergistic effect of IFN-γ on the action of TNF-α when both cytokines were added together. Hence HBEC appear to respond in a similar manner as HUVEC. Retinal microvascular endothelial cells resemble HBEC in that they form a tight continuous monolayer with restricted permeability, the so-called blood-retinal barrier. Similarly to HBEC, Crane et al. (2000) reported no effect of INF-γ on CCL2 production, although in their case a concentration 5 times lower than in our study was used.

Using immunocytochemical techniques, intracellular CCL2 could not be detected upon cytokine stimulation in HBEC (Harkness et al., 2003). In HUVEC cells, CCL2 appears to be distributed in small vesicles throughout the cells when stimulated with a combination of IFN-γ and TNF-α (Oynebraten et al., 2004).

2.4.4.2.CCL5

TNF-α induced CCL5 production by HBEC, whereas IFN-γ had no effect. When the two chemokines were added together, they displayed an important synergistic effect on CCL5 production. By contrast, an increase in CCL5 expression by HBEC induced by IFN-γ has been reported previously (Shukaliak and Dorovini-Zis, 2000). The possibility that IFN-γ added to the culture medium may be inactive due to either degradation of the recombinant protein in the medium or to loss of receptor expression in cultured cells is highly unlikely since IFN-γ alone induced the expression of other chemokines such as CXCL10. Thus, it is possible that other factors such as those mentioned above for CCL2 are responsible for the differences in the effect of IFN-γ observed by these studies. More surprisingly, the anti-inflammatory cytokine TGF-β also exerted a synergistic effect together with TNF-α on CCL5 production by HBEC. CCL5 staining was not detected when the cells were stimulated with cytokines, suggesting that the chemokine is not stored nor re-internalised after 48 h stimulation.
2.4.4.3. CXCL10

CXCL10 expression is strongly induced by IFN-γ in many cell types and this was also the case in HBEC. By contrast, TNF-α did not induce a significant increase in CXCL10 expression. In addition, as demonstrated for CCL5, there was a synergistic effect when both TNF-α and IFN-γ were combined, where the production of CXCL10 was maximal. Endothelial cells from non-CNS vascular beds secrete CXCL10 in response to TNF-α, when stimulated under the same conditions and using the same concentration and detection methods (Hillyer et al., 2003). These results are also in agreement with those of Salmaggi et al. (2002) who have shown that TNF-α at a concentration of 200U/ml was able to induce CXCL10 production by HUVEC, but not by HBEC. This effect was further increased when IFN-γ and TNF-α were combined. This suggests a unique response pattern of the HBEC for TNF-α. The intracellular distribution of CXL10 in HBEC was somewhat different from other chemokines, in particular when the cells were stimulated with cytokines. In the latter case, CXCL10 appeared to be localised mainly within perinuclear vesicles. This could be due to either internalisation of CXCL10 produced by the cells and degradation by the proteasome system or the presence of de novo protein in the Golgi apparatus. Hillyer and Male (2005) have demonstrated that when human recombinant CXCL10 was added to HUVEC there was a rapid internalisation of the chemokine and this may be the case for CXCL10 secreted by HBEC. However, the pattern of staining looks different (Hillyer and Male, 2005) and indicates that the CXCL10 in the present study represents an intracellular stock.

2.4.4.4. CXCL8

In HBEC, CXCL8 was up-regulated by TNF-α alone whereas IFN-γ tended to decrease the basal secretion of CXCL8. This effect was more pronounced when IFN-γ was applied in combination with TNF-α, since a significant decrease on the TNF-α effect on CXCL8 expression was observed. The inhibitory effect of IFN-γ on the action of TNF-α has been
previously reported in other cell types including endothelial cells. In fact, this effect appears to be cell type specific. In some cell types such as monocytes IFN-γ is reported to suppress the effect of TNF-α (Schnyder-Candrian et al., 1995), whereas in other cell types such as a human gastric cell line, MKN 45, IFN-γ enhances TNF-α CXCL8 production (Yasumoto et al., 1992). The decrease in CXCL8 by IFN-γ in endothelial cells was found to be not only at the protein level, but also at the mRNA level (Nyhlen et al., 2004). The mechanism which underlines this process remains unclear but it most certainly occurs by interference of TNF-α signal transduction pathways that drive CXCL8 expression by IFN-γ. As reviewed by Hoffmann et al. (2002), CXCL-8 expression is regulated both at the transcriptional and post-transcriptional levels. To induce maximal secretion of CXCL8 there must be an inhibition of nuclear respiratory factor (NRF), a transcription factor that acts as a repressor of CXCL8. In addition, both Nuclear Factor Kappa B (NF-κB) and activator protein-1 (AP-1) need to be activated for efficient transcription of CXCL8 whereas the mRNA becomes stabilised through activation of the p38 MAP kinase pathway. TNF-α activates NF-κB and AP-1 through a protein kinase C dependant pathway (Kim and Rikihisa, 2002). The activation of those two transcription factors by TNF-α is inhibited in the presence of IFN-γ in endothelial cells, suggesting that NF-κB and AP-1 are targets for IFN-γ to counterbalance the TNF-α effect (Borgmann et al., 2002). Overall, the suppression of CXCL8 production by IFN-γ may be considered as a protective effect against neutrophil infiltration, since CXCL8 is the major chemoattractant for these cells. IFN-γ is specific to a Th1 mediated response, whereas in general there is poor neutrophil infiltration in MS brains. Furthermore, in EAE models such as BALB/c and C57BL/6, which are strains of mice that are resistant to EAE when immunised with myelin basic protein, the knock-out mice for either IFN-γ or its receptor rapidly develop a progressive lethal disease, where there is a major infiltration of neutrophils throughout the brain (Tran et al., 2000b).
TGF-β decreased the effect of TNF-α on CXCL8 production, also it represented only a partial inhibition. This may be due to the low concentration used of 25ng/ml. There is evidence that TGF-β may not exert its inhibitory effects in the presence of high levels of pro-inflammatory cytokines (Condos et al., 1998).

2.4.4.5. Variation in CXCL8 expression among HBEC cultures from different donors

By contrast with the other chemokines, there was a high variability in the secretion of CXCL8 by HBEC from different donors both at the basal level and in cytokine-stimulated cells. This variability in CXCL8 expression by HBEC has been observed by others in response to the HIV-1 protein tat (Hofman et al., 1999), and in other endothelial cells types in response to cytokines (Hillyer et al., 2003). These differences are not only in the amount of chemokine released in the supernatant, but also in the level of CXCL8 localised within the cells in vitro. In one case, CXCL8 was located in rod-like shaped vesicles probably corresponding to Weibel-Palade bodies, whereas for one other donor CXCL8 was diffuse within the cytoplasm. CXCL8 has been identified in Weibel-Palade bodies in HUVEC (Utgaard et al., 1998). The method of HBEC isolation is supposed to isolate primarily the microvascular fraction of cerebral blood vessels, which are devoided of Weibel-Palade bodies (Dorovini-Zis and Huynh, 1992), only present in larger vessels such as arteries or venules. However, in some cultures, vWF was located in Weibel-Palade bodies of endothelial cells, whereas in others it was located diffusely in the cytoplasm. This suggests the presence of HBEC of both microvascular and macrovascular origin in primary cultures. Indeed, it has been suggested that pure microvascular cultures are hard to obtain (Pachter and Song, 2005), and that when placed in culture, HBEC from larger microvessel fragments grow at a faster rate than HBEC from smaller vessels (Spatz et al., 1997). In addition, the type of blood vessel where HBEC originate may influence their phenotype. While in capillary and postcapillary venules, continuous tight junctions are present, arteries
have endothelial tight junctions containing focal discontinuities and collecting veins contain focal leaky regions (Nagy et al., 1984). HBEC from different types of blood vessels also have different immunological functions, as leukocyte extravasation has been proposed to occur mainly at the level of postcapillary venules (Villringer et al., 1991; Battastini et al., 2003). These observations may suggest that phenotypic and functional variations are responsible for the differences in staining of CXCL8 observed among different batches of HBEC. It is thus possible that CXCL8 is stored in HBEC originating from larger vessels, whereas HBEC from capillaries do not express CXCL8 at basal level.

Another factor that might influence intracellular location and/or expression of CXCL8 in cultured HBEC is the number of cell divisions since isolation. This type of effect has been demonstrated for vWF, for which expression by HBEC tends to decrease as the number of passages increases (Craig et al., 1998). In the present study, HBEC at passage 2 were routinely used. However, the number of cell divisions since isolation may still be different. This is because the yield of capillary fragments and hence the number of dividing cells after isolation is dependant on a number of factors (post-mortem time, quantity of tissue, cause of death among others).

Another hypothesis underlying differences in CXCL8 intracellular distribution would involve inter-individual variation among different donors. Variability of CXCL-8 production between brain endothelium from different donors cannot be excluded and it is possible that the state of activation of endothelial cells at the moment of death may influence subsequent chemokine production in culture. The cells producing highest levels of CXCL8 at the basal level originated from a donor who died of sepsis. Sepsis results in an overwhelming systemic inflammation, which is caused by the excessive release of cytokines into the circulation, mainly TNF-α, IL-1β, IL-6 and CXCL8 (Blackwell and Christman, 1996). On the other hand, it has also been proposed that endothelial cells store
CXCL8 in Weibel-Palade bodies, after a prolonged stimulation with inflammatory mediators (Wolff et al., 1998), and when cells are subject to a preceding inflammatory stimulation, CXCL8 can be released without de novo protein synthesis in HUVEC. This could be the case in HBEC isolated from a patient with sepsis in which the high levels of CXCL8 release may be due to prior priming with circulating cytokines. However, as the cells went through a number of cell divisions, this possibility is unlikely.

Finally, differences in chemokine receptor expression between different batches may determine differences in CXCL8 internalization. These may involve CXCL8 specific receptors such as CXCR1 or CXCR2 or promiscuous receptors that bind many chemokines such as DARC. DARC is a seven-transmembrane-segment receptor which binds several chemokines of the CC and CXC families and serves to clear these chemokines from the blood. Among the diverse chemokines binding to DARC are CXCL8, CCL5 and CCL2, but not CXCL10 (Comerford and Nibbs, 2005). Some individuals lack the expression of this receptor on erythrocytes, which confer upon them a resistance to the malarial parasite, but these individuals are immunologically normal. The clearance of CXCL8 from the blood in these individual is ensured by the presence of the DARC receptor on postcapillary venule endothelial cells (Hadley et al., 1994). CXCL8 can be transported across an endothelial cell monolayer via DARC (Lee et al., 2003a). Thus it could be hypothesised that HBEC, which could arise from different sources (capillaries, venules, arterioles) could show a different pattern of CXCL8 expression depending on their level of expression of DARC. For example, the endothelial cells that are positive for CXCL8 at basal level could originate from venules that express the DARC receptors that would allow them to internalize the chemokines secreted by the endothelial cells. This, however, is unlikely because of the lack of DARC on brain endothelial cell in situ. Indeed, DARC in the brain was located by immunohistochemistry on Purkinje cells in the cerebellum and not on endothelial cells (Horuk et al., 1996). Furthermore, the staining for CCL5 and CCL2, also
ligands of DARC, was negative indicating the absence of functional DARC on HBEC. It is also relevant to consider that the levels of secreted chemokines were relatively different among the different chemokines. CXCL8 was detected at very high level in the supernatant of unstimulated cells, whereas CCL2 was relatively low. In order to answer this question, it would have been of interest to investigate chemokine concentration in the supernatant and to correlate this with the intracellular staining in the same cells. Differences in pattern of binding of CXCL8 to the vascular bed have also been observed. Rot et al. (1996, communicated results but not shown) reported substantial differences in CXCL8 binding pattern among organs and also within the same organ in normal pig. For example CXCL8 binding to endothelial cells was present only in venules of the pericardium, in small veins and other blood vessels in the heart muscle but not in the lung (Rot et al., 1996). In ischemic brain, there is an important infiltration of neutrophils suggesting that the endothelial cells are able to bind CXCL8 under certain circumstances (Garau et al., 2005).

2.4.5. Effect of differentiating factors used to induce BBB phenotype in cultured endothelium on chemokine expression

The use of differentiating factors known to induce BBB properties in vitro induced a decrease of the intracellular staining of CXCL10 under basal conditions, and of increasing the release of CXCL8 in the supernatant when exposed to a combination of the two cytokines IFN-γ and TNF-α. It would have been of interest to stimulate the endothelial cells with ACM alone especially as astrocyte factors have a great influence in the inflammatory response capability of the brain. Astrocytic factors have been shown to induce ramification of macrophages on hippocampal slices, and reduced their expression of MHC class II and LFA (Hailer et al., 1998). This effect was partly inhibited by neutralizing antibodies directed toward TGF-β (Hailer et al., 1998). Nevertheless, ACM did not induce any changes in the pattern of expression of chemokines by HBEC according to Prat et al. (2001). It is much more probable that the decrease in CXCL10 and the

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increase in CXCL8 observed in the present study are directly related to a cAMP mediated effect on the signalling pathway of IFN-γ. The IFN-γ signal transduction pathway involves mainly activation of signal transducer and activator of transcription 1 (STAT1) as reviewed by Stark et al. (1998) and the cAMP pathway blocks STAT1 (Sengupta et al., 1996). It is possible that a similar signalling scenario occurs in HBEC. First, IFN-γ would stimulate the secretion of CXCL10 via STAT1. An increase in intracellular cAMP would then lead to inhibition of STAT1 and hence a decreased expression of CXCL10.

2.4.6. Polarized release of chemokines by HBEC.

Cells in culture form two-dimensional monolayers when grown on plastic substrates and therefore are far removed from their natural three-dimensional environment in vivo. In a physiological context, it is possible that chemokines may be released either on the luminal side into the blood or the abluminal side into the brain parenchyma. HBEC grown on filters constitute an in vitro model more closely resembling the in vivo situation and allowing the possibility of determining polarized release of chemokines. HBEC release of CCL2 into the upper chamber, corresponding to the luminal side, is five-fold higher compared to that into the lower chamber when grown on 3μm diameter pore tissue culture inserts under stimulation of TNF-α (Weiss et al., 1998). The results presented here using 0.4 μm pores demonstrated that CCL2 release into the apical side at basal level is 50% higher than into the basolateral side, whereas three quarters of the total CXCL8 released was found in the upper chamber at basal level. These data suggest that chemokines are preferentially released onto the luminal side rather than contributing to the brain inflammatory reaction within the brain parenchyma. However, the role of luminal released chemokines by HBEC in orchestrating leukocyte infiltration is still unclear.

Chemokines released into the circulation would be rapidly degraded by proteases or their action neutralized by decoy chemokine receptors such as DARC expressed on erythrocytes
(reviewed by Comerford and Nibbs (2005)). However, it is possible that secreted chemokines bind to the endothelial surface either on the secretory cell itself or on endothelial cells further along the capillary wall. In order to address this question, the localisation of chemokines on/in the endothelial cells by immunocytochemical methods was investigated. CCL5 stained negatively on the endothelial cells either at the basal level or after cytokine stimulation. CCL2 is released at basal level into the supernatant but no chemokine staining was detected at the light microscopy level, neither on the surface of the cells nor within the cytoplasm. It is possible that the immunocytochemical methods lack sensitivity to detect the chemokines. Yet Hillyer et al (2005) have shown that there is little if any binding of recombinant CCL2 on the surface of the different endothelial cells, which would be in agreement with our own observations. It is also possible that the cells lose their property to bind the chemokines in vitro, as seems to be the case for CXCL8 (Rot et al., 1996). As for CXCL10, we used three different antibodies, and two of these antibodies showed a negative staining. One antibody showed positive staining for CXCL10 on unstimulated cells. There are reasons to hypothesise that this staining might not be specific (see chap 4 for explanations).

No papers in the literature report on chemokine staining on HBEC in vitro under basal conditions, with the exception of Hofman et al. (1999), who showed a very low staining for CXCL8 (less that 3% of the culture). The same group also reported that CXCL8 can be up-regulated at the cellular level by endothelin-1, but not on all the cells (50% to 70%) and the staining was mainly around the cell membrane (Hofman et al., 1998). CCL2 in a rat brain endothelial cells line shows an intracellular localisation (Harkness et al., 2003). However there are few studies looking at intracellular chemokines on other endothelial cell types for CXCL8, where CXCL8 was detected in the Weibel-Palade bodies at basal level in human microvascular endothelial cells from the intestine and nasal polyps endothelial cells but not in HUVEC (Utgaard et al., 1998).
2.4.7. Conclusion

The isolation and purification of HBEC was improved using a technique which has been developed to create a pure endothelial cell preparation for rat brain tissue. These cells are proven to be more sensitive than rat brain endothelial cells to puromycin treatment. This has allowed us to obtain a rather pure brain endothelial preparation, and not to lose any cells due to contaminants, and has proven to be a considerable improvement in research on primary HBEC. Its use has already helped to obtain a human brain endothelial cell line (see chap 4). We further demonstrate that HBEC can produce large amounts of CXCL10 and CXCL8, and to a lesser extent CCL2 and CCL5 under cytokine stimulation. Furthermore, a possible explanation was provided for the fact that neutrophils are absent from MS lesions in that there is regulation of CXCL8 secretion by IFN-γ. Interestingly, chemokine production in the brain can be influenced by the same factors that can trigger BBB properties.
Chapter 3: Chemokine receptor expression by human brain endothelial cells
3.1 Introduction

Chemokine receptors, first documented in 1989 (Samanta et al., 1989), have been shown to participate in several important functions such as chemotaxis, transport and transcytosis of chemokines, clearance of excess chemokines and angiogenesis. Many cells displaying a high expression of chemokine receptors are located in the parenchyma of MS brains. However, immunohistochemical studies in chemokine receptor expression on human brain showing positive reactivity within the neurovascular unit do not differentiate between brain endothelial cells and perivascular cells, such as pericytes, or perivascular macrophages or even the end feet of parenchymal cells such as astrocytes (Decleves et al., 2000). Yet the expression of chemokine receptors by HBEC could be crucial as they may play a role in chemotaxis, transcytosis, clearance of excess chemokines, and angiogenic or angiostatic responses.

Previously, in vitro studies have shown that endothelial cells from different vascular beds express a variety of chemokine receptors (Berger et al., 1999; Murdoch et al., 1999; Hillyer et al., 2003). In addition, chemokine receptor expression may vary from one endothelial cell type to another (Hillyer et al., 2003). Nevertheless, few studies have reported the expression of chemokine receptors by HBEC, probably due to the difficulties encountered when trying to isolate them and limited tissue availability. Berger et al. (1999) have shown the expression in vitro of CCR2A, CCR3, CCR4, CCR5 and CXCR1, CXCR3 and CXCR4 in HBEC in primary culture by immunocytochemistry and by FACS analysis.

In this part of the present study, the expression of chemokine receptors that have been previously shown to be up-regulated in lesions such as CCR1, CCR2, CCR5, CXCR2 and CXCR3 was investigated (Balashov et al., 1999; Mennicken et al., 1999; Sorensen et al., 1999; Huang et al., 2000; Jee et al., 2002; Sorensen et al., 2002; Trebst et al., 2003). These
receptors are of particular interest since their chemokine ligands are also expressed by endothelial cells such as CCL2, CCL5, CXCL8 and CXCL10. In addition, CXCR1 was also investigated since it is the receptor for CXCL8 chemokine together with CXCR2, and is strongly expressed by other endothelial cell types (Murdoch et al., 1999; Hillyer et al., 2003).

In view of the paucity of data concerning CNS endothelium and chemokine receptor expression, in this study, attempts were made to characterise the expression of some chemokine receptors in vitro, at the transcript level by RT-PCR and at the protein level by immunocytochemistry, and in vivo in MS brain, by immunohistochemistry and at the electron microscopy level combined with immunogold detection techniques.
3.2 Material and methods

3.2.1. RT-PCR

RT mix: 1X ImProm-II reaction buffer (25mM Tris-HCl, pH 8.3 at 25°C, 37.5mM KCl and 5mM DTT), 3mM of MgCl₂, 0.5mM deoxyribonucleoside triphosphates (dNTPs), 1U/μl of rRNasin and 2 μl of Improm-II™ Reverse transcriptase (Promega UK, Southampton, UK)

PCR mix: 1X PCR buffer (50mM KCl, 10mM Tris-HCl, pH 9.0 at 25°C, and 0.1% Triton® X-100), 1.5mM MgCl₂, 0.2mM of dNTPs, 1 μM of each primer (forward and reverse) and 1 U of Tag polymerase (Promega UK, Southampton, UK)

Loading Dye: 6X : 0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll® 400, 10mM Tris-HCl, pH 7.5, and 50mM EDTA (Promega UK, Southampton, UK)

TAE buffer: 40mM tris acetate/1mM EDTA (Sigma-Aldrich, Dorset, UK), PH 8

RNA was isolated from primary HBEC grown to confluence in a F25 cm² flask coated with collagen as described in section 2.2.1, 2.2.2, 2.2.4. Total RNA was extracted using 1 ml of TRIzol LS (Invitrogen, Renfrewshire, UK) according to the manufacturer’s protocol. The cells were scraped off the flask in the 1 ml TRIzol reagent and incubated at RT for 5 min. 2.6 ml of chloroform (Sigma-Aldrich, Dorset, UK) was added and after a vigorous shake, samples were centrifuged (HAWK 15105 from Sanyo) at 12,000g for 15 min at 4°C. This centrifugation step allows the separation of protein, DNA and RNA. The RNA is located in the colourless upper aqueous phase. The aqueous phase was collected and the RNA was precipitated by addition of 0.66 ml isopropyl alcohol (BD chemicals, Dorset, UK) for 10 min at RT. The isopropyl supernatant was removed after a centrifugation of 12,000 g for 10 min at 4°C. The pellet left containing the RNA was washed with 1.33 ml
of 75% ethanol (B&D chemicals, Dorset, UK). After another centrifugation step, the pellet was dried, re-suspended in 30 μl of distilled water and stored at -80°C. A 1 μl aliquot was taken and added to 500 μl of distilled water. The concentration of RNA in each sample was calculated with the following formula

\[ C_{RNA}(\text{ng/ml}) = \text{Abs}_{260nm} \times 40 \]

After determining the OD at a wave length of 260nm in a UV spectrophotometer (Spectronic Genesys 5, Milton Roy, Essex, UK) and correcting the dilution factor.

For the reverse transcription (RT) procedure, the protocol given by the supplier, Promega (ImProm-II™ Reverse transcriptase) was followed. 2 μg of RNA was mixed with 1 μg of random primer at 70°C for 5 min then annealed on ice. Reverse transcription was carried out by addition of 40 μl RT mix at 25°C for 5 min followed by 60 min at 40°C. To terminate the reaction, the mix was incubated for further 15 min at 70°C. The negative control for each sample consisted of RNA samples annealed with random primers with Reverse Transcriptase and without the Reverse Transcriptase. The negative control ensures that there is no contamination with genomic DNA. The resulting cDNA was either stored at -20°C, or taken directly into the PCR amplification procedure.

The PCR amplification steps were performed as advised by Promega which supplies the Taq DNA polymerase. 4 μl of cDNA from the RT reaction was added to 20 μl of PCR mix. This PCR reaction mix was placed into the PCR machine (iCycler, Bio-Rad Laboratories, Hertfordshire, UK) PCR conditions were 5 min at 94°C, followed by 40 cycles (94°C, 45 seconds (sec); Tm, see table 3.1, 45sec, 72°C, 60 sec) finishing with 7 min at 72°C. The reaction ended by a final 72°C incubation for 7 min. In addition to the chemokine receptor mRNA detection, each sample was run in parallel with cyclophilin primers, acting as a positive control.
The product of the RT-PCR was mixed with a loading Dye and run in a 1.5% agarose gel in TAE buffer at the voltage of 5 V/cm. The bands were visualised under UV light with Alphalmager 1220 (Alpha Innotech Corporation, distributor GRI, Essex, UK). The expected length of the RT-PCR products is listed in table 3.1.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Tm (°C)</th>
<th>Length (bp)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR1 F</td>
<td>ACG AAA GCC TAC GAG AGT G GGT GAA CAG GAA GTC TTG G</td>
<td>50</td>
<td>240</td>
<td>Designed by I. Romero</td>
</tr>
<tr>
<td>CCR1 R</td>
<td>GAT TAC GGT GCT CCC TGT C GCC ACA GAC ATA AAC AGA ATC</td>
<td>50</td>
<td>496</td>
<td>Designed by I. Romero</td>
</tr>
<tr>
<td>CCR2 F</td>
<td>GCT GAG ACA TCC GTT CCC CTA CA GGT GAC CGT CCT GGC TTT TA</td>
<td>58</td>
<td>477</td>
<td>(Lazzarino et al., 2000)</td>
</tr>
<tr>
<td>CCR2 R</td>
<td>GTG ATG CTG GTC ATC TTA TAC AG TTG TTT GGA TGG TAA GCC TGG</td>
<td>52</td>
<td>230</td>
<td>Designed by I. Romero</td>
</tr>
<tr>
<td>CXCR1 F</td>
<td>CGA AGG ACC GTC TAC TCA TC AGT GTG CCC TGA AGA AGA GC</td>
<td>53</td>
<td>519</td>
<td>Designed by I. Romero</td>
</tr>
<tr>
<td>CXCR1 R</td>
<td>GGA GCT GCT CAG AGT AAA TCA C GCA CGA GTC ACT CTC GTT TTC</td>
<td>53</td>
<td>200</td>
<td>(Jinquan et al., 2000)</td>
</tr>
</tbody>
</table>

Table 3.1: Sequence of the primers used for RT-PCR with corresponding melting temperatures (Tm) and length of expected PCR products (in number of base pairs, bp)

3.2.2. Immunocytochemistry

After seeding, the cells were grown for one week on Lab-Tek™ II Chamber slide (Nunc, UK) coated with collagen. They were fixed with 4% paraformaldehyde in PBS for 10 min on ice, and the membrane was permeabilised with 0.1% Triton-X100 in PBS for 5 min. After 3 rinses with PBS, the non specific binding sites were blocked using 10% goat serum in PBS for 1 h at RT. The primary antibodies for the chemokine receptors were then incubated for 1 h at RT. The antibodies, from R&D systems, were fluorescently labelled and are listed in table 3.2. To ensure that the cells in primary culture had an endothelial phenotype and were not contaminating cells from the isolation procedure, cells were also incubated with an antibody specific for vWF as an endothelial cell marker. For double
labelling, after the excess anti-chemokine receptor antibodies were washed off, the same protocol as described in chapter 2, section 2.2.6.1 was followed. Briefly, the second primary antibody (vWF, 1/200, Sigma-Aldrich, Dorset, UK) was applied for 1 h at RT. After 3 washes with PBS, the goat anti rabbit secondary antibody (1/100, Chemicon, Hampshire, UK) conjugated to either phycoerythrin or fluorescein was added to the cells and incubated for 1 h. After 3 final washes, the slides were mounted with a glass coverslip with Dako Fluorescent Mounting Medium (DakoCytomation Ltd, Cambridgeshire, UK) and viewed with a fluorescence microscope (Olympus BX61).

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Type</th>
<th>Label</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human CXCR1</td>
<td>Monoclonal mouse IgG2A, clone #42705</td>
<td>Phycoerythrin</td>
<td>R&amp;D, Oxfordshire, UK</td>
</tr>
<tr>
<td>Human CXCR2</td>
<td>Monoclonal mouse IgG2A, clone #48311</td>
<td>Phycoerythrin</td>
<td>R&amp;D, Oxfordshire, UK</td>
</tr>
<tr>
<td>Human CXCR3</td>
<td>Monoclonal mouse IgG1, clone #48311</td>
<td>Phycoerythrin</td>
<td>R&amp;D, Oxfordshire, UK</td>
</tr>
<tr>
<td>Human CCR1</td>
<td>Monoclonal mouse IgG2B, clone #53504</td>
<td>Phycoerythrin</td>
<td>R&amp;D, Oxfordshire, UK</td>
</tr>
<tr>
<td>Human CCR2</td>
<td>Monoclonal mouse IgG2B, clone #48607</td>
<td>Phycoerythrin</td>
<td>R&amp;D, Oxfordshire, UK</td>
</tr>
<tr>
<td>Human CCR3</td>
<td>Monoclonal mouse IgG2A, clone #61828</td>
<td>Fluorescein</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>Human CCR4</td>
<td>Polyclonal rabbit IgG</td>
<td>No label</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Human CCR5</td>
<td>Monoclonal mouse IgG2B, clone #45502</td>
<td>Fluorescein</td>
<td>R&amp;D, Oxfordshire, UK</td>
</tr>
<tr>
<td>Mouse IgG1</td>
<td>Normal mouse IgG1, clone #11711</td>
<td>Phycoerythrin / Fluorescein</td>
<td>R&amp;D, Oxfordshire, UK</td>
</tr>
<tr>
<td>Mouse IgG2A</td>
<td>Normal mouse IgG2A, clone #20102</td>
<td>Phycoerythrin / Fluorescein</td>
<td>R&amp;D, Oxfordshire, UK</td>
</tr>
<tr>
<td>Mouse IgG2B</td>
<td>Normal mouse IgG2B, clone #20116</td>
<td>Phycoerythrin / Fluorescein</td>
<td>R&amp;D, Oxfordshire, UK</td>
</tr>
</tbody>
</table>

Table 3.2: List of antibodies for the chemokine receptors used for Immunocytochemical analysis.
3.2.3. Immunohistochemistry

0.1M Phosphate Buffer (PB): 78mM sodium phosphate dibasic, 23mM sodium phosphate monobasic, pH

Mowiol mounting solution: 12 % mowiol in 60mM PB and 30% glycerol

Fresh tissue, from the same origin as the tissue used for the isolation of HBEC (section 2.2.3), of 1 cm³ was fixed using 2% PAF and 3.75% acrolein in 0.1M PB for 1 h and left overnight in 2% PAF only. The sections were cut at 50μm using a Vibratome (Leica Microsystems, Buckinghamshire, UK).

In order to characterise the type of lesions, the sections were stained for CD68 and MHC class II. As reported in Bo et al. (1989), active lesions are CD68 positive/MHC class II positive, chronic active lesions are CD68 positive/MHC class II positive in the edge of the lesion and CD68 positive/MHC class II negative in the centre, and chronic inactive lesions are CD68 negative/MHC class II negative.

All sections were permeabilized with 0.1% Triton X100 in PBS for 1 h. Non-specific binding sites were blocked using 10% normal rabbit serum for 1 h. The primary antibodies (CD68, 1/20 from Dako Corporation, Cambridgeshire, UK and MHC class II 1/200 from Serotec Ltd, Oxfordshire, UK) were incubated on the slide overnight at RT. After 3 washes with PBS, the secondary antibody (rabbit anti-mouse streptavidin conjugated, Dako, 1/100) was left to bind for 1 h. The excess of secondary antibody was washed off by 3 times rinsing with PBS and the avidin-biotinylated peroxidase (Vectastain ABC Kit, Vector Laboratories, Peterborough, UK) was added on the sections for 1 h. 10 mg of 3,3’-Diaminobenzidine (DAB), dissolved in 20 ml of PBS with 20 μl of hydrogen peroxide, was used to reveal the presence of peroxidase for 3 min. The reaction was stopped by 3
washes with PBS. Nuclei were stained using Harris’ Hematoxylin for 5 min, the excess was washed off under running tap water, and to intensify the staining, the slides were plunged in acetic acid (3%) for 10 sec, and further rinsed in running tap water. Thereafter, the sections were dehydrated in a series of ethanol baths (50%, the 75%, 90%, 95%, and 100% ethanol and a final clearing step in 100% xylene) and mounting with DPX (Distrene, Plasticiser, Xylene) (B&D chemicals, Dorset, UK).

For sections labelled for vWF/GFAP and CXCR1/CXCR3, an antigen retrieval step was included, essentially as described by Shi et al. (1991). Sections were left for 20 min in a 0.01M citrate buffer, pH 6, at 95°C using a heating block. All sections were then permeabilized with 0.1% Triton X100 in PBS for 1 h. Non-specific binding was blocked using 10% normal goat serum for 1 h. Sections were incubated with mouse anti-CXCR1/CXCR3 (10μg/ml, R&D, Oxfordshire, UK) for 1 h and, after 3 washes, the secondary goat anti-mouse IgG (1/100, Chemicon, Hampshire, UK) conjugated to the fluorochrome cy3 was left for 1 h at RT. Sections were then incubated with a rabbit polyclonal anti-human vWF (1/800, Sigma, Dorset, UK) or anti-GFAP (1/100, Chemicon, Hampshire, UK) antibodies in PBS overnight at RT. The secondary antibody used was a goat anti-rabbit conjugated to FITC (1/200, Chemicon, Hampshire, UK), and was incubated for 1 h. Sections were mounted using mowiol and viewed on the fluorescence microscope (Olympus, BX61).

3.2.4. Immunogold labelling and electron microscopy

0.1M Phosphate Buffer (PB): 78mM sodium phosphate dibasic, 23mM sodium phosphate monobasic, pH 7.4

0.1M Tris-buffered saline (TS): 100mM Trizma base, 154mM saline, pH 7.6.

Blocking solution: 0.5% BSA in TS
0.01M Phosphate Buffer Saline (PBS): 7.7mM sodium phosphate dibasic, 2.3mM sodium phosphate monobasic, 300mM sodium chloride, pH 7.4.

Cryoprotectant solution: 25% sucrose, 3.5% glycerol in PB, pH 7.4

Incubation/washing buffer: 0.8% BSA and 0.1% gelatine in PBS

Immunogold labelling was performed essentially as described by Rodriguez et al. (2001). For chemokine receptor staining, fresh tissue was fixed using 2% PAF and 3.75% acrolein in 0.1M PB for 1 h and left overnight in 2% PAF. The sections were cut at 50µm using a Vibratome (Leica Microsystems, Buckinghamshire, UK). The floating sections were then treated with 1% sodium borohydride in PB for 30 min and then freeze-thawed to permeabilize the tissue. For this procedure, the sections were incubated in Cryoprotectant solution and then rapidly immersed in dichlorofluoromethane (Air Products, Warwickshire, UK), followed by liquid nitrogen and then three successive PB washes. Blocking solution was added for 30 min, and sections were incubated with primary mouse antibody (mouse anti-human CXCR1, 5µg/ml or mouse anti-human CXCR3, 1µg/ml, R&D, Oxfordshire, UK) for 24 h at RT and a further 24 h at 4°C in 0.1% BSA in TS. Non-specific binding of the secondary antibody was blocked with the incubation/washing buffer for 10 min after a rinse with PBS. Sections were then incubated for 2 h with goat anti-mouse secondary antibody (dilution 1/50) conjugated to colloidal gold (British Biocell International, Wales, UK) in the incubation/washing buffer. After a 5 min wash with the incubation/washing buffer and three rinses with PBS, bound gold particles were fixed using 2% glutaraldehyde in PBS for 10 min followed by a wash in a 0.2M citrate buffer solution. The sections were reacted with a silver enhancement solution (British Biocell International, Wales, UK). The silver enhancement reaction was stopped by two washes in the citrate buffer and three washes with PB. The immunogold stain tissue was post-fixed for 1 h in 2% osmium tetroxide in PB for electron microscopy viewing. They were then dehydrated in a series of ethanol solutions (concentration 50%, 70%, 80%, 95% for 5 min,
and twice in 100% for 10 min) and twice in propylene oxide for 10 min before being embedded in Epon 812 between sheets of Aclar plastic. Epon polymerisation was carried out by incubation at 60°C over 48 h, and a sample of the tissue was mounted on the tips of Epon Block. Ultra-thin sections of 70 nm were cut with a diamond knife (Diatone, TAAB, Dorset, UK) and collected on copper mesh grids. The ultra thin cutting was made by Frances Coyle. The sections on grids were counterstained with uranyl acetate for 20 min and lead citrate for 6 min, and examined with a Joel JEM1010 electron microscope attached to a Gatan Bioscan digital camera (Joel, Hertfordshire, UK). Micrographs at X8000 magnification were taken, and scanned with Epson Perfection 4870 Photo, visualised with Photoshop 5.5 programme.

For quantification of the gold particle density (number of gold particles/μm²), the negative film of the photograph was scanned and used to count the number of gold particles on the blood vessels present in one section and reported on the area of the endothelial cells, that was determined on the digital images, using NIH image programme on a Macintosh G4. More over, the localisation of the gold particle in the cells was recorded as follows: cytoplasmic, luminal or baso-lateral and was reported as a percentage of the total number of gold particle.

3.2.5. Statistical analysis

Significance was determined by a one way or two way ANOVA, followed by a post hoc Tukeys t-test. P<0.05 was considered significant.
3.3 Results

3.3.1. Expression of chemokine receptors by HBEC in vitro

Figure 3.1 shows that at the mRNA level, HBEC express CCR1, CCR5, CXCR1, CXCR2 and CXCR3 but not CCR2 as detected by RT-PCR.

At the protein level CCR3, CCR5 (fig 3.2), and CXCR1, CXCR2 and CXCR3 (fig 3.3) were expressed by HBEC as shown by immunocytochemistry. CCR1 or CCR2 (fig 3.2) could not be detected, suggesting they are not expressed constitutively in vitro. Chemokine receptor staining was compared to the level of fluorescence obtained with isotype-matched negative control antibodies conjugated to TRITC or FITC (fig 3.4). The staining for CXCR1, CCR3 and CXCR3 was very strong whereas that for CXCR2 and CCR5 was much weaker. Chemokine receptors appeared to be localised mainly intracellularly rather than on the cell surface, in particular around the nucleus. Their distribution within the cell appeared uniform inside the cytoplasm and not specifically within a vesicular compartment. The pattern of chemokine receptor expression did not differ between cells originating from MS brains and those from epileptic patient brains (data not shown). However, the intensity of the staining could not be directly compared as the experiments involving cells from different donors were not carried out at the same time. For each chemokine receptor, cells were also labelled for vWF to confirm the endothelial cells identity (fig 3.2-4).

3.3.2. Characterisation of MS lesions

All tissue sections were characterised according to the method of Bo et al. (1994). CD68 is a marker for microglia and macrophages and according to Bo et al. (1994), only CD-68 positive inactivated microglia, which are small ramified cells are detected in NAWM
(normal appearing white matter) (fig 3.5). Resting microglia are slightly positive for MHC class II antigen in NAWM (fig 3.5).

Generally, the staining for CD68 shows a different pattern in MS lesions (fig 3.5). Microglial cells were much more rounded, and with an amoeboid form, representing an activated stage of the microglia. In addition, the antibody specific for CD68 also stained infiltrated macrophages in lesions.

There were additional distinctive immunohistochemical features that differentiated active lesions, chronic active lesions and chronic silent lesions. In the active lesions, all CD68 positive macrophage/microglia cells were also MHC class II positive. However, in chronic active lesions, the centre of the lesion was negative for MHC class II, and the edges were still MHC class II positive, suggesting that an active immune response was still occurring. By contrast, in chronic silent lesions, staining for amoeboid cells for CD68 was observed whereas the staining for MHC class II on these amoeboid cells was absent (not shown).

Using these criteria, a number of chronic active and chronic silent lesions in brain tissue from 6 different MS patients were characterised. The description of each individual case, including age, gender, disease type, post-mortem time, cause of death and lesion activity is shown in table 3.3. In all 6 patients, active lesions were rarely identified. It has been shown that active lesions, either chronic or acute, are mostly present at early stages of the disease (Sanders et al., 1993). In support of this finding, active lesions were exclusively detected in brain tissue from patients that died at an early age (34 and 44 years old) whereas chronic active lesions were detected in a 50 year old patient. In the remaining three cases, only demyelinated chronic silent lesions, or no lesions at all could be identified. As only a small sample of tissue was obtained from the MS Tissue Bank for each individual patient,
whether the pattern of lesions described above is representative of the whole brain remains to be determined.

For the study combining immunogold techniques and transmission electron microscopy, only three cases shown in bold in table 3.3 were analyzed corresponding to those with chronic active lesions and also with the best preserved ultrastructure of the blood vessels which facilitated interpretation of the results. All tissues from the 6 cases were analyzed for CXCR1/CXCR3 expression by immunofluorescence (sections 3.3.3).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Post mortem (h)</th>
<th>Cause of death</th>
<th>lesion activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>Female</td>
<td>13</td>
<td>Multiple sclerosis</td>
<td>Chronic active lesion</td>
</tr>
<tr>
<td>2</td>
<td>34</td>
<td>Female</td>
<td>15</td>
<td>Pneumonia</td>
<td>Active/chronic active lesions</td>
</tr>
<tr>
<td>3</td>
<td>59</td>
<td>Female</td>
<td>32</td>
<td>Respiratory failure, cardiac arrest</td>
<td>No demyelinated lesions</td>
</tr>
<tr>
<td>4</td>
<td>70</td>
<td>Female</td>
<td>21</td>
<td>Aspiration pneumonia, sepsis, multiple sclerosis</td>
<td>Silent chronic lesion</td>
</tr>
<tr>
<td>5</td>
<td>44</td>
<td>Female</td>
<td>36</td>
<td>Multiple sclerosis</td>
<td>Active/chronic active/chronic silent lesions</td>
</tr>
<tr>
<td>6</td>
<td>71</td>
<td>Female</td>
<td>36</td>
<td>bronchopneumonia, multiple sclerosis</td>
<td>No demyelinated lesions</td>
</tr>
</tbody>
</table>

Table 3.3: Case description of brain tissue from the MS patients investigated in this study.

3.3.3. Expression of chemokine receptors by HBEC in situ in normal appearing white matter.

CXCR1 and CXCR3 appeared to be expressed at high levels by HBEC in culture. A detailed analysis of the expression of these two chemokine receptors was therefore performed in situ in MS brain sections.

50μm thickness sections were double labelled with either vWF and CXCR1 or CXCR3 to determine the cellular location of the receptors. Cells that were positive for vWF appeared
to also express CXCR1 and CXCR3 (fig 3.6/3.7) but not all. Moreover, the thickness of the section does not allow a clear identification co-localisation, as some of the fluorescence detected for CXCR1 and CXCR3 could originate from the astrocytes foot processes that surround the vessels and are closely juxtapose to endothelial cells, and endothelial cells are thin. In addition, other cells within the brain parenchyma were also positively labelled for CXCR1 and CXCR3.

Since human astrocytes have been previously shown to express CXCR1 and CXCR3 (Flynn et al., 2003), double labelling with CXCR1/3 and GFAP, a specific marker for astrocytes was carried out. Since astrocytes are bigger cells and easier to identify by immonohistochemistry, a clear co-localisation of CXCR1 and CXCR3 and GFAP was observed indicating that astrocytes (fig 3.8/3.9) processes and the end-feet surrounding the vessels were strongly positive. Cells enclosed within the astrocytic end-feet were also positively labelled although the endothelial nature of those cells could not be ascertained.

3.3.4. Expression of chemokine receptors by HBEC in situ in MS lesions.

CXCR1 and CXCR3 were also expressed by many cells in MS lesion (fig 3.6-3.9). The positive staining was more diffuse throughout the lesion, probably due to astrogliosis or leukocyte infiltration. There did not appear to be any difference in the intensity of the label between the edge and the centre in a chronic active lesion (not shown). There were no clear results indicating that the staining on the astrocytes or in vessels were stronger for either of the two chemokine receptors investigated here. Small rounded cells in the perivascular space of infiltrated vessels were strongly positive for CXCR3, which could correspond to activated T lymphocytes (fig 3.10).
3.3.5. Ultrastructure of MS brain at the electron microscopy level

MS lesions are characterised by the loss of myelin around axons, appearance of gliosis, and presence of leukocytes around some but not all the vessels (fig 3.11 A). Some of these cells in electron microscopy show electron dense material accumulating in vacuoles, a feature that is characteristic of phagocytosis of myelin by macrophages or microglia (fig 3.11 B). The centre of the lesions present fewer cell nuclei suggesting a decline in the number of cells and axons denuded of myelin (fig 3.11 C). On the edge of lesions, some fibres were still myelinated, but the myelin sheet appeared thinner. These observations apply for both chronic active and chronic silent lesions. These two types of lesion could not be differentiated at the electron microscopical level.

3.3.6. Subcellular localisation of CXCR1 and CXCR3 by immunogold labelling and electron microscopy

3.3.6.1. CXCR1

CXCR1 was detected on cells other than HBEC in the vessels. Some polynuclear cells, which were highly CXCR1 positive, were located inside the vessel (fig 3.12). It is possible that these cells are neutrophils, as they are known to be CXCR1 positive (Ley, 1996), and neutrophils are likely to be found in the blood stream as they represent ~ 70% of the leukocyte population (Weber-Dabroska et al., 2002).

3.3.6.2. CXCR1 in endothelial cells

From each block of tissue, a number of vessels (table 3.4) characterised by the presence of endothelial cells connected together by electron-dense tight junctions, surrounded by a basal lamina, and containing a lumen were investigated (fig 3.13). The number of gold particles present on the luminal or abluminal membranes or within the cytoplasm of endothelial cells was counted and reported as a percentage of the total number of gold particles present in the endothelial cells. 87% of the gold particles labelling CXCR1 the
NAWM were located in the cytoplasm whereas 3% were found on the abluminal membrane and 9% on the luminal membrane (table 3.4).

The preferential location within the cytoplasm was observed for all three donors with a similar percentage of gold particles in the cytoplasm on HBEC in NAWM. For the localisation on the membrane there was more variability between the three donors (table 3.4) although there was a slightly higher distribution on the luminal side versus the abluminal side. In the MHC class II positive and the MHC class II negative lesions, there were some differences compared with the NAWM, but these differences were not consistent, suggesting that CXCR1 expression and localisation is unchanged on brain endothelial cells in MS in lesion. There were no changes in the overall number of gold particle in the NAWM versus the lesion (fig 3.15).

<table>
<thead>
<tr>
<th>NAWM</th>
<th>Nb of vessel (n=number of total particles/vessel)</th>
<th>% of GP in cytoplasm</th>
<th>% of GP on luminal membrane</th>
<th>% of GP on Abluminal membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>8 (n=11)</td>
<td>87.2</td>
<td>5.7</td>
<td>7.2</td>
</tr>
<tr>
<td>Case 2</td>
<td>2 (n=11.5)</td>
<td>87.3</td>
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<td>87.2</td>
<td>11.6</td>
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<tr>
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<td>87.2</td>
<td>8.9</td>
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<tr>
<td>MHC class II+ lesion</td>
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<td>5 (n=1.8)</td>
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<td>0</td>
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<tr>
<td>Case 2</td>
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<td>13.6</td>
<td>7</td>
</tr>
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<td>12.8</td>
<td>3.8</td>
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<tr>
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<td></td>
<td>86.6</td>
<td>9.8</td>
<td>3.6</td>
</tr>
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<td>MHC class II- lesion</td>
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<td>6 (n=3.33)</td>
<td>94.29</td>
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<td>2.9</td>
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<td>52.2</td>
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</tr>
<tr>
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<td>77.1</td>
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<td>9</td>
</tr>
<tr>
<td>MEAN</td>
<td></td>
<td>73.7</td>
<td>19.7</td>
<td>6.6</td>
</tr>
</tbody>
</table>

Table 3.4: Percentage of gold particles (GP) labelling CXCR1 according to membrane or intracellular localisation counted on the number of vessels found in one section for each lesion type and NAWM per case. Significant differences were determined by ANOVA followed by a post hoc t-test.
3.3.6.3. CXCR3

Leukocytes within vessels were not as positive for CXCR3 as CXCR1 and some were even negative for CXCR3 (fig 3.14 A). All cells with high granule content, probably macrophages or neutrophils, were also negative (fig 3.14 B). In the parenchymal space, some glial cells, possibly astrocytes according to their morphological appearance, were contained a higher gold particle density for CXCR3 (fig 3.14 C).

3.3.6.4. CXCR3 in endothelial cells

The intracellular distribution of CXCR3 within endothelial cells was different to that of CXCR1. 65% of gold particles were located in the cytoplasm in NAWM whereas 14.3% of the gold particles were on the luminal membrane, and 20% were on the abluminal membrane (table 3.5). The proportion of gold particles in the cytoplasm of HBEC was greater than control for MHC class II negative lesions, indicating that CXCR3 may have been internalized by HBEC. The proportion of CXCR3 positive gold particles on the abluminal surface of HBEC, higher than that observed for CXCR1, was lower in chronic active lesions (MHC class II positive) but not in chronic silent lesions (MHC class II negative) compared to NAWM. The overall number of gold particles in the MHC class II negative lesion also decreased ($P=0.013$, $n=18$ for NAWM and $n=17$ for the MHC class II lesion) (fig 3.15).
<table>
<thead>
<tr>
<th></th>
<th>Nb of vessel (n=number of total particles/vessel)</th>
<th>% of GP in cytoplasm</th>
<th>% of GP on luminal membrane</th>
<th>% of GP on Abluminal membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NAWM</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
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<td>28.57</td>
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<td>31.4</td>
<td>11.82</td>
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<td>8.22</td>
<td>23.54</td>
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<td><strong>64.8</strong></td>
<td><strong>14.3</strong></td>
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</tr>
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<td><strong>MHC class</strong></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>II+ lesion</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>24.8</td>
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</tr>
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<td>6 (n=27)</td>
<td>70.1</td>
<td>10.6</td>
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</tr>
<tr>
<td>Case 3</td>
<td>5 (n=6.6)</td>
<td>70.6</td>
<td>18.3</td>
<td>10</td>
</tr>
<tr>
<td><strong>MEAN</strong></td>
<td></td>
<td><strong>70.0</strong></td>
<td><strong>17.4</strong></td>
<td><strong>4.5</strong> (P=0.033)</td>
</tr>
<tr>
<td><strong>II- lesion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case 1</td>
<td>4 (n=3.5)</td>
<td>74.3</td>
<td>12.5</td>
<td>18.75</td>
</tr>
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<td>Case 2</td>
<td>8 (n=5)</td>
<td>87.7</td>
<td>3.6</td>
<td>26.3</td>
</tr>
<tr>
<td>Case 3</td>
<td>6 (n=5.8)</td>
<td>71.7</td>
<td>22.3</td>
<td>7.1</td>
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<td><strong>MEAN</strong></td>
<td></td>
<td><strong>79.0</strong> (P=0.049)</td>
<td><strong>12.3</strong></td>
<td><strong>17.8</strong></td>
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Table 3.5: Percentage of gold particles (GP) labelling CXCR3 according to membrane or intracellular localisation counted on the number of vessels found in one section for each lesion type and NAWM per case. Significant differences were determined by ANOVA followed by a post hoc t-test.
**Fig 3.1: Chemokine receptor mRNA expression by HBEC**

Chemokine receptor mRNA expression by primary human brain endothelial cells was determined by standard semi-quantitative RT-PCR. mRNA for the chemokine receptors CCR1, CCR5, CXCR1, CXCR2 and CXCR3 was detected in primary human brain endothelial cells (+). Cyclophilin was used as positive control and to ensure equal cDNA loading for each sample. Negative controls (-) of samples incubated in the absence of RT were included. Data were obtained from 2 experiments.
<table>
<thead>
<tr>
<th>Chemokine receptors</th>
<th>vWF</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR1 (IgG2b)</td>
<td></td>
</tr>
<tr>
<td>CCR2 (IgG2b)</td>
<td></td>
</tr>
<tr>
<td>CCR3 (IgG2a)</td>
<td></td>
</tr>
<tr>
<td>CCR4 (polyclonal rabbit IgG)</td>
<td></td>
</tr>
<tr>
<td>CCR5 (IgG2b)</td>
<td></td>
</tr>
</tbody>
</table>

**Fig 3.2: Immunocytochemical detection of CCR receptors and vWF in HBEC.**
HBEC were grown on collagen-coated Labtek chamber slides, fixed, permeabilised with Triton X-100 and then processed for detection of CCR receptor by immunocytochemistry using specific fluorescent conjugated antibody (left column). Cells were then double labelled with an antibody for vWF, an endothelial cell marker (right column). Antibody isotype indicated for each receptor. Results are representative of three different experiments done with a single well per treatment. Scale bar=50 μm
Fig 3.3: Immunocytochemical detection of CXCR receptors and vWF in HBEC.
HBEC were grown on collagen-coated Labtek chamber slides, fixed, permeabilised with Triton X-100 and then processed for detection of CXCR receptor by immunocytochemistry using specific fluorescent conjugated antibody (left column). Cells were then double labelled with an antibody for vWF, an endothelial cell marker (right column). Antibody isotype indicated for each receptor. Results are representative of three different experiments done with a single well per treatment. Scale bar=50 μm
Fig 3.4: Immunocytochemical detection of isotope matched controls for CCR and CXCR receptors and vWF in HBEC.

HBEC were grown on collagen-coated Labtek chamber slides, fixed, permeabilised with Triton X-100 and then processed for detection of isotype control by immunocytochemistry using specific fluorescent conjugated antibody (left column). Cells were then double labelled with an antibody for vWF, an endothelial cell marker (right column). Results are representative of three different experiments done with a single well per treatment. Scale bar=50 μm.
Fig 3.5: Immunohistochemical detection of CD68 or MHC class II in NAWM and in MS active lesions counter-stained with hematoxylin.

Fixed 50 μm thick sections cut with a vibratome were permeabilised with Triton X-100 and then processed for detection of CD68, a marker for microglia (arrows), or MHC class II by immunohistochemistry. Sections were then counterstained with haematoxylin to visualise cell nuclei in NAWM (left column) and in MS lesions (right column). Results are representative of experiments from six donors done in duplicate. Scale bar=50 μm
Fig 3.6: Immunohistochemical detection of CXCR1 and vWF in human NAWM and in MS lesions.
Fixed 50 μm thick sections cut with a vibratome were permeabilised with Triton X-100 and then processed for detection of CXCR1 receptor (in red) and vWF (in green) by immunohistochemistry in NAWM (left column) and in MS lesions (right column). Merged images show the localisation of CXCR1 within the neurovascular unit. Results are representative of experiments from six donors done in duplicate. Scale bar=20 μm.
Fig 3.7: Immunohistochemical detection of CXCR3 and vWF in human NAWM and in MS lesions.

Fixed 50 μm thick sections cut with a vibratome were permeabilised with Triton X-100 and then processed for detection of CXCR3 receptor (in red) and GFAP (in green) by immunohistochemistry in NAWM (left column) and in MS lesion (right column). Merged images show the localisation of CXCR3 within the neurovascular unit. In the NAWM, some vessels were clearly CXCR3 positive (arrow), but for some others it is less obvious (star). Results are representative of experiments from six donors done in duplicate. Scale bar=20 μm
Fig 3.8: Immunohistochemical detection of CXCR1 and GFAP in human NAWM and in MS lesions.

Fixed 50 μm thick sections cut with a vibratome were permeabilised with Triton X-100 and then processed for detection of CXCR1 receptor (in red) and GFAP (in green) by immunohistochemistry in NAWM (left column) and in MS lesions (right column). Merged images show the localisation of CXCR1 on astrocytes. Results are representative of experiments from six donors done in duplicate. Scale bar=20 μm
Fig 3.9: Immunohistochemical detection of CXCR3 and GFAP in human NAWM and in MS lesions.
Fixed 50 μm thick sections cut with a vibratome were permeabilised with Triton X-100 and then processed for detection of CXCR3 receptor (in red) and GFAP (in green) by immunohistochemistry in NAWM (left column) and in MS lesions (right column). Merged images show the localisation of CXCR3 on astrocytes. Results are representative of experiments from six donors done in duplicate. Scale bar=20 μm
Fig 3.10: Immunohistochemical detection of CXCR3 in human MS chronic active lesion.
Fixed 50 μm thick sections cut with a vibratome were permeabilised with Triton X-100 and then processed for detection of CXCR3 receptor by immunohistochemistry in a MS chronic active lesion. Results show one experiment (done in duplicate). Scale bar=50 μm
Fig 3.11: Electron micrographs of a lesion within the brain of a MS patient.
Fixed 50 μm thick sections cut with a vibratome and ultra cut to 70 nm were processed to be analysed by electron microscopy. Lesions were characterised by the absence of myelin surrounding the axons (A) and some blood vessels were surrounded by infiltrated cells most probably leukocytes (B). Some of the cells within the brain parenchyma contained vesicles (dashed arrow) with myelin degradation products (C). It is possible to distinguish nuclei (NU), erythrocyte in capillary (REY) and tight junction (TJ). Scale bar = 5 μm.
**Fig 3.12:** Digital electron micrograph of a CXCR1 positive leukocyte inside a vessel from a MS patient.

Fixed 50 μm thick sections cut with a vibratome were processed for detection of CXCR1 by immunohistochemistry using silver-enhanced gold particles, ultra-thin cut to 70 nm and visualised with an electron microscope. Scale bar = 1μm.

**Fig 3.13:** Electron micrograph of a CXCR3 labelled vessel in a MHC class II negative lesion.

Fixed 50 μm thick sections cut with a vibratome were processed for detection of CXCR1 by immunohistochemistry using silver-enhanced gold particles, ultra thin cut to 70 nm and visualised with an electron microscope. The black arrow indicates a silver-enhanced gold particle on the membrane and the grey arrow indicates a gold particle in the cytoplasm of a brain endothelial cell in a capillary. It is also possible to distinguish a tight junction (TJ), the nucleus of the endothelial cells (NU) and an erythrocyte (ERY) located in the lumen (LU). Scale bar = 1μm.
Fig 3.14: Digital electron micrograph of CXCR3 positive cells within MS brain.
Fixed 50 μm thick sections cut with a vibratome were processed for detection of CXCR3 by immunohistochemistry using silver-enhanced gold particles, ultra thin cut to 70 nm and visualised with an electron microscope. Granulated leukocytes (A) and other types of leukocyte (B) were negative for CXCR3. In the parenchyma of the brain, other cell types that resembled astrocytes were highly positive for CXCR3 (the arrows indicate gold particles). Scale bar= 1μm
3.4 Discussion

While the distribution of chemokine receptors in different populations of leukocytes has been well documented and reviewed (Olson and Ley, 2002; Kim, 2004), their expression on other cell types is still under investigation. Yet, evidence suggests that their role is more than just in leukocyte trafficking. For example, CXCR4 is known to be crucial for development, as CXCR4 knock-out mice are lethal at embryonic stage presenting haematopoietic signs, heart defects, and brain abnormality (Zou et al., 1998).

Several studies have reported chemokine receptor expression on endothelial cells, in particular on HUVEC as these cells are relatively easily to obtain and isolate. HUVEC express a wide range of CXCR chemokine receptors, whereas fewer CCR chemokine receptors are present on these cells (Gupta et al., 1998). Their functional role has been suggested to be as regulators of angiogenesis (Luster et al., 1995; Simonini et al., 2000), since chemokines modulate endothelial proliferative responses (Li et al., 2003a).

Here, the expression of a number of chemokine receptors in vitro on HBEC at the mRNA level by RT-PCR and at the protein level by immunocytochemistry and immunogold electron microscopy was studied. This last technique permits the identification of the subcellular distribution of the chemokine receptor within the cells. We have focused our attention on the chemokine receptors that are shown to be involved in MS namely CXCR1, CXCR3 CCR1, CCR2 and CCR5.
3.4.1. Expression of CXCR chemokine receptors on human brain endothelial cells *in vitro*

In this study the expression of CXCR1, CXCR2 and CXCR3 by HBEC at the mRNA level and at the protein level was demonstrated. These receptors appeared to be mainly intracellular.

*In vitro* the environment to which the cells are exposed is very different to that *in vivo*. Most especially the very rich growth factor medium used for HBEC might influence the expression of chemokine receptors by these cells. Furthermore other resident cells of the brain could modulate the expression of the chemokine receptors *in vivo*, as it is well known for instance that astrocyte factors influence the phenotype of endothelial cells (Rauh *et al.*, 1992).

The intracellular localisation of the chemokine receptors might reflect the presence of chemokines in the culture medium and their binding to the receptor. Indeed, it has been shown that high concentrations of CXCL8 when bound to CXCR1 receptor result in its internalization and subsequent recycling (Matityahu *et al.*, 2002). As shown in the previous chapter, CXCL8 can be released constitutively by HBEC. As for CXCR3, CXCL10 is not expressed at basal level, but CXCR3 binds two other ligands, CXCL9 and CXCL11, which could be constitutively produce by HBEC. Indeed, CXCL9 has been shown to be released by HBEC basally whereas CXCL10 is only released after IFN-γ treatment (Salmaggi *et al.*, 2002).

HBEC were labelled for CXCR3 both at sub-confluence, when they are still dividing, and at confluence, when they are at a quiescent stage. In each case, CXCR3 was expressed at a similar level. These results are in contrast with the observations by Romagnani *et al.*
(2001) who showed that the expression of CXCR3 is dependent on the cell cycle, and prominent in the S-phase and G2-M phase in endothelial cells.

Our findings are in agreement with Berger et al. (1999) in terms of CXCR1 and CXCR3 expression in vitro, but not of CXCR2. A low expression of CXCR2 was detected in HBEC, and this expression was supported by RT-PCR analysis. In their study, Berger et al. (1999) used a different set of antibodies for CXCR2 which may account for the differences from our results. Another possibility is the presence of VEGF in the culture medium. This growth factor upregulates the expression of CXCR4 in HUVEC (Salcedo et al., 1999), and could have the same effect on CXCR2 on HBEC as both receptors share angiogenic properties. Effectively, the present culture medium used in this study contains growth factors such as VEGF, IGF, EGF, and FGF, whereas Berger et al. (1999) only used FCS. CXCR2 has been reported to be expressed some other endothelial cell types at low levels (Salcedo et al., 2000; Hillyer et al., 2003). In addition, Murdoch et al. (1999) detected the transcript for CXCR2 but could not detect its protein in HUVEC. CXCR2 expression has also been reported on murine brain endothelial cells (Otto et al., 2000).

3.4.2. Expression of CCR chemokine receptors on HBEC in vitro

By contrast with CXCR receptors, there is less evidence for CCR receptor expression on endothelial cells in vitro. Berger et al. (1999) showed the expression of CCR2A, CCR3 and a weak expression of CCR5 on HBEC. Andjelkovic et al. (1999), using freshly isolated human brain microvessels, demonstrated the expression of CCR1, CCR2 and CCR5 by immunocytochemistry.

In this study, mRNA transcripts for CCR1 and CCR5 were detected in HBEC. The expression at the transcriptional level of CCR3 and CCR4 was not investigated. At the
protein level, CCR3 and CCR5 were shown to be expressed, but CCR1 staining was negative.

The presence of mRNA transcript of CCR1 in RNA isolated from HBEC could arise either from contaminating cells in the preparation, or from the fact that the expression of CCR1 protein at basal level is inhibited by post-transcriptional mechanisms. The expression of CCR1 is in agreement with Berger et al. (1999), also Andjelkovic et al. (1999) was able to show positive staining for CCR1 on microvessels. As the microvessels are freshly isolated, it is possible that the culture conditions may regulate the expression of CCR1. Alternatively, pericytes and/or perivascular cells but not endothelial cells, may express CCR1.

CCR2 was not detected in HBEC at either the transcript or the protein level. Yet it has been demonstrated to be expressed on HBEC by other groups (Andjelkovic et al., 1999; Berger et al., 1999) and to be functional in endothelial cells, as CCL2, the ligand for CCR2, induces an increase in the permeability of an endothelial cell monolayer (Stamatovic et al., 2005). The antibodies used by the two other groups were different from ours, but that does not account for the lack of mRNA detection.

CCR5, which is increased in lesions in MS, is the receptor for CCL3, CCL4 and CCL5. Its low expression by HBEC has also been described by others (Berger et al., 1999) as well as on others endothelial cell types (Hillyer et al., 2003).

3.4.3. **Expression of CXCR1 in situ**

CXCR1 is an ELR positive chemokine receptor of the CXCR family. It is expressed on neutrophils (Godaly et al., 2000) and has high affinity for the ligands CXCL8 and CXCL6. The angiogenic effect of CXCR1 has been well described *in vitro*, and its expression on
endothelial cells is well established (Simonini et al., 2000). CXCR1 expression has not yet been described in the human brain, but has been shown to be expressed \textit{in vitro} by some resident cells of the brain such as astrocytes and microglia (Flynn et al., 2003). CXCR1 is also expressed on cholinergic neurons \textit{in vitro} and CXCL8 induces a decrease in the \( \text{Ca}^{2+} \) current in those cells (Puma et al., 2001). In rat brain, mRNA of CXCR1 was widely distributed in different regions as determined by \textit{in situ} hybridization although the cell types expressing CXCR1 were not investigated (Danik et al., 2003).

In this study, CXCR1 was shown to be expressed in human brain by a number of cell types, such as endothelial cells, or cells that were associated with the microvessels, using immunohistochemistry. In addition there were other cell types that were labelled for CXCR1. Some of these cells were also labelled by GFAP antibodies, suggesting a CXCR1 positive population of astrocytes. Despite the fact that no previous studies showed CXCR1 expression in the brain, our data are supported by previous \textit{in vitro} work showing the expression of CXCR1 by cultured human astrocytes and microglia (Flynn et al., 2003). The GFAP negative population of CXCR1 brain-resident cells may consist of microglial cells, although co-staining with antibodies specific of microglial markers such as CD68 may be needed to confirm this.

\subsection*{3.4.4. Expression of CXCR3 \textit{in situ}}

CXCR3 is an ELR negative member of the CXCR receptor family. It is the only known receptor for CXCL9, CXCL10 and CXCL11. CXCR3 expression receptor is a hallmark of activated T-lymphocytes. It has also been reported to be expressed \textit{in situ} by CNS resident cells, such as a subpopulation of neurons, astrocytes and endothelial cells (Xia et al., 2000; Goldberg et al., 2001; Van Der Meer et al., 2001). CXCR3 was also found to be expressed on endothelial cells in other organs (Romagnani et al., 2001; Hillyer et al., 2003).
The present study showing the expression of CXCR3 co-localising with vWF and GFAP, is in accordance with previous findings (Van Der Meer et al., 2001). However, other studies did not demonstrate the expression of CXCR3 by astrocytes. Sorensen et al. (2002) and Kieseier et al. (2002) failed to show the expression of CXCR3 in NAWM of MS brains, and its expression was exclusively associated with infiltrating lymphocytes in lesions. The antibodies used in these studies are of different sources. By contrast, Goldberg et al. (2001), using the same antibody from R&D as in our study, obtained similar results. Whether different antibodies recognize different domains of the same protein, being more or less accessible, still remain to be determined. Different patterns of staining using two different antibodies for CXCR3, the 49801.111 clone (the same used in our study) and the 1C6 clone, has been discussed in the context of human glomerulonephritis (Segerer et al., 2004). The clone 49801.111 revealed a wider variety of CXCR3 positive cells including smooth muscle cells compared to the 1C6 clone. The clone 49801.111 was able to recognize the new variant of CXCR3 receptor called the CXCR3-B (Lasagni et al., 2003), which differs in its 52 amino acid N-terminal domain from the more classical form, the CXCR3-A. The CXCR3-B form has been shown to be expressed by non-CNS endothelial cells in vivo (Lasagni et al., 2003). Recognition of different epitopes by antibodies could explain the absence of positive labelling for CXCR3 in HBEC and other cell types of NAWM (Sorensen et al., 2002), in contrast with the present findings. Indeed another group, using the same CXCR3 antibody as the one used here, detected CXCR3 positive glial cells in NAWM (Simpson et al., 2000b). However, our results suggest that the variant form of CXCR3, CXCR3-B, is expressed in the CNS, by the endothelial cells and by astrocytes. The primers used to detect CXCR3 mRNA recognised both forms of the receptor and hence could not give further information as to which type of CXCR3 was expressed by HBEC.
In MS lesions, CXCR3 was expressed by endothelial cells and by astrocytes, as well as on rounded cells in the perivascular space, most likely corresponding to infiltrating leukocytes. CXCR3 expression in MS lesions was more diffuse compared to NAWM. In response to inflammatory conditions, astrocytes in lesions becomes reactive (Holley et al., 2003) giving rise to gliosis. It is possible that a hypertrophic astrocytic reaction involving CXCR3 positive astrocytes would be responsible for a more diffuse staining for CXCR3 in MS lesions.

3.4.5. Subcellular localisation of CXCR1 and CXCR3 by immunogold labelling and electron microscopy

No previous studies have reported the localization of CXCR3 and CXCR1 in situ by immunogold labelling. An in vitro study Salcedo et al. (2000), as well as our observations on brain tissue, indicate that CXCR1 and CXCR3 chemokine receptors are expressed by endothelial cells and are partially located intracellularly. The number of gold particles was low on the endothelial cells compared to other cell types resembling astrocytes, although these results are obtained from post-mortem tissue where cytolytic damage may have differentially affected chemokine receptor expression by different cell types.

In this study, there is a trend towards a decrease of CXCR3 expression by HBEC in lesions. This decrease is higher in the MHC class II negative lesion than in the MHC class II positive. There has been debate about whether or not there is an increase in the number of blood vessels in the lesions. Kirk et al. (2004) showed an increase of the number of vessels, but it has been argued that this effect could originate from the shrinkage of the tissue related to myelin loss. The elevation in MS lesions of VEGF (Proescholdt et al., 2002), an important factor in vascularisation, would support the idea of an increase in the density of blood vessels in the lesions. As a result, it is possible that when angiogenesis is promoted, angiostatic receptors such as CXCR3 could be down-regulated as is the case in MS lesions.
To answer this question, it would be interesting to study the effect of growth factors on CXCR3 expression.

In terms of subcellular localization, there appears to be an increase in the intracellular pool of CXCR3, and a decrease in the abluminal membrane localization in MS lesions. The fact that CXCL10 induces the internalisation of its receptor might account for the observed increase in the intracellular pool of CXCR3 (Campanella et al., 2003) as CXCL10 is increased in MS lesions in astrocytes (Sorensen et al., 2002) and could explain the decrease of the receptor on the abluminal side but not on the luminal side. One group has suggested that CCR2 acts as a means of transport for its chemokine ligand, CCL2 across the BBB (Andjelkovic and Pachter, 2000). It is possible that the same case applies for CXCR3 and its ligand, as they are greatly increased in the lesions, mainly in the active lesions. However, experiments carried out at the Open University suggest that CXCR3 does not transport CXCL10, as there is no inhibition by CXCL11 of CXCL10 trancytosis across non-brain human endothelial cells (D. Male, personal communication). Whether this is the case for HBEC remains to be determined.

3.4.6. Conclusion

In this study, CCR1, CCR5, CXCR1, CXCR2 and CXCR3 are expressed by HBEC. CXCR1 and CXCR3 are strongly expressed \textit{in vivo}. These two chemokine receptors are also present \textit{in situ}, on endothelial cells but also on other cell types. Both CXCR1 and CXCR3 are located on the luminal and abluminal surface of the HBEC, although the highest proportion of receptors was located intracellularly.
4. Chapter 4: Isolation and characterisation of a human brain endothelial cell line: HCMEC/D3
4.1 Introduction

The unique features of the BBB that restrict passage of cells and molecules from the blood into the brain are mainly associated with the expression of specific proteins, such as those at the tight junctions or efflux transporters, by HBEC. The specific pattern of protein expression differentiates HBEC from endothelial cells of other vascular beds. In vitro reconstruction models of the BBB are crucial for the understanding of the biochemical and immunological properties of brain microvascular endothelial cells. Primary cells and immortalised cell lines originating from different animal species such as bovine, rat and porcine endothelial cells are mostly used (reviewed by Deli et al. (2005)). Studies on HBEC are limited by the availability of brain tissue, and also by technical difficulties in the isolation method to yield highly pure endothelial cell cultures. Primary HBEC are rapidly overgrown by contaminating cells with a higher division rate such as astrocytes. In addition, HBEC in culture rapidly de-differentiate, lose their unique barrier phenotype and undergo senescence after a small number of passages further limiting their application to in vitro studies of the human BBB (Grant et al., 1998).

Immortalisation of a cell type allows unlimited access to material derived from a single cell clone. A number of brain endothelial cells from different species have been successfully created, such as the GP8 rat brain endothelial cell line (Greenwood et al., 1996) or the t-BBEC-117 bovine brain endothelial cell line (Sobue et al., 1999). In both cases, the cells were transfected with SV40 large T antigen to promote proliferation and avoid senescence. Primary cells in culture have a limited proliferative lifespan before they undergo permanent growth arrest, known as replicative senescence. When replicative senescence is bypassed by transformation with viral oncogene such as simian virus 40 large T antigen (SV40 LT), cells can gain additional lifespan.
Attempts to immortalise HBEC have proven more difficult and SV40 large T transfection is not sufficient for the cells to express a stable phenotype. Stins et al. (1997) described an immortalised human brain endothelial cell line, expressing brain endothelial markers such as occludin but with an unstable phenotype (Callahan et al., 2004). Gu et al. (2003) transfected HBEC with the catalytic component of human telomerase reverse transcriptase (hTERT) to prevent telomere shortening following cell division, and obtained a stable cell line with a TEER of about 20 ohms.cm$^2$. When adult somatic cells divide, their telomeres shorten by 10 to 200 bp per division (Von Zglinicki et al., 2000) because the cells do not contain functional telomerase. However, these authors did not investigate the expression of junctional molecules characteristic of HBEC.

A new human brain endothelial cell line, named hCMEC/D3, derived through expression of hTERT and SV40 large T antigen via a lentiviral vector transduction system was obtained from primary HBEC isolated as described in Chapter 2. In this chapter, some characteristics and properties of this novel human brain endothelial cell line are described and the cells are compared to other isolated cell clones in terms of expression of several brain endothelial cells markers, such as junctional proteins. In addition, chemokine and chemokine receptor expression by hCMEC/D3 cells are then analysed in order to verify the validity of this cell line to study in vitro leukocyte infiltration across the BBB during inflammation.
4.2 Material and methods

4.2.1. Isolation of primary HBEC

Primary HBEC were isolated from a temporal lobe resection carried out at King's College Hospital as described in Chapter 2 section 2.2.3. Cells were allowed to grow for 2 weeks at which point they were sent to our collaborator's lab at the Institute Cochin in Paris (Dr. B. Weksler and Dr P.-O. Couraud) for immortalisation.

4.2.2. Immortalisation of HBEC

This part of the work was carried out at the Institut Cochin in Paris by Dr. B. Weksler. At passage 0, primary HBEC were incubated overnight with a DNA flap lentiviral vector encoding human Telomerase Reverse Transcriptase (hTERT) (100ng p24units/ml) in normal growth medium. Following overnight incubation, the cells were washed and left to grow in normal growth medium for a further 2 days. Cells were then incubated with a low concentration of DNA flap lentiviral vector encoding SV40 large T antigen (20 ng p24units/ml) overnight. After washing, cells were grown at 37 °C in normal growth medium until sub-confluence. A week following lentiviral transduction with replication-defective lentiviral vectors, the absence of viral replication on HBEC was confirmed by detection of p24 in culture supernatants by ELISA. Two attempts at immortalising HBEC were made. On the second successful attempt, cloning was performed by me during a three month period at the Institut Cochin as described below.

4.2.3. Cloning

Individual cell clones were isolated by limiting dilution cloning. Sub-confluent lentivirally transduced cells were trypsinised and plated onto a 96 well collagen-coated plate at a density of 30 cells/plate to ensure the clonality of cells growing from one well. Each clone
was initially screened according to their morphological appearance, their growth capacity and their inhibition contact properties assessed by observations on the morphological appearance of cells at confluence. Approximately 15 clones were selected. The selected clones were then further selected according to their stable expression of endothelial cell markers such as vWF, PECAM-1, β-catenin and ZO-1. One clone, termed hCMEC/D3, met all the above criteria and was selected for further investigation.

4.2.4. Cell culture

Both primary cultures of human brain endothelial cell lines and immortalised cell clones were cultured as described in Sections 2.2.1, 2.2.2 and 2.2.4

4.2.5. Transendothelial electrical resistance (TEER)

hCMEC/D3 cells were seeded on collagen-coated clear polyester membrane Transwell® filters (0.4 μm pore size, 1 cm² surface area, Corning Costar Ltd, Buckinghamshire, UK) at a density of 10⁵ cells/insert and grown to confluence (1-2 days). The medium was carefully aspirated, and replaced by sterile PBS. TEER across the cell monolayer and the insert was measured with an Endohm and a measurement chamber (model EVOM, World Precision Instruments, Sarasota, USA). The endohm chamber was sterilized with ethanol and rinse with PBS. The filter was placed in the steril chamber and a cap was placed aboved. Both the cap and the chamber contain each a voltage-sensitive silver/silver chloride pellet in the center plus an annular current electrode. The symmetrically apposing circular disc electrodes, situated above and beneath the membrane, allow a uniform current to flow across the membrane. The electrical resistance of a filter pre-coated with collagen was measured and the value obtained was subtracted from the resistance obtained with the hCMEC/D3 monolayer on inserts. TEER values are given in Ω·cm².
4.2.6. Paracellular permeability

**Permeability buffer:** Dulbecco’s Modified Eagle Medium (Invitrogen, Renfrewshire, UK), 10mM HEPES and 2% FCS.

hCMEC/D3 cells were seeded on collagen-coated clear polyester membrane Transwell® filters (0.4 μm pore size, 1 cm² surface area, Corning Costar Ltd, Buckinghamshire, UK) at a density of 10⁵ cells/insert and grown to confluence (1-2 days). The cells were washed once with sterile permeability buffer. The filters were transferred to a 12 well plate containing 1.5 ml/well of permeability medium. 0.5ml of 2mg/ml FITC-dextran of 70KDa (FD70) (Sigma-Aldrich, Dorset, UK) dissolved in permeability buffer was added in the upper chamber. The filters were transferred every 5 min into a fresh well containing 1.5 ml permeability buffer for 30 min from the beginning of the experiment. The fluorescence diffusing to the bottom well was measured at 535nm with a fluorescence reader (model Flite, Wallar, USA). The cleared volume (in ml) represents the cumulative volume of FD70 solution passing through the cell monolayer. It is calculated as follows

\[
\text{Cleared volume} = \frac{(F_s - F_b)}{F_i}
\]

whereby \(F_s\) is the fluorescence per ml of the well (arbitrary units/ml), \(F_i\) is the fluorescence of the initial solution (arbitrary units) and \(F_b\) corresponds to the background fluorescence of the permeability buffer (arbitrary units/ml). The cleared volume was plotted against time. The slope of the curve allows calculation of the permeability coefficients (Pe in cm.min⁻¹) of the endothelial cell monolayer using the following equation:

\[
\frac{1}{PS} = \frac{1}{m_e} - \frac{1}{m_f} \quad \text{and} \quad Pe = \frac{PS}{S}
\]

\(m_e\) and \(m_f\) are the slopes of the curves corresponding to endothelial cells on filters and to filter only, respectively, in ml/min. \(PS\) is the permeability-surface area product of the endothelial cell monolayer (ml.min⁻¹) and \(S\) is the surface area of the filter (1 cm²). Fig 4.1 shows an example to illustrate how \(m_e\) and \(m_f\) are calculated.
Fig 4.1: Typical curve representing cleared volume of FITC-dextran 70KDa (FD70) vs time of an hCMEC/D3 monolayer (squares) and a collagen-coated filter (circles). hCMEC/D3 were grown on Transwell filters until confluence. A FD70 solution was added to the upper chamber and the amount of fluorescence crossing the hCMEC/D3 monolayer determined every 5 min. The slope of the curves for the filter without cells (mf) and for the filter with confluent hCMEC/D3 cells (me) were used to calculate the permeability coefficient (Pe).

4.2.7. FACS analysis

Chemokine receptor expression by hCMEC/D3 cells of CCR1-6 and CXCR1-5 was determined by flow cytometric analysis under basal conditions and following stimulation with a combination of 10ng/ml IFN-γ and 500ng/ml TNF-α for 24 h. The concentrations of cytokines selected for this experiment were of the same order as those used for other experiments in Weksler et al. (2005). Cells were first washed with HBSS without Ca²⁺ and Mg²⁺ for 1 min and then aspirated. 0.25% w/v porcine Trypsin-EDTA was added for 5 min at 37 °C. Once the cells were detached from the extracellular matrix, trypsin was neutralised by addition of normal culture medium and the cells were spun at 300 g for 5 min. The cells were fixed by suspending them in a solution of 4% PAF in PBS for 10 min.
on ice, and permeabilized for 1 min in 0.1% triton-X100 in PBS at RT. Non-specific
binding of antibodies was blocked by incubating cells with 10% goat serum for 30 min at
RT. Around 500,000 cells per tube were incubated with a labelled antibody diluted to 1/3
in 30µl at RT for CCR1-3, CCR5-6 and for CXCR1-CXCR5 using the antibodies listed in
table 4.1. CCR4 (diluted to 1/100) was an unlabelled antibody thus requiring the addition,
after the first antibody, of a FITC-conjugated goat anti-rabbit antibody (dilution of 1/200,
from Chemicon, Hampshire, UK) for 1 h at RT. Between each step, the cells were rinsed
with PBS and centrifuged at 300g for 5 min. After a final wash, the cells were re-
suspended in 0.5 ml PBS and the labelled fluorescence on the cells was read using
FACSCalibur with CellQuest™ software (Becton Dickinson, Oxfordshire, UK). The
median fluorescence of 10,000 cells per sample was read and used for statistical analysis
after having subtracted the median fluorescence for the isotype controls or for cells
incubated with secondary antibody alone.
<table>
<thead>
<tr>
<th>Antigen</th>
<th>Type</th>
<th>Label</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human CXCR1</td>
<td>Monoclonal mouse IgG2A, clone #42705</td>
<td>Phycoerythrin</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>Human CXCR2</td>
<td>Monoclonal mouse IgG2A, clone #48311</td>
<td>Phycoerythrin</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>Human CXCR3</td>
<td>Monoclonal mouse IgG1, clone #48311</td>
<td>Phycoerythrin</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>Human CXCR4</td>
<td>Monoclonal mouse IgG2A, clone #12G5</td>
<td>Phycoerythrin</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>Human CXCR5</td>
<td>Monoclonal mouse IgG2B, clone #51505</td>
<td>Phycoerythrin</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>Human CCR1</td>
<td>Monoclonal mouse IgG2B, clone #53504</td>
<td>Phycoerythrin</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>Human CCR2</td>
<td>Monoclonal mouse IgG2B, clone #48607</td>
<td>Phycoerythrin</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>Human CCR3</td>
<td>Monoclonal mouse IgG2A, clone #61828</td>
<td>Fluorescein</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>Human CCR4</td>
<td>Polyclonal rabbit IgG</td>
<td>No label</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Human CCR5</td>
<td>Monoclonal mouse IgG2B, clone #45502</td>
<td>Fluorescein</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>Human CCR6</td>
<td>Monoclonal mouse IgG2B, clone #53103</td>
<td>Phycoerythrin</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>Mouse IgG1</td>
<td>Normal mouse IgG1, clone #11711</td>
<td>Phycoerythrin / Fluorescein</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>Mouse IgG2A</td>
<td>Normal mouse IgG2A, clone #20102</td>
<td>Phycoerythrin / Fluorescein</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>Mouse IgG2B</td>
<td>Normal mouse IgG2B, clone #20116</td>
<td>Phycoerythrin / Fluorescein</td>
<td>R&amp;D</td>
</tr>
</tbody>
</table>

Table 4.1: List of antibodies specific for the chemokine receptors used for FACS and immunocytochemistry analysis.

### 4.2.8. Capture ELISA

Chemokine levels in culture supernatants after 48 h cytokines stimulation were determined as described in Section 2.2.5.

### 4.2.9. Immunocytochemistry

Cells grown to confluence on collagen-coated Permanox Labtek slides were fixed, permeabilised and incubated with antibodies listed in Table 4.1 as described in Section 2.2.6.1
4.2.10. Western blotting

Laemmli’s buffer: 50mM Tris-HCl, 2% SDS, 0.1% bromophenol blue, 40% glycerol, 1 mM sodium orthovanadate and 50mM DTT

Running gel: 33% v/v Protogel (30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide) (National Diagnostics, North Yorkshire, UK), 0.1% w/v SDS, 0.37M tris/HCl pH 8.8, 0.03% v/v TEMED and 0.1% w/v APS (Sigma-Aldrich, Dorset, UK).

Stacking gel: 16.7% v/v Protogel (30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide) (National Diagnostics, North Yorkshire, UK), 0.03% w/v SDS, 0.12M tris/HCl pH 6.8, 0.03% v/v TEMED and 0.03% w/v APS (Sigma-Aldrich, Dorset, UK).

Running buffer: 25mM Tris, 192mM glycine, 1%SDS (National Diagnostics, North Yorkshire, UK)

Transfer buffer: 25mM Tris, 192mM glycine (National Diagnostics, North Yorkshire, UK), 20% ethanol

Blocking buffer: PBS, 0.2% tween 20 and 2 % dry milk

Washing solution: PBS, 0.2% tween 20

hCMEC/D3 cells were grown to confluence in collagen-coated 90 mm diameter Petri dishes either in normal medium or in differentiation media. At confluence, cells were either left in their respective culture medium or stimulated with 50ng/ml of TNF-α and 100ng/ml of IFN-γ in normal medium for 48 h (according to what has been used for chemokine expression on HBEC). Cells were then washed with ice cold PBS containing 1mM sodium orthovanadate, lysed in 250μl Laemmli’s buffer and lysed cells were scraped into an Eppendorf tube with a sterile scraper (Greiner Laboratories Limited, Gloucestershire, UK). In order to inhibit protein degradation induced by the presence of released intracellular proteases, the samples were heated at 95°C for 5 min, a temperature
at which most protein loses enzymatic activities due to denaturation. The samples were then stored at -20°C until further analysis.

On each lane of the stacking gel, a 15μl sample was loaded and run in parallel with molecular weight markers ranging from 14.4 to 97.4 kDa or from 45 to 200 kDa (Bio-rad Laboratories, Hertfordshire, UK), at a constant voltage of 50V until the sample arrived at the interface between the stacking and the running gels and then at 150V until the visible bromophenol blue ran out of the gel. Electrophoresed proteins in the gel were transferred onto a Protan® nitrocellulose transfer membrane (Schleicher & Schuell UK Ltd, Greater London, UK), using a wet blotting system (Trans-blot cell systems, Bio-Rad Laboratories, Hertfordshire, UK) at constant amperage of 15 mAmperes overnight at 4°C. The membrane was quickly washed with Ponceau Red solution (0.1% Ponceau (w/v) in 5% acetic acid (v/v), Sigma-aldrich, Dorset, UK) to verify equal loading of samples on each lane.

Nitrocellulose membranes were incubated with blocking buffer for 1 h, followed by incubation with the primary antibody (in blocking buffer) for 1 h, at RT (see Table 4.2 for details of the primary antibodies used). After 5 consecutive washes with washing solution, the secondary antibody was added for 1 h, at RT. The secondary antibody was horseradish peroxidase conjugated anti-mouse IgG or anti-rabbit IgG according to the primary used at a dilution of 1/5000 or 1/20000 respectively (Pierce Biotechnology, Cheshire, UK). The membrane was washed 5 times with washing buffer followed by a rinse with PBS only for 10 min and developed using the ECL™ Western blotting detection reagents system (Amersham Pharmacia, Buckinghamshire, UK) for 1 min. Membranes were then exposed to Hyperfilm (Amersham Pharmacia, Buckinghamshire, UK) and developed in a Kodak X-Omat automated processor (Rochester, USA).
<table>
<thead>
<tr>
<th>Antigen specificity</th>
<th>Host species</th>
<th>Concentration</th>
<th>Expected size</th>
<th>Gel %</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>PECAM-1</td>
<td>Mouse IgG1</td>
<td>0.4μg/ml</td>
<td>130 kDa</td>
<td>6%</td>
<td>Serotec</td>
</tr>
<tr>
<td>Beta-catenin</td>
<td>Mouse IgG1</td>
<td>0.4μg/ml</td>
<td>94 kDa</td>
<td>10%</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Rabbit</td>
<td>0.5μg/ml</td>
<td>225 kDa</td>
<td>6%</td>
<td>Zymed</td>
</tr>
<tr>
<td>Occludin</td>
<td>Mouse IgG1-κ</td>
<td>0.5μg/ml</td>
<td>65 kDa</td>
<td>10%</td>
<td>Zymed</td>
</tr>
<tr>
<td>Claudin-3</td>
<td>Rabbit</td>
<td>0.5μg/ml</td>
<td>22 kDa</td>
<td>15%</td>
<td>Zymed</td>
</tr>
<tr>
<td>Claudin-5</td>
<td>Mouse IgG1</td>
<td>1μg/ml</td>
<td>22-24 kDa</td>
<td>15%</td>
<td>Zymed</td>
</tr>
<tr>
<td>CXCL10</td>
<td>Rabbit</td>
<td>0.2μg/ml</td>
<td>8.5 kDa</td>
<td>15%</td>
<td>Peprotech</td>
</tr>
<tr>
<td>CXCL10</td>
<td>Goat</td>
<td>0.1μg/ml</td>
<td>8.5 kDa</td>
<td>15%</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>CXCL8</td>
<td>Rabbit</td>
<td>0.2μg/ml</td>
<td>8.9 kDa</td>
<td>15%</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>CCL2</td>
<td>Mouse IgG2B</td>
<td>0.1μg/ml</td>
<td>8.6 kDa</td>
<td>15%</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>CCL5</td>
<td>Rabbit</td>
<td>0.1μg/ml</td>
<td>7.8 kDa</td>
<td>15%</td>
<td>R&amp;D</td>
</tr>
</tbody>
</table>

Table 4.2: List of antibodies used in Western-Blot. The final concentration of the antibody is given when the company gave the initial concentration otherwise the dilution factor is reported.

4.2.11. Statistical analysis

Statistical significance was determined by a one way or two way ANOVA, followed by a post hoc Tukeys t-test, or a student t-test. \( P<0.05 \) was considered significant.
4.3 Results

4.3.1. Characterisation of initial endothelial clones: morphology and expression of junctional proteins

Following immortalisation, around 100 cell clones were obtained by limited dilution cloning from which 15 were initially selected based on their non-transformed morphology and their contact inhibited properties. Four different clones were further selected based upon their morphology which closely resembled those of primary HBEC (section 2.3.1). These clones were termed hCMEC/3G, hCMEC/3C8, hCMEC/14D1 and hCMEC/15A2. Cells were then screened for the expression of endothelial cell markers including PECAM-1, β-catenin, ZO-1 and vWF (figs 4.2 and 4.3). hCMEC/3G cells did not express β-catenin and ZO-1 was not located at the cell-cell junctions (fig 4.2). Rare expression of PECAM-1 and β-catenin was detected located at the cellular junction, whereas no ZO-1 was detected for the clone hCMEC/3C8 (fig 4.2). Based on these observations, these two clones were not analysed further. Both hCMEC/14D1 and hCMEC/15A1 cells expressed PECAM-1, β-catenin and ZO-1 at the cell-cell junctions and cells also showed intracellular staining for vWF (fig 4.3). Although these two clones appeared suitable as HBEC lines, their limited replicative lifespan and lack of stability in terms of expression of immortalising genes such as hTERT prevented their further use. Indeed, large polynucleated cells appeared in culture from passage 11, indicative of the presence of cells undergoing senescence (not shown). The number of cells displaying this phenotype in culture increased with passage, while their growth rate considerably decreased and their morphology became more heterogeneous.

The hCMEC/15A2 cell clone was further transduced with hTERT and sub-cloned by limited dilution cloning in order to obtain a more phenotypically stable cell line. A new
clone, termed hCMEC/D3, showed a morphology reminiscent of both the parental hCMEC/15A2 clone and primary cells (fig 4.4) and was stable for at least 15 further passages (from passage 20 to 35). Similarly to hCMEC/15A2 cells, hCMEC/D3 cells expressed PECAM-1, β-catenin and ZO-1 at the cell-cell junctions (fig 4.5) as did the primary HBEC (section 2.3.2). The expression of these junctional proteins remained stable over several passages (fig 4.6). Claudin-5 could also be faintly detected at the cell borders while claudin-3 was distributed diffusely within the cytoplasm (fig 4.5).

4.3.2. TEER and paracellular permeability to FD70 of hCMEC/D3 monolayers

hCMEC/D3 monolayers grown to confluence on Transwell filters were tested for their permeability to FD70. The permeability coefficient of control hCMEC/D3 monolayers was on average of $2.67 \times 10^{-5} \pm 1.20 \times 10^{-5}$ cm/min (table 4.3). This permeability coefficient was of the same magnitude as the values obtained with primary HBEC (table 4.3). TEER was on the contrary low, close to 10 ohms.cm$^2$, reflecting a high ionic permeability, compared to HBEC. These results indicate that hCMEC/D3 constitutes an in vitro BBB model which is potentially suitable for the study of paracellular permeability, especially for molecules of high molecular weight.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Permeability Coefficient to FD70 (cm/min)</th>
<th>TEER (ohms-cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCMEC/D3</td>
<td>$2.67 \times 10^{-5}$ (±$2.09 \times 10^{-5}$)</td>
<td>9.66±3.33</td>
</tr>
<tr>
<td>HBEC (passage 2)</td>
<td>$4.58 \times 10^{-5}$</td>
<td>23.5 ± 3.53</td>
</tr>
</tbody>
</table>

Table 4.3: Permeability coefficient and TEER for hCMEC/D3 cells and HBEC. Results presented are the mean ± S.E.M. of three different experiments for hCMEC/D3 and two different experiments for HBEC.
4.3.3. Effect of BBB promoting factors on TEER and expression of junctional proteins by hCMEC/D3 cells

The influence of BBB promoting factors on the barrier phenotype of hCMEC/D3 cells was investigated. The expression of junctional proteins such as β-catenin, PECAM-1, ZO-1, occludin, claudin-3 and claudin-5 was determined by immunocytochemistry and Western blotting on hCMEC/D3 cells grown in differentiation medium and compared to control conditions. In the presence of differentiation medium, a change in the morphology of hCMEC/D3 cells was observed by phase contrast microscopy. The cells in normal medium were more elongated, whereas the cells in differentiation medium were more of a cobblestone shape (fig 4.7). Furthermore, a more regular and continuous cortical distribution was observed for β-catenin and ZO-1 in cells in differentiation medium compared to cells grown under basal conditions (fig 4.7). Differentiation medium did not induce redistribution of tight junctional proteins such as occludin and claudin-3 from the cytosol to the cell junctions. Nevertheless, all tight junction proteins investigated were expressed by hCMEC/D3 cells as shown by immunoblotting. Both occludin and claudin-3 were up-regulated by treatment with differentiation medium whereas the expression of other tight junction proteins already present at the cell-cell junction such as β-catenin and ZO-1 and claudin-5 remained unchanged (fig 4.8). In parallel studies, hCMEC/D3 cells were grown to confluence on Transwell filters and the TEER hCMEC/D3 monolayers was determined. There was a slight but significant ($P=0.001$, $n=3$) increase in TEER when cells were grown with Differentiation medium compared to control cells (fig 4.9). Altogether, these data suggest that, as with HBEC (Torok et al., 2003), factors such as astrocytic-conditioned media and elevated intracellular cAMP levels are able to induce a tighter barrier phenotype in hCMEC/D3 cells.
4.3.4. Cytokine-regulation of adhesion molecule expression by hCMEC/D3 cells.

ICAM-1, VCAM-1 and PECAM-1 are known to be expressed by endothelial cells either under basal conditions or following stimulation by cytokines (dos Santos et al., 1996). Flow cytometric analysis was used to determine the expression of these three adhesion molecules by hCMEC/D3 cells both constitutively and following incubation with 25ng/ml TNF-α for 48 h. ICAM-1 and PECAM-1 were strongly expressed at the basal level, while only a faint expression of VCAM-1 was detected (fig 4.10). When stimulated with TNF-α, there was a seven-fold increase in ICAM-1 ($P=0.001$, $n=4$) expression and a nine-fold increase in VCAM-1 ($P=0.044$, $n=4$) expression. By contrast TNF-α induced significant decrease in PECAM-1 expression ($P=0.038$, $n=4$) by 30 % compared to control cells (fig 4.11). Weksler et al. (2005) also demonstrated the expression of ICAM-2, CD40, CD44 and MHC class II by hCMEC/D3 which followed a similar pattern of expression to that by primary HBEC (Bo et al., 1996; Omari and Dorovini-Zis, 2003), reinforcing the potential of hCMEC/D3 as a cell line suitable for immunological studies.

4.3.5. Constitutive and cytokine-induced chemokine production by hCMEC/D3 cells

Constitutive levels of CCL2, CCL5, CXCL8 and CXCL10 were measured in culture supernatants of confluent hCMEC/D3 cells for 48 h (fig 4.12). None of the chemokines determined were within the lower detection limit in the culture medium of control cells (~0.5ng/ml). Following treatment with IFN-γ for 48 h, the release of CXCL10 into the supernatant was induced to 38+/−10.8ng/ml and a small, but non significant increase in CCL2 was detected (2.4+/−1.3ng/ml), although CXCL8 and CCL5 levels remained below the detection limit of the assay. By contrast, TNF-α induced the production of all chemokines tested, CCL2, CXCL8, ($P=0.0001$, $P=0.003$, $n=3$) CCL5, and CXCL10 (no statistical analysis due to the low number of experiments), to different concentrations (fig 4.12).
The combination of IFN-γ and TNF-α triggered a synergistic effect rather than an additive one for CCL5 and CXCL10. For example, stimulation with INF-γ or TNF-α alone led to culture supernatant levels of 38ng/ml and 60ng/ml, respectively, but when the two cytokines were combined, the CXCL10 level rose to 231ng/ml (fig 4.12). By contrast, IFN-γ had an inhibitory effect on CXCL8 production induced by TNF-α, leading to an almost two-fold decrease. Finally, IFN-γ did not have any effect on the production of CCL2 when added to TNF-α (fig 4.12).

TGF-β alone (25ng/ml) had no effect on the chemokine production by hCMEC/D3 cells. When used in combination with other cytokines, TGF-β induced an inhibitory effect on TNF-α stimulation of CXCL8 \((P=0.027, n=3)\) and CCL5 production by hCMEC/D3 cells leading to a four-fold and a 1.50-fold decrease respectively (fig 4.12).

### 4.3.6. Intracellular expression and compartmentalisation of chemokines in hCMEC/D3

CCL2, CCL5 and CXCL8 staining in the cytoplasm of hCMEC/D3 cells was negative, suggesting no intracellular stock of these chemokines at the basal level. Stimulation of cells with cytokines did not induce any changes in the pattern of chemokine staining (fig 4.13).

CXCL10 labelling in endothelial cells \textit{in vitro} was conducted with three different antibodies originating from different species (fig 4.14). A mouse anti-human CXCL10, a rabbit anti-human CXCL10 and a goat anti-human CXCL10 were used. Similarly to the pattern of staining for primary HBEC, the three antibodies gave different patterns of staining. While untreated cells were faintly stained with the rabbit anti-human CXCL10, cells were not stained by the two other antibodies. When hCMEC/D3 cells were stimulated
with the cytokines, there was a strong staining around the nucleus for the three antibodies. In addition small vesicles could be observed with both the mouse and the rabbit anti-human CXCL10. The Western-blot analysis, using both the rabbit anti-human CXCL10 and the biotinylated goat anti-human CXCL10, revealed no band of the correct size (8.5kDa) for unstimulated cells, whereas a strong band appeared when the cells were stimulated with cytokines (fig 4.15). A number of bands of different molecular weight than the expected one of CXCL10 were also visible within the immunoblot (fig 4.15), suggesting that the antibody, in addition to CXCL10, binds non-specifically to a number of other proteins. This might explain why there was positive staining for CXCL10 by immunocytochemistry on unstimulated hCMEC/D3 cells. It is also possible that a band above that corresponding to CXCL10 could be the pro-peptide form of CXCL10 of a higher molecular size (about 14.5 kDa) and that this antibody, as opposed to the two others can recognise both CXCL10 forms.

4.3.7. Constitutive and cytokine-induced chemokine receptor expression

Chemokine receptors are important in inflammatory conditions and play a role in angiogenesis. In order to confirm that chemokine receptor expression by hCMEC/D3 cells was unchanged by lentiviral transduction of immortalising genes, the expression of CCR1-6 and CXCR1-5 was investigated by flow cytometric analysis under basal conditions and following stimulation with a combination of 10ng/ml IFN-γ and 500ng/ml TNF-α for 24 h. The concentrations of cytokines selected for this experiment were of the same order as those used for other experiments in Weksler et al. (2005). hCMEC/D3 cells expressed all CXCR receptors tested, with a high expression of CXCR1 and CXCR3 and a lower expression of CXCR2, CXCR4 and CXCR5 (fig 4.16). There was a small but significant ($P=0.023$, $n=3$) up-regulation in CXCR3 expression following cytokine treatment (fig 4.18). As for the CCR receptors, the expression of CCR3, CCR4, CCR5 and CCR6 could be detected while hCMEC/D3 cells were negative for CCR1 and CCR2 (fig 4.17).
Following stimulation by cytokines, CCR1, CCR3 and CCR4 were slightly, but significantly up-regulated ($P=0.044$, $P=0.038$, $P=0.002$, $n=3$) (fig 4.18).
Fig 4.2: Immunocytochemical detection of β-catenin, ZO-1 and PECAM-1 in hCMEC/3G and hCMEC/3C8 clones.

hCMEC/D3 were grown on collagen-coated Labtek chamber slides, fixed, permeabilised with Triton X-100 and then processed for detection of β-catenin, ZO-1 and PECAM-1 by immunocytochemistry. The immunocytochemical detection of PECAM-1 for the hCMEC/3G clone was not performed. Results are from one single experiment with single well. Scale bar=50μm.
Fig 4.3: Immunocytochemical detection of β-catenin, vWF, ZO-1 and PECAM-1 on hCMEC/3G and hCMEC/3C8 clones.
hCMEC/D3 were grown on collagen-coated Labtek chamber slides, fixed, permeabilised with Triton X-100 and then processed for detection of β-catenin, ZO-1 and PECAM-1 by immunocytochemistry. The cells were co-labelled with vWF located in the cytoplasm and β-catenin located at the cell-cell junctions. Results are from one single experiment with a single well. Scale bar=50μm.
Fig 4.4: Phase contrast micrographs of cultured hCMEC/D3.
hCMEC/D3 were grown on collagen-coated flasks until confluence. Phase contrast images were taken with a light microscope with a x 10 objective (left image) and a x 20 objective (right image) showing monolayers of contact-inhibited closely apposed fusiform cells. Scale bar = 100 μm.
Fig 4.5: Immunocytochemical detection of β-catenin, PECAM-1, ZO-1, Claudin-5 and Claudin-3 labelling in hCMEC/D3

hCMEC/D3 were grown on collagen-coated Labtek chamber slides, fixed, permeabilised with Triton X-100 then processed for detection of β-catenin, PECAM-1, ZO-1, Claudin-5 and Claudin-3 by immunocytochemistry. Results are representative of three different experiments with a single well. Scale bar=50μm.
Fig 4.6: Immunocytochemical detection β-catenin, ZO-1 and PECAM-1 in hCMEC/D3 at passage 20 (left) and passage 27 (right).
hCMEC/D3 were grown on collagen-coated Labtek chamber slides, fixed, permeabilised with Triton X-100 then processed for detection of β-catenin, ZO-1 and PECAM-1 by immunocytochemistry. Results are from one experiment. Scale bar=50μm.
Fig 4.7: Immunocytochemical detection β-catenin, ZO-1, Occludin and claudin-3 on hCMEC/D3 cells grown in normal medium (left) and in differentiation medium (right).

hCMEC/D3 were grown on collagen-coated Labtek chamber slides, fixed, permeabilised with Triton X-100 then processed for detection of β-catenin, ZO-1, occludin and claudin-3 by immunocytochemistry. Results are representative of three different experiments done with a single well. Scale bar=50μm.
**Fig 4.8: Immunoblotting of β-catenin, PECAM-1, ZO-1, Occludin, Claudin-3 and Claudin-5 in hCMEC3 cells grown in normal medium (left) or in differentiation medium (right).**

hCMEC/D3 cells were grown to confluence in normal medium or in differentiation medium on collagen-coated Petri dishes. Cells were lysed in Leammli’s buffer and lysates were subjected to electrophoresis on a 6%, 10% or 15% polyacrylamide gel. Electrophoresed proteins were then transferred onto nitrocellulose membrane. Equal loading was checked using Ponceau red. The blots were probed with an anti β-catenin, PECAM-1, ZO-1, occludin, claudin-3 or claudin-5 antibody and an appropriate HRP-conjugated secondary antibody. The signal was then revealed by chemiluminescence. The blots are representative of experiments repeated twice.
Fig 4.9: Effect of BBB promoting factors on the TEER of hCMEC/D3 monolayers.

hCMEC3 cells were grown on collagen-coated Transwell filters and the electrical resistance was measured once the cells reached confluence (1-2 days). The TEER of cells grown in differentiation medium was significantly higher than control monolayer ($P<0.05$). Significance was tested by $t$-test. Results presented are the mean ± S.E.M. of three different experiments from single insert.
Fig 4.10: Basal and TNF-α-induced ICAM-1, VCAM-1 and PECAM-1 expression in hCMEC/D3 cells by FACScan analysis.

hCMEC/D3 cells were fixed with 4% PAF, permeabilised with TritonX-100, labelled with antibodies specific for ICAM-1, VCAM-1 and PECAM-1, and analysed by FACScan. Representative FACScan histograms show the expression of unstimulated hCMEC/D3 cells (black line) and hCMEC/D3 cells stimulated with 25 ng/ml TNF-α for 48 h (grey line). Cells not labelled with primary antibodies served as negative controls and are represented as grey filled histograms. The histograms are representative of experiments repeated four time.

Fig 4.11: Basal and TNF-α-induced ICAM-1, VCAM-1 and PECAM-1 expression in hCMEC/D3 cells by FACScan analysis.

hCMEC/D3 cells were fixed with 4% PAF, permeabilised with TritonX-100, labelled with antibodies specific for ICAM-1, VCAM-1 and PECAM-1, and analysed by FACScan. Results are expressed as the median fluorescence for ICAM-1, VCAM- and PECAM-1 expression by unstimulated hCMEC/D3 cells and hCMEC/D3 cells stimulated with 25 ng/ml TNF-α for 48 h. Significance was tested on normalised values with a *t*-test (*=P<0.05). Results presented are the mean ± S.E.M. of four different experiments.
**Fig 4.12:** Chemokine release into culture medium by hCMEC/D3 cells following stimulation by cytokines for 48 h.

Supernatants from confluent hCMEC/D3 monolayers were collected after 48 h stimulation with TNF-α (50ng/ml), IFN-γ (100ng/ml) and TGF-β (25ng/ml) or a combination of them and assayed by sandwich ELISA for chemokine production. Results presented are the mean ± S.D. of three different experiments for CXCL8 and CCL2, two for CXCL10 and CCL5 (one well per treatment). Significance was tested by ANOVA followed by a post hoc t-test for CXCL8 and CCL2, no statistic analysis could be performed for CXCL10 and CCL5 as the number of experiment was too small. * significant difference ($P < 0.05$) versus the control; Δ significant difference ($P < 0.05$) of sample treated with a combination of cytokines versus TNF-α treated sample.
Fig 4.13: Immunocytochemical detection of CXCL8, CCL2 and CCL5 in hCMEC/D3 cells.
hCMEC/D3 cells were grown on collagen-coated Labtek chamber slides, fixed, permeabilised with Triton X-100 and then processed for detection of CXCL8, CCL2 and CCL5 by immunocytochemistry in unstimulated cells (left column) or TNF-α (50ng/ml) and IFN-γ (100ng/ml) stimulated cells (right column). Results are representative of three different experiments. Scale bar = 50µm.
Fig 4.14: Immunocytochemical detection of CXCL10 in hCMEC/D3 cells

hCMEC/D3 cells were grown on collagen-coated Labtek chamber slides, fixed, permeabilised with Triton X-100 and then processed for detection of CXCL10 by immunocytochemistry using three different antibodies, a mouse anti-human CXCL10, and rabbit anti-human CXCL10 and a goat anti-human CXCL10 in unstimulated cells (left column) or TNF-α (50ng/ml) and IFN-γ (100ng/ml) stimulated cells (right column). Results are representative of three different experiments. Scale bar = 50μm.
Fig 4.15: Immunoblotting of CXCL10, CXCL8, CCL2 and CCL5 in hCMEC/D3 cells at basal level and following stimulation with IFN-γ and TNF-α.
hCMEC/D3 cells were grown to confluence on collagen-coated Petri dishes then stimulated for 48 h with 100ng/ml IFN-γ and 50 ng/ml TNF-α or left unstimulated. Cells were lysed in Laemmli’s buffer and lysates were subjected to electrophoresis on a 15% polyacrylamide gel. Electrophoresed proteins were then transferred onto nitrocellulose membranes. The blots were probed with anti-CXCL10, CXCL8, CCL2 and CCL5 antibodies and appropriate HRP-conjugated secondary antibodies. The signal was then revealed by chemiluminescence. The blots are representative of experiments repeated twice.
Fig 4.16: Basal and TNF-α and IFN-γ-induced chemokine receptor expression in hCMEC3 cells by FACScan analysis.

hCMEC3 cells were fixed with 4% PAF, permeabilised with TritonX-100, labelled with FITC- or PE- labelled antibodies specific for CXCR1-5, and analysed by FACScan. Representative FACScan histograms show the expression of unstimulated hCMEC/D3 cells (black line) and hCMEC/D3 cells stimulated with 500 ng/ml TNF-α and 10ng/ml IFN-γ for 24 h (grey line). Cells labelled with FITC- or PE- labelled isotope-matched antibodies are represented as grey filled histograms. The histograms are representative of experiments repeated three times.
Fig 4.17: Basal and TNF-α and IFN-γ-induced chemokine receptor expression in hCMEC3 cells by FACSscan analysis.

hCMEC3 cells were fixed with 4% PAF, permeabilised with TritonX-100, labelled with FITC- or PE- labelled antibodies specific for CCR1-6, and analysed by FACSscan. Representative FACSscan histograms show the expression of unstimulated hCMEC/D3 cells (black line) and hCMEC/D3 cells stimulated with 500 ng/ml TNF-α and 10ng/ml IFN-γ for 24 h (grey line). Cells labelled with FITC- or PE- labelled isotope-matched antibodies are represented as grey filled histograms. The histograms are representative of experiments repeated three times.
Fig 4.18: Summary of chemokine receptor in hCMEC3 at constitutive level and when stimulated with TNF-α and IFN-γ for 24 h.
Results are expressed as the mean fluorescence for CXCR1-5 and CCR1-6 expression by unstimulated hCMEC/D3 cells (white filling) and hCMEC/D3 cells stimulated with 500 ng/ml TNF-α and 10ng/ml IFN-γ (grey filling) for 24 h. Significance was tested on normalised values by ANOVA with a post hoc t-test (*=P<0.05). Results presented are the mean ± S.E.M. of three different experiments.
4.4 **Discussion**

In this study, the hCMEC/D3 cell line, a stable human brain endothelial cell line, was obtained by transduction of hTERT to prolong stable lifespan and of SV40 large T antigen to foster proliferation, using a new generation of lentiviral vectors that are efficient on slowly proliferating cells such as HBEC. This cell line was characterised, in the first instance for its properties to create a low permeability barrier, indicating functional retention of the physiological properties of the BBB. The expression of junctional proteins, such as β-catenin, PECAM-1, ZO-1, claudin-5 and claudin-3 was demonstrated by immunocytochemistry. The expression of junctional proteins, despite being slightly lower than in HBEC, appeared stable for a high number of passages. The expression of some junctional proteins such as occludin and claudin-3 was up-regulated in the presence of ACM and cAMP elevating agents, known to have barrier-phenotype promoting effects on brain primary endothelial cells (Rubin *et al.*, 1991). Furthermore, on a functional level, hCMEC/D3 cells showed low paracellular permeability to FD70, of the same magnitude as primary HBEC. However, hCMEC/D3 cells showed a low value of TEER.

Endothelial cells from different organs display different patterns of expression of inflammatory molecules such as adhesion molecules, chemokines and chemokine receptors (Hillyer *et al.*, 2003). The hCMEC/D3 cell line was therefore further characterised for its level of adhesion molecule expression, chemokines and chemokine receptors as well as for its response to cytokines such as IFN-γ, TNF-α and TGF-β. Constitutive and cytokine-induced expression of adhesion molecules by hCMEC/D3 cells follow a similar pattern to that observed in HBEC. CXCL8, CCL2, CCL5 and CXCL10 levels of expression was within the same range as that of primary HBEC both at the basal level and when stimulated with cytokines alone or with a combination of cytokines, with the exception of CXCL10.
which, in contrast to primary endothelium, was induced by TNF-α. Expression of chemokine receptors by hCMEC/D3 cells was also similar to primary brain endothelium, although there was little increase in chemokine receptor expression following pro-inflammatory cytokine treatment.

4.4.1. Expression of endothelial junctional proteins by hCMEC/D3 cells in normal medium and in differentiation medium.

Confluent monolayers of hCMEC/D3 cells showed a subcellular localisation of BBB endothelial junction-associated molecules similar to primary HBEC. Nevertheless, the level of expression of junctional proteins appeared generally lower, and the junctional staining was neither as strong nor as continuous (section 2.3.3). In general, cultured HBEC lose their capacity to express certain tight junctional proteins over a number of passages, as shown by the results from section 2.3.3, where occludin and claudin-5 were not uniformly expressed by all cells, and junctional localisation of claudin-3 was rare. In cryofracture studies, the plasma membrane cleaves between the acyl tails of membrane phospholipids, leaving a monolayer on each half of the specimen. The E-face is the inner face of the outer lipid monolayer. The P-face is the inner lamella viewed from outside the cell. Claudin-3 forms tight junctions associated with the P-face (Furuse et al., 1999). BBB endothelial cells \textit{in vivo} reveal a P-face/E-face ratio of about 55/45 (Kniesel et al., 1996). Microvascular brain endothelial cells cultured \textit{in vitro} form tight junctions, which are associated with the E-face (Wolburg et al., 1994). This effect was shown to be associated with a lower expression of claudin-3 (Liebner et al., 2000), which at the time the authors considered to be claudin-1. However, it was later demonstrated that the claudin-1 antibody used cross-reacted with claudin-3 (Wolburg et al., 2003). In non-blood–brain barrier endothelial cells such as pulmonary vessels, tight junctions are almost completely associated with the E-face (Schneeberger, 1982). Therefore it appears as if brain endothelial cells in culture dedifferentiate towards a non-CNS endothelial phenotype. This
phenomenon reinforces the idea that either the absence of factors present within the brain extracellular milieu or the culture conditions in vitro induce dedifferentiation of brain endothelial cells.

A number of factors present in the growth medium could be responsible for dedifferentiating endothelial cells in vitro such as IGF-1 known to enhance dedifferentiation in human neuroblastomas (Weber et al., 2003) or EGF that induce dedifferentiation in renal epithelial cells (Zhuang et al., 2005). FCS and serum-derived factors such lysophosphatidic acid and VEGF have been shown to alter the characteristics of brain endothelium (Nitz et al., 2003). Indeed, TEER values of porcine brain endothelial cells grown in serum were lower than those of cells cultured without serum (Nitz et al., 2003). Furthermore, ZO-1, occludin and claudin-5 staining in these cells were redistributed from the cell periphery to the perinuclear cytoplasm in the presence of serum (Nitz et al., 2003). In our study, hCMEC/D3 cells were cultured for much longer periods than primary cells in the presence of serum, which could account for the lower levels of junctional proteins when compared to primary HBEC.

Brain-derived factors absent in the culture medium may include those secreted by surrounding astrocytes that induce an increase in intracellular cAMP of endothelial cells. The combination of ACM and agents such as forskolin which activates adenylate cyclase and thus elevates levels of intracellular cAMP has been shown to be most effective in the improvement of barrier properties in cultured endothelial cells as it induced lower paracellular permeability and higher expression of tight junctional proteins (Rubin et al., 1991; Wolburg et al., 1994; Rist et al., 1997). Wolburg et al. (1994) showed that bovine brain endothelial cells grown in ACM and cAMP expressed higher amounts of occludin and claudin-3 despite their failure to induce a more junctional localisation of these proteins. This is one indication that the ability to respond to astrocyte-derived factors and rise in
intracellular cAMP is retained by transformed cell lines such as hCMEC/D3 cells. This is in agreement with Hamm et al. (2004) who showed no differences in the subcellular localisation of occludin, claudin-3 and claudin-5 between bovine brain endothelial cells in co-culture with rat astrocytes and cells which were first grown in the presence of rat astrocytes, but then the astrocytes were removed for 1 to 4 days. Astrocytes may be crucial for inducing the formation of tight junction but not for maintaining them in their proper subcellular localisation, as removal of astrocytes with 3-chloropropanediol *in vivo* in rats induced only a transient loss of junctional proteins such as ZO-1 and claudin-5 (Willis et al., 2004). Co-culture with rat astrocytes induced no differences in ZO-1 and β-catenin localisation in bovine brain endothelial cells (Hamm et al., 2004). However, in this study ZO-1 and β-catenin staining was more continuous when cells were grown in the presence of ACM and cAMP elevating agents.

Increased intracellular cAMP may therefore be one of the important contributing factors maintaining tight junction structure. Indeed there is a slight increase in ZO-1 expression at the junction in porcine brain endothelial cells when grown in ACM, but the elevation is markedly higher in the presence of both ACM and cAMP (Torok et al., 2003). Furthermore, increase in cAMPi has been shown to increase the gene expression of claudin-5 in porcine brain endothelial cells (Ishizaki et al., 2003). Alternatively, the up-regulation in tight junctional protein expression in response to cAMP might not be associated with an increase in TEER and a lower permeability. Indeed, improved electrical resistance levels are readily reversible since resistance decreases rapidly when cAMP levels return to normal (Rubin et al., 1991). Thus, the effects of cAMP on TEER are likely to be independent of gene transcription (Rubin et al., 1991). It is possible that cAMP regulates reorganisation of the cell cytoskeleton as untreated endothelial cell cultures contain actin stress fibers throughout their cytoplasm, but after cAMP treatment stress fibers become less abundant and the belt of filamentous actin at sites of cell junctions...
becomes more apparent (Rist et al., 1997). Other cells from the CNS might be important for inducing barrier properties, such as neurones (Tontsch and Bauer, 1991) or pericytes (Hayashi et al., 2004). In addition, pericytes have been reported to support survival of endothelial cells and to promote angiogenesis by secreting VEGF (Darland et al., 2003; Erber et al., 2004).

4.4.2. TEER and paracellular permeability to FITC-dextran 70kDa of hCMEC/D3 monolayers

As mentioned previously in this Chapter and in Chapter 2, cerebral endothelial cells, when cultured, lose their characteristics and resemble peripheral endothelial cells. Furthermore, immortalisation of a cell type is often associated with dedifferentiation. hCMEC/D3 is a cell line that retains expression of tight junctional molecules such as occludin and claudin-5 despite their not being consistently detected at cell-cell contacts. In order to evaluate the tightness of the junctions of the hCMEC/D3 cell line, the permeability of a confluent hCMEC/D3 monolayer to FD70 was investigated as well as the resistance reflecting the ionic permeability. The permeability to FD70 obtained with hCMEC/D3 monolayers showed that despite the fact that the expression of occludin, claudin-5 and claudin-3 was not continuous at the cell-cell junctions, hCMEC/D3 showed lower permeability characteristics than a non brain endothelium monolayer such as HUVEC (Weksler et al., 2005) or than a brain endothelial cell line from other species such as the rat line GPNT (Romero et al., 2003). The expression of other tight junctional proteins at the junction such as JAM-1 which also participates in permeability regulation (Martin-Padura et al., 1998) may have contributed to the lower paracellular permeability of hCMEC/D3 cells compared to other cell lines.
The hCMEC/D3 cell line shows low TEER values which are not in agreement with the low permeability to paracellular marker observed. In this study, the low junctional localisation of the claudins and occludin probably results in the low resistance values given by hCMEC/D3 monolayers. Indeed, electrical resistance across cell monolayers reflect their ionic permeability. In epithelial cells of mice carrying a null mutation for occludin, alteration of occludin expression did not affect the transepithelial resistance (Saitou et al., 2000). Occludin has been suggested to have a more important role in regulating paracellular permeability rather than in establishing the tight junction structure and properties (Balda et al., 2000). By contrast, localisation of claudins at the junctions is considered to be directly related to electrical resistance. Epithelial cells treated with clostridium perfringens enterotoxin which binds to claudin-3 and claudin-4 show low electrical resistance values (Sonoda et al., 1999). Claudins have been suggested to seal paracellular diffusion of tracers across the tight junction (Matter and Balda, 2003). This is further supported by the slightly higher resistance obtained with HBEC when treated with BBB promoting factors, in which there is an increased expression of occludin and claudin-3. In conclusion, hCMEC/D3 monolayers possess functional intercellular junctions which result in low permeability especially to hydrophilic proteins with a molecular mass higher than 4 KDa (Weksler et al., 2005), whereas the hCMEC/D3 cell line is a less valuable BBB model for small hydrophilic molecules and ions.

4.4.3. Expression of adhesion molecules by hCMEC/D3 cells

ICAM-1 is a major protein in the regulation of leukocyte adhesion to endothelial cells, and blocking ICAM-1 results in an inhibition of leukocyte infiltration (Alter et al., 2003). ICAM-1 has been reported to be expressed on brain endothelial cells and to be up-regulated by stimulation with cytokines in vitro (Fabry et al., 1992; Wong and Dorovini-Zis, 1992) as well as in vivo (Henninger et al., 1997; Komatsu et al., 1997). hCMEC/D3
cells, similarly to primary HBEC, express high constitutive levels of ICAM-1 which are up-regulated by TNF-α stimulation.

VCAM-1 is expressed under basal conditions at very low levels by hCMEC/D3 cells. In primary HBEC, it has been shown that VCAM-1 is expressed at a low level (Stins et al., 1997) similarly to primary rat brain endothelial cells (dos Santos et al., 1996). VCAM-1 expression by hCMEC/D3 cells was induced by TNF-α after 48 h stimulation at a concentration of 25ng/ml. TNF-α has been shown to have a transient effect on VCAM-1 expression by primary rat brain endothelial cells, with a maximum increase between 12 and 24 h and a subsequent decline to basal levels (dos Santos et al., 1996). The rapid increase in VCAM-1 expression in response to TNF-α has been also described in human endothelium after 4 h (Stins et al., 1997). Thus it is possible that the small effect of TNF-α in up-regulating VCAM-1 is associated with either the low concentration used (25ng/ml) or the time of investigation (48 h). Larger effects on VCAM-1 expression was observed on hCMEC/D3 cells after 24 h cytokine stimulation and when the concentrations of cytokines used were higher (Weksler et al., 2005).

After 48 h of stimulation with TNF-α, there was a small decrease in the expression of PECAM-1. PECAM-1 is a junctional protein, and TNF-α increases the permeability of the BBB (section 5.3.2) (Deli et al., 1995). Hence, it is possible that cytoskeletal reorganization following increases in endothelial permeability by TNF-α results in decreased PECAM-1 expression despite its possible importance in transmigration. This observation has been previously reported on HUVEC (Shaw et al., 2001). Another protein, which acts both as an adhesion molecule important in transmigration as well as a junctional protein is JAM-1, and its expression is also decreased following stimulation with cytokines (Shaw et al., 2001).
4.4.4. Chemokine expression by hCMEC/D3 cells

hCMEC/D3 cells showed overall little difference in the release of CXCL8, CXCL10, CCL2 and CCL5 into cell culture supernatants compared with primary HBEC using the same conditions as described in section 2.3.4.

CCL2 was detected in the supernatants of primary HBEC albeit at low levels, but is not released by hCMEC/D3 cells. This difference between primary and immortalised cells may be a direct consequence of promoting proliferation by introducing immortalising genes into cells. Effectively transformed BMEC also expressed little if any CCL2 at basal levels (Vanderkerken et al., 2002; Hillyer et al., 2003), whereas primary BMEC have been shown to express low levels of CCL2 in the culture medium (Pellegrino et al., 2005). It is therefore possible that the rate of cellular proliferation could affect chemokine production, with rapidly dividing cells producing less CCL2. However, Harkness et al. (2003) showed that addition of dexamethasone, a brain endothelial cell differentiating agent that decreases proliferation rate, to the culture medium decreased basal CCL2 production by an immortalised rat brain endothelial cell line (Harkness et al., 2003). In this study chemokine production was assessed in confluent monolayers of contact-inhibited cells and the same culture medium and growth factors were used for both primary and immortalised endothelial cells, hence it is highly unlikely that proliferation rate has influenced CCL2 production. Moreover, no CCL2 was detected intracellularly, and this was confirmed by Western-blotting which is a more sensitive method. Alternatively, differences in CCL2 production between primary and immortalised cells may be the result of the clonal nature of cell lines, whereas primary cultures consist of a heterogeneous population of cells.

As described in section 2.3.4, there are variations in CXCL8 production by primary HBEC isolated from different donors. CXCL8 production by hCMEC/D3 cells is well within the
observed range for primary cells both at basal and stimulated levels i.e. no CXCL8 is present at basal level whereas low levels are detected when stimulated with cytokines. Similarly to some batches of primary HBEC, no CXCL8 was detected by immunocytochemistry either at basal level or when the cells were stimulated with cytokines for 48 h whereas a faint band for CXCL8 was detected in lysates of cytokine stimulated cells by western-blotting, suggesting low levels of intracellular CXCL8.

CCL5 and CXCL10 are both inducible chemokines and are not expressed at the basal level in primary HBEC. This is also the case for hCMEC/D3 cells. Cytokine stimulation induced a similar response in CCL5 secretion in both primary cells and hCMEC/D3 cells, whereas hCMEC/D3 cells, but not primary cells, released CXCL10 into the culture medium in response to TNF-α. It has been previously shown that most other endothelial cell types such as BMEC, SVEC, DMVEC, HUVEC and LMVEC (Briones et al., 2001; Hillyer et al., 2003) also produced CXCL10 in response to TNF-α stimulation. The absence of an inductive effect by TNF-α in HBEC might constitute a specific property of brain endothelium that differentiates it from other vascular beds. However, as discussed earlier, the hCMEC/D3 cell line may represent an intermediate phenotype between brain and non-brain endothelium, thus it is possible that TNF-α-induced CXCL10 production ensues as a result of brain endothelial cell dedifferentiation.

4.4.5. Chemokine receptor expression by hCMEC/D3 cells

The expression of chemokine receptors on primary HBEC was difficult to quantify by techniques such as FACS analysis due to the low numbers of cells produced by the isolation method. Hence it was not possible to compare their expression between hCMEC/D3 cells and primary HBEC. Nevertheless the pattern of chemokine receptor expression was similar for both primary and immortalised cells. Similarly to primary cells, CCR1 and CCR2 were not detected at the protein level in hCMEC/D3 cells, whereas
CCR3 and CCR5 were both present at low levels. By immunocytochemistry, CCR4 could not be detected on primary HBEC, whereas FACS analysis showed a low expression of CCR4 in hCMEC/D3 cells (table 4.4). CCR4 has been reported to be expressed by primary HBEC at low level by immunocytochemistry (Berger et al., 1999). It is possible that in our case, the antibody was not sensitive enough to detect CCR4 by immunocytochemistry, while FACS analysis was a more sensitive technique. In the CCR receptor family CCR6 was also detected on hCMEC/D3. This is the first report of CCR6 expression by brain endothelial cells. CCR4 and CCR6 expression has been previously reported on DMVEC, LMVEC and SVEC, but not HUVEC (Hillyer et al., 2003). Only CCR1 and CCR3 expression increased when stimulated with 500ng/ml of TNF-α and 10ng/ml of IFN-γ after 24 h. Transcript levels of CCR1 and CCR3 are increased in neutrophils when stimulated with IFN-γ (Bonecchi et al., 1999). CCR2, as demonstrated for HBEC was absent and its expression could not be induced by cytokine simulation. CCR5 expression was unaffected by cytokine stimulation. This shows a special regulation of chemokine receptor expression by endothelial cells. Indeed in the case of leukocytes, CCR5 has been shown to be up-regulated in response to 10ng/ml IFN-γ and TNF-α on T cells (Juffermans et al., 2000).

CXCR1, CXCR2 and CXCR3 are expressed in a similar pattern by both primary human brain endothelial cell lines and hCMEC/D3 cells (table 4.4). CXCR4 was also expressed by hCMEC/D3 cells but only at low levels in primary cells. CXCR4 has been reported on primary HBEC (Berger et al., 1999; Mukhtar et al., 2002; Mukhtar et al., 2005), and is of particular interest as a co-receptor for HIV virus, and thus could be participating in the entry mechanism of the virus into the brain. Interestingly, CXCR5 was also detected by FACS analysis. This chemokine receptor is important in the development of normal lymphoid tissue and is mainly located on B cells and some T cells. This is the first time that CXCR5 was described on HBEC in vitro. In vivo, CXCR5 expression has been previously reported to be associated with blood vessels in primary CNS lymphoma (Smith...
et al., 2003). In this study, the authors demonstrate that CXCL13, the only known ligand of CXCR5, is produced within the tumour. It is possible that CXCL13 activation of CXCR5 on brain endothelial cells may induce vascularisation of the tumour. However, it is unlikely as CXCL13 has been previously shown to have the opposite effect and to inhibit the angiogenic action of growth factors such as FGF-2 on HUVEC (Spinetti et al., 2001) and there is no evidence supporting an angiogenic effect for CXCL13. When compared among cells originating from different vascular beds, CXCR5 expression is variable, which may determine the nature of the response to CXCL13. CXCR5 expression has been demonstrated on SVEC, while little if any CXCR5 was present on HUVEC and none on BMEC, DMEC and LMVEC (Hillyer et al., 2003).

4.4.6. Conclusion

In this study we have obtained a stable human brain endothelial cell line, termed hCMEC/D3, by transduction of hTERT and SV40 large T antigen using highly efficient lentiviral vectors. A comprehensive characterisation of this cell line showed that cells retained expression of junctional proteins, specific to HBEC, which contribute to low permeability of large hydrophilic molecules. This cell line also represents an interesting model to study the role of HBEC in inflammation and infiltration of leukocytes into the CNS, as the expression of adhesion molecules, chemokines and chemokine receptors closely resembles the expression pattern of primary HBEC (table 4.4).
<table>
<thead>
<tr>
<th>Junctional Proteins</th>
<th>HBEC</th>
<th>hCEMC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>PECAM-1</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>β-Catenin</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>ZO-1</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Claudin-5</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>Claudin-3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Occludin</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>Permeability Coefficient to FD70 (cm/min)</td>
<td>$4.58 \times 10^{-5}$ (±$1.51 \times 10^{-5}$)</td>
<td>$2.67 \times 10^{-5}$ (±$2.09 \times 10^{-5}$)</td>
</tr>
<tr>
<td>TEER (ohms.cm$^{-2}$)</td>
<td>$23.5 \pm 3.53$</td>
<td>$9.66 \pm 3.33$</td>
</tr>
</tbody>
</table>

Chemokine released (ng/ml)

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Basal</th>
<th>IFN-γ</th>
<th>TNF-α</th>
<th>TGF-β</th>
<th>IFN-γ+ TNF-α</th>
<th>IFN-γ+ TGF-β</th>
<th>TGF-β+ TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL8</td>
<td>72(±27.2)</td>
<td>42(±12.5)</td>
<td>308(±60.5)</td>
<td>30(±13.1)</td>
<td>162(±31.1)</td>
<td>53(±23.7)</td>
<td>134(±23.5)</td>
</tr>
<tr>
<td>CXCL10</td>
<td>0</td>
<td>100(±16.5)</td>
<td>8(±2)</td>
<td>0</td>
<td>200(±50.9)</td>
<td>115(±26.8)</td>
<td>12(±4)</td>
</tr>
<tr>
<td>CCL2</td>
<td>12(±3.8)</td>
<td>10(±2.7)</td>
<td>34(±8)</td>
<td>15(±4.6)</td>
<td>36(±7.2)</td>
<td>16(±4.5)</td>
<td>34(±6.9)</td>
</tr>
<tr>
<td>CCL5</td>
<td>0</td>
<td>0</td>
<td>5(±3)</td>
<td>0</td>
<td>43(±2.8)</td>
<td>2(±0.9)</td>
<td>22(±4.7)</td>
</tr>
</tbody>
</table>

Chemokine expression

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Basal</th>
<th>IFN-γ+ TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL8</td>
<td>±</td>
<td>± CXCL8</td>
</tr>
<tr>
<td>CXCL10</td>
<td>±</td>
<td>CXCL8/CXCL10</td>
</tr>
</tbody>
</table>

Chemokine receptor expression

<table>
<thead>
<tr>
<th>CCR receptor</th>
<th>CCR3/CCR5</th>
<th>CCR3/CCR4/CCR5</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR receptor</td>
<td>CXCR1/CXCR2/CXCR3</td>
<td>CXCR1/CXCR2/CXCR3</td>
</tr>
</tbody>
</table>

Table 4.4: Summary of junction protein expression, permeability to FD70, TEER, chemokine release chemokine expression and chemokine receptor expression of HBEC and hCEMC3.
5. Chapter 5: Effect of CXCL10 on the immunological and barrier function of human brain endothelial cells and astrocytes
5.1 Introduction

CXCL10, because of its chemotactic effect on T lymphocytes, which are themselves involved in many autoimmune disorders, is of particular interest in MS. Indeed, CXCL10 is found to be up-regulated in CSF (Mahad et al., 2002; Scarpini et al., 2002), and induced in MS lesions (Sorensen et al., 1999; Simpson et al., 2000b; Sorensen et al., 2002). Furthermore, HBEC, the first cells to be contacted by leukocytes in the transmigration process, express high levels of CXCR3, the only receptor for CXCL10 (Berger et al., 1999).

The gene for CXCR3, unlike those for all other chemokine receptors, is located on the human chromosome Xq13 (Loetscher et al., 1998). CXCR3 has three ligands, CXCL9, CXCL10 and CXCL11, the latter binding CXCR3 with the highest affinity (Cole et al., 1998). The three ligands have different binding sites on the extracellular loop of the receptor, allowing different responses. The N-terminus and the first extracellular loop mediate CXCL10 and CXCL11 responses, while the third extracellular loop is important for CXCL10 and CXCL9-induced chemotaxis (Xanthou et al., 2003). Similarly, specific intracellular domains are involved in response to different ligands. For instance, CXCL9 and CXCL10 induced-CXCR3 internalisation is mediated by the C-terminus and β-arrestin1 binding, while the third intracellular loop is required by CXCL11 (Colvin et al., 2004). The C-terminus and a DRY sequence in the third trans-membrane domain are important for the chemotactic response induced by the three chemokines (Colvin et al., 2004).

CXCR3 is a 364 amino acid G-protein coupled receptor with seven transmembrane domains (Lasagni et al., 2003). In addition, two alternative variants of CXCR3 have recently been identified (Lasagni et al., 2003; Ehlert et al., 2004). The CXCR3-B variant is
a 416 amino acid receptor containing a longer N-terminus on the extracellular side, and differs from the original CXCR3, currently named CXCR3-A, in its first 52 amino acid residues. CXCR3-B is a receptor for CXCL4 as well as for the other three known ligands, CXCL9, CXCL10 and CXCL11. The other variant is the result of alternative splicing via exon skipping (Ehlert et al., 2004). This variant, termed CXCR3-alt, only has a predicted four or five transmembrane domain and an altered C-terminal domain (Ehlert et al., 2004). Cells transfected with CXCR3-alt displayed a chemotactic response towards CXCL11 only, and higher concentrations of the chemokine were needed to generate a response in comparison to the normal receptor variant (Ehlert et al., 2004).

Binding of a ligand leads to the activation of a range of signalling pathways. Binding of CXCL11, which is the most potent agonist, activates p42/p44 MAP kinase and Akt/phosphatidylinositol 3 kinase (Smit et al., 2003). The pathway responsible for the migration towards CXCL11 is mediated by Goi and a phospholipase C-dependant pathway (Smit et al., 2003). In human glomerular mesangial cells and pericytes, CXCR3 activation induces chemotactic properties, and also induces proliferation (Romagnani et al., 1999; Bonacchi et al., 2001). In these cells, the MAP kinase pathway induces these two effects, as PD98059, an inhibitor of p42/p44 MAP kinase partially inhibits the migration and completely inhibits the proliferative effect of CXCL10 on pericytes (Bonacchi et al., 2001). By contrast, CXCL10 has an anti-proliferative effect on endothelial cells in agreement with its angiostatic role (Luster et al., 1995). In endothelial cells, there is no increase in the calcium influx in response to CXCL10 (Salcedo et al., 2000). Lasagni et al. (2003) hypothesised that the different proliferative responses to CXCL10 between endothelial cells and non-endothelial cells may be the result of the exclusive expression of a different variant receptor by endothelial cells, specifically CXCR3-B which signals though a different pathway than the typical Goi pathway induced by CXCR3-A. Indeed, CXCR3-B transfected cells when stimulated with CXCL10, decreased their proliferation activity, and
this effect was not blocked by *Bordatella pertussis* toxin, but was linked to an increase in the intracellular cAMP (Lasagni *et al.*, 2003).

In this study, the role of CXCR3, strongly expressed by hCMEC/D3 cells (fig 4.16) on the endothelial barrier function and infiltration of leukocytes into the brain, was investigated following CXCL10 stimulation. The paracellular permeability of hCMEC3/D3 monolayers in response to CXCL10 was assessed, as well as the adhesion of a monocytic cell line, THP1, on endothelium activated by CXCL10. Furthermore, the functional expression of CXCR3 was confirmed by investigating the activation of signalling pathways in hCMEC/D3 cells in response to CXCL10.
5.2 **Material and methods**

5.2.1. **Western-blot**

**Stripping buffer:** 100mM β-mercapto-ethanol (Sigma Aldridge, Dorset, UK), 2%w/v SDS, 62.5mM Tris/HCL pH 6.8.

hCMEC/D3 cells were grown to confluence on sterile 90 cm² petri-dishes (Greiner Laboratories Limited, Gloucestershire, UK) and rested in serum-free medium for 48 h prior to stimulation. CXCL10 (100ng/ml) was used to stimulate the cells at 0, 2, 5, 15, 30 and 60 min. Following stimulation, cells were lysed in Laemmli’s buffer and processed as described in section 4.2.9. The antibodies used are reported in table 5.1.

Following blotting with antibodies specific for the phosphorylated form of the signalling proteins, each nitrocellulose membrane was stripped and incubated with the antibody against the non-phosphorylated form of the protein in order to verify equal loading of samples on different lanes. This was achieved by washing the blot for 15 min in PBS followed by immersion in stripping buffer for 30 min at 50°C. After a final wash in PBS, the membrane was blocked and re-probed as previously described in section 4.2.9.
<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Antigen specificity</th>
<th>Host species</th>
<th>Concentration</th>
<th>Expected size</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospho-P42/44 MAP kinase (pY1007, pY1008)</td>
<td>Rabbit</td>
<td>0.1µg/ml</td>
<td>42 and 44 kDa</td>
<td>Biosource International</td>
<td></td>
</tr>
<tr>
<td>P42 MAP kinase</td>
<td>Mouse IgG2a</td>
<td>1µg/ml</td>
<td>42 kDa</td>
<td>Upstate Biotechnology</td>
<td></td>
</tr>
<tr>
<td>Phospho-p38 MAP kinase (Thr180/Tyr182)</td>
<td>Rabbit</td>
<td>1/1000</td>
<td>43 kDa</td>
<td>Cell Signaling Technology</td>
<td></td>
</tr>
<tr>
<td>p38 MAP kinase</td>
<td>Rabbit</td>
<td>1/1000</td>
<td>43 kDa</td>
<td>Cell Signaling Technology</td>
<td></td>
</tr>
<tr>
<td>Phospho-SAPK/JNK (Thr183/Tyr185)</td>
<td>Mouse IgG1</td>
<td>1/2000</td>
<td>46 and 54 kDa</td>
<td>Cell Signaling Technology</td>
<td></td>
</tr>
<tr>
<td>SAPK/JNK</td>
<td>Rabbit</td>
<td>1/1000</td>
<td>46 and 54 kDa</td>
<td>Cell Signaling Technology</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.1: List of antibodies used for Western-blotting. The final concentration of the antibody is given where the company gave the initial concentration, otherwise the dilution factor is reported.

5.2.2. TEER measurement

TEER was measured from confluent hCMEC/D3 monolayers as explained in Section 4.2.4.

5.2.3. Paracellular permeability

Paracellular permeability to FD70 of a confluent hCMEC/D3 monolayer in response to CXCL10 stimulation (100ng/ml for 48h) was proceded as in section 4.2.5. The concentration of CXCL10 was chosen according to the amount of CXCL10 secreted by HBEC (section 2.3.4.4).

5.2.4. FACS analysis

Adhesion molecule expression in response to CXCL10 was assessed by FACS analysis as in section 4.2.6.
5.2.5. Adhesion of THP1 cells to hCMEC/D3 monolayers

5.2.5.1. Culture conditions and maintenance of THP1 cells

THP1 medium was composed of RPMI medium, 20% Foetal calf serum, 50U/ml penicillin and 50μg/ml fungizone (Invitrogen, Renfrewshire, UK). THP1 cells were grown in suspension and after reaching a cell density of 10^6 cells/ml, cells were split 1:2.

5.2.5.2. Cell adhesion assay

hCMEC/D3 cells were seeded onto 96 well plates at a density of 2x10^5 cells per well (Greiner, Gloucestershire, UK) and grown to confluence. hCMEC/D3 cells were stimulated with 100ng/ml CXCL10 for 6, 24 and 48 h and with 25ng/ml TNF-α for 48 h, in a serum and growth factor free medium. The concentrations of TNF-α selected for this experiment were of the same order as those used for other experiments in Che et al., (2002). The concentration of CXCL10 was chosen according to the amount of CXCL10 secreted by HBEC (section 2.3.4.4). THP1 cells were labelled with radioactive chromium-51 (^51Cr) administered from an aqueous sodium chromate stock solution (37 MBq, 1mCi, Amersham Biosciences, Buckinghamshire, UK). Briefly. 10^6 THP1 cells were incubated in 100 μl of RPMI medium (Invitrogen, Renfrewshire, UK) with 100 μCi of ^51Cr at 37°C for 1 h. The excess of ^51Cr was removed by 2 consecutive washes with RPMI medium by spinning the cells at 300xg for 5 min. hCMEC/D3 cells were then washed four times with warm medium and 2x10^5 ^51Cr-labelled THP1 cells were added in 200 μl per well. After 1 h incubation at 37°C, loosely adherent or unattached THP1 were washed 4 times with warm medium, and the hCMEC/D3 monolayer plus the adherent THP1 cells were lysed in 150μl of 1% Triton X-100 for 10 sec. The cell lysates were transferred into Eppendorf tubes and the radioactivity was read on a 1470 automatic γ-counter Wallac (Perkin Elmer Lifesciences, Cambridgeshire, UK). The percentage of adherent THP1 in each sample was calculated from the radioactivity of a control sample using the following formula:
(cpm in the binding lymphocyte lysate / total cpm in input lymphocyte lysate) x 100.

5.2.6. Capture ELISA

Detection of CCL2, CCL5 and CXCL8 release in response to CXCL10 stimulation was determined using capture ELISA as described in section 2.2.5.

5.2.7. Statistical analysis

Significance was determined by a one way or two way ANOVA, followed by a post hoc Tukeys t-test. $P<0.05$ was considered significant.
5.3 Results

5.3.1. Signal transduction pathways activated by CXCR3 in hCMEC/D3 cells

In order to assess whether CXCR3 expression on hCMEC/D3 cells was functional and whether CXCL10 could trigger the activation of signalling pathways in hCMEC/D3 cells, levels of phosphorylation of p42/44 MAP kinase, JNK kinase and p38 MAP kinase was analysed by western-blotting. Phosphorylation of the three kinases was observed when hCMEC/D3 cells were stimulated with 100ng/ml CXCL10 (fig 5.8). The level of p42/44 and p38 MAP kinase phosphorylation was stronger than JNK kinase phosphorylation. Phosphorylation of p42/44 MAP kinase was observed after 5 min and reached a maximum at 15 min, whilst p38 kinase phosphorylation occurred slightly earlier, with a maximum phosphorylation at 5 min. JNK phosphorylation increased after 5 min and this level of phosphorylation was maintained at 15 min. At 30 min the level of phosphorylation of JNK was back to basal level (fig 5.1). In conclusion, CXCL10 stimulation of hCMEC induced activation of several signalling pathways including the three MAP kinases, p42/44 MAP kinase, p38 MAP kinase and JNK kinase.

5.3.2. Effect of CXCL10 on the permeability of hCMEC/D3 monolayers

CXCL10-induced effects in the permeability to FD70 of hCMEC/D3 monolayers were determined in order to assess the role of this chemokine in the inflammatory-induced changes in barrier function observed in MS. The permeability of hCMEC/D3 monolayers was assessed following 3 h and 48 h stimulation with CXCL10 (100ng/ml). As a positive control TNF-α–induced permeability changes were recorded in parallel (fig 5.2 & 5.3). Cleared volume versus time in response to CXCL10 stimulation for 3 h and 48 h or TNF-α, for 3 h was not significantly different to that of control cells (fig 5.2). As a result, there were no significant differences in the permeability coefficient of the endothelial monolayer
Stimulation of hCMEC/D3 monolayers with 25ng/ml TNF-α for 48 h resulted in a ten fold increase in the permeability coefficient of the hCMEC3 monolayer to FD70 \((P=0.007, n=3)\) (fig 5.3). The TEER values of hCMEC/D3 cells also showed an decrease when the cells were treated with TNF-α for 48 h but not for 3 h, whereas no changes in TEER were observed for CXCL10 at either 3 or 48 h (fig 5.4).

5.3.3. **Effect of CXCL10 on the adhesion of THP1 monocytic cells to hCMEC/D3 monolayers: in vitro adhesion assays and adhesion molecule expression**

In order to determine whether CXCL10 could modulate the adhesion of leukocytes to endothelial cells by direct activation of the endothelial cells, hCMEC/D3 were stimulated with CXCL10 for 3 h, 24 h and 48 h, prior to addition of THP1 cells and the percentage of cells adhering to the hCMEC/D3 monolayer was counted. As a positive control, the cells were stimulated for 48 h with TNF-α (25ng/ml) in the same way. The percentage of adherent THP1 cells to unstimulated hCMEC/D3 monolayer was 5.98±0.99% \((n=3)\). CXCL10 did not increase the adhesion of THP1 cells to hCMEC/D3 monolayers following 3, 24 and 48 h treatment, whereas 150% greater THP1 adherence to the TNF-α stimulated hCMEC/D3 monolayer was observed \((P=0.007, n=3)\) (fig 5.5).

Expression of adhesion molecules on hCMEC/D3 cells were measured by FACS analysis after 3 h and 48 h stimulation with CXCL10 (100ng/ml). In direct relation to the adhesion assay results, ICAM-1, VCAM-1 and PECAM-1 were expressed at variable levels by hCMEC/D3 (fig 5.6), and were unaffected by CXCL10 stimulation for 3 h (fig 5.7) or for 48 h (fig 5.8). Taken together, these data suggest that CXCL10-induced adhesion of leukocytes to the endothelium is not a result of the direct activation of CXCR3 by CXCL10 on endothelial cells.
5.3.4. **CXCL10-induced chemokine production by hCMEC/D3 cells**

Release of CXCL8, CCL2 and CCL5 into the culture medium in response to CXCL10 (100ng/ml) stimulation for 48 h was quantified by sandwich ELISA. Basal production of CXCL8 and CCL2 was extremely low, whilst CCL5 was not detected. CXCL10 stimulation did not induce up-regulation of these three chemokines (table 5.1). In conclusion, 100ng/ml CXCL10 did not modulate the production of CXCL8, CCL2 and CCL5 in hCMEC/D3 after 48 h.

<table>
<thead>
<tr>
<th>Chemokines</th>
<th>Basal chemokine production (ng/ml)</th>
<th>CXCL10 stimulated (100ng/ml for 48 h) chemokine production (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL8</td>
<td>0.17 ± 0.07</td>
<td>0.23 ± 0.1</td>
</tr>
<tr>
<td>CCL2</td>
<td>0.39 ± 0.06</td>
<td>0.31 ± 0.03</td>
</tr>
<tr>
<td>CCL5</td>
<td>Under limit detection (60pg/ml)</td>
<td>Under limit of detection (60pg/ml)</td>
</tr>
</tbody>
</table>

Table 5.2: CCL2, CCL5 and CXCL8 production at basal levels and in response to CXCL10 stimulation (100ng/ml) for 48 h on hCMEC/D3 monolayer.
Fig 5.1: Time course of p42/44 MAP kinase, p38 MAP kinase and JNK phosphorylation induced by CXCL10 in hCMEC/D3 cells. Confluent hCMEC/D3 cells were stimulated with 100 ng/ml CXCL10 for 1, 5, 15, 30 and 60 min or unstimulated. For all phosphorylated proteins the nitrocellulose filters were dehybridised and reprobed with an antibody recognising the non-phosphorylated form of the protein to ensure equal loading. The Western blots shown here are representative of 2 (for p42/44 MAP kinase and p38 MAP kinase) and show one for SAPK/JNK.
Fig 5.2: Clearance of FD70 through hCMEC/D3 monolayers stimulated with CXCL10 or TNF-α.

hCMEC/D3 cells were grown on Transwell filters until confluence. (A) Cells were stimulated with 100 ng/ml CXCL10 for 3 h (pink line) or 48 h (green line) before the permeability to FITC-dextran was determined. (B) Cells were stimulated with 25 ng/ml of TNF-α for 3h (pink line) or 48 h (green line) before the permeability to FD70 was determined. The cleared volume was calculated every five minutes for 30 minutes and was plotted against time to obtain clearance of FITC-dextran through the endothelial monolayer. Data are the means of duplicates from one representative experiment.

Fig 5.3: Permeability coefficient of hCMEC/D3 monolayers to FD70 stimulated with CXCL10 or TNF-α.

hCMEC/D3 cells were grown on Transwell filters until confluence. Cells were stimulated with 100 ng/ml CXCL10 or 25ng/ml of TNF-α for 3 h or 48 h before the permeability to FD70 was determined. Significance was tested on normalised values by ANOVA with a post hoc t-test (*=P<0.05). Results presented are the mean ± S.E.M. of three different experiments with duplicate wells.
**Fig 5.4: Effect of CXCL10 or TNF-α on the TEER of hCMEC/D3 monolayers.**

hCMEC/D3 cells were grown on Transwell filters until confluence. Cells were stimulated with 100 ng/ml CXCL10 or 25ng/ml of TNF-α for 3 h or 48 h before the TEER was determined. Results presented are the means of two different experiments with duplicate wells.
Fig 5.5: Effect of CXCL10 stimulation of hCMEC/D3 monolayers on the adhesion of ThP1 cells.
hCMEC/D3 cells were grown on 96 well plates until confluence. hCMEC/D3 cells were stimulated with CXCL10 (100 ng/ml) for 3, 24 or 48 h, or TNF-α (25ng/ml) for 48 h and the adhesion of 51Cr labelled ThP1 was determined. Significance was tested by ANOVA with a post hoc t-test (*=P<0.05). Results are from one representative experiment of three ± S.E.M. of triplicate wells.
Fig 5.6: Basal and CXCL10-induced ICAM-1, VCAM-1 and PECAM-1 expression by hCMEC/D3 cells by FACScan analysis.
hCMEC/D3 cells were fixed with 4% PAF, permeabilised with TritonX-100, labelled with antibodies specific for ICAM-1, VCAM-1 and PECAM-1, and analysed by FACScan. Representative FACScan histograms show the expression of unstimulated hCMEC/D3 cells (black line) and hCMEC/D3 cells stimulated (grey line) with 100 ng/ml CXCL10 for 3 h (left) or 48 h (right). Cells not labelled with primary antibodies served as negative controls and are represented as grey filled histograms. The histograms are representative of experiments repeated three times.
**Fig 5.7:** Basal and CXCL10-induced ICAM-1, VCAM-1 and PECAM-1 expression by hCMEC/D3 cells by FACScan analysis.

Results are expressed as the median fluorescence for ICAM-1, VCAM-1 and PECAM-1 expression by unstimulated hCMEC/D3 cells and hCMEC/D3 cells stimulated with 100 ng/ml CXCL10 for 3 h. Significance was tested on normalised values by ANOVA with a post hoc t-test (*=P<0.05). Results presented are the mean ± S.E.M. of three different experiments.

**Fig 5.8:** Basal and CXCL10-induced ICAM-1, VCAM-1 and PECAM-1 expression by hCMEC/D3 cells by FACScan analysis.

Results are expressed as the median fluorescence for ICAM-1, VCAM-1 and PECAM-1 expression by unstimulated hCMEC/D3 cells and hCMEC/D3 cells stimulated with 100 ng/ml CXCL10 for 48 h. Significance was tested on normalised values by ANOVA with a post hoc t-test (*=P<0.05). Results presented are the mean ± S.E.M. of three different experiments.
5.4 Discussion

In this chapter, the role of CXCL10, one of the ligands of CXCR3 that is highly expressed by both primary HBEC and hCMEC/D3 cells was investigated. CXCL10 facilitates the infiltration of leukocytes into the brain by acting on the permeability properties and the immunological properties of the endothelial cells. Brain endothelial cells, the main component of the BBB, form a physical barrier protecting the brain and restricting the infiltration of immune cells. Furthermore, they express lower levels of adhesion molecules, such as VCAM-1 at resting levels (dos Santos et al., 1996), therefore, we hypothesized that CXCL10, present in the MS brain at high levels, would modulate the immunological and permeability characteristics of the brain endothelium.

CXCR3 was first confirmed to be an active receptor on hCMEC/D3 since phosphorylation of three different MAP kinase pathways; p42/44 MAP kinase, p38 MAP kinase and SAPK/JNK, at different time points, was observed following stimulation with CXCL10 when stimulated with CXCL10. As p38 and/or p42/44 MAP kinase and/or SAPK/JNK are involved in physiological responses of endothelial cells that have been shown to promote the infiltration of leukocytes such as release of chemokines (Hashimoto et al., 1999; Marin et al., 2001), adhesion molecules expression (Pietersma et al., 1997; De Cesaris et al., 1999) and increase in permeability (Nwariaku et al., 2003), changes of permeability, chemokine release or adhesion molecule expression by hCMEC/D3 in response to CXCL10 was investigated.

CXCL10, at a concentration of 100ng/ml had no effect on the permeability to FD70 of an hCMEC/D3 monolayer, and did not induce any changes in the TEER, which could imply that the opening of the BBB in MS is not directly correlated with CXCL10 expression in MS lesion. In contrast, TNF-α, which has previously been reported to affect the integrity
of the BBB by translocation of junctional proteins (Ozaki et al., 1999), is here confirmed to increase the permeability and decrease TEER of hCMEC/D3 monolayer at a concentration of 25ng/ml for 48 h.

hCMEC/D3 adhesive properties toward THP1 cells also remained unchanged in response to CXCL10 treatment, and the level of adhesion molecules, ICAM-1, VCAM-1 and PECAM-1 was not altered. Furthermore, there was no apparent up-regulation, induced by CXCL10, of the release of three different chemokines that can be expressed by HBEC as determined previously (section 2.3.4).

5.4.1. **Signal transduction pathways induced by CXCL10 stimulation in hCMEC/D3 cells**

Stimulation of hCMEC/D3 cells with CXCL10 at 100ng/ml induced phosphorylation of 3 different types of MAP kinases, p42/44 MAP kinase, p38 MAP kinase and SAPK/JNK. The activation of these kinases was short lived; about 10 min for p38 MAP kinase and SAPK/JNK, and a longer activation for p42/44 MAP kinase. This suggests that CXCR3, the only receptor for CXCL10 which is expressed on hCMEC/D3 cells, is coupled to a signalling pathway and may induce a physiological response. In these cells, the p42/44 MAP kinase phosphorylation response to CXCL10 has already been described in other cell types such as pericytes (Bonacchi et al., 2001) starting 15 min after stimulation and is maximal at 60 min, in astrocytes (personal communication, S. Maru) with the same pattern of stimulation as the one observed with hCMEC/D3, as well as in neurons after 15 min stimulation (Xia et al., 2000). p42/44 MAP kinase activation is generally associated with proliferation, but also regulates cell differentiation, migration and survival, amongst other processes, as reviewed by Ebisuya et al. (2005). Cell proliferation in response to CXCL10 has been reported in several cell types such as pericytes (Bonacchi et al., 2001) and astrocytes (personal communication, S. Maru) however not for endothelial cells, on which
it could exert an angiostatic effect and to inhibit the angiogenic effect of growth factors such as bovine FGF (Angiolillo et al., 1995). It would have been interesting to confirm this effect on hCMEC/D3. In fact, the duration and magnitude of the p42/44 MAP kinase activity has been suggested to regulate physiological responses. For example, a strong activation of p42/44 MAP kinase activity causes cell-cycle arrest (Roovers and Assoian, 2000), as it stimulates the cyclin dependent kinase inhibitor p21, causing a decrease in the activity of cyclin dependent kinase, and in turn, the arrest of the cell in G1 of the cell cycle (Sewing et al., 1997). The transient activation of p42/44 MAP kinase in brain endothelial cells might be too small to induce any proliferative responses. However added to VEGF, which has been already shown to induce a strong p42/44 MAP kinase activation (Wong and Jin, 2005), CXCL10 might enhance the level of phosphorylation of p42/44 MAP kinase enough to stimulate the cyclin dependent kinase inhibitor p21, hence inhibiting the VEGF proliferative effect.

p38 MAP kinase and SAPK/JNK phosphorylation in response to CXC10 stimulation has not been reported before. The p38 MAPK signalling pathway plays an important role in inflammation and other physiological processes. The p38 MAP kinase pathway is activated in response to TNF-α stimulation of endothelial cells (Hashimoto et al., 1999). In the case of TNF-α, p38 MAP kinase activation leads to an increase in the permeability of HUVEC monolayer via formation of stress fibres (Kiemer et al., 2002), causes the up-regulation of CXCL8 (Hashimoto et al., 1999), and increases the surface expression of VCAM-1 but not ICAM-1 (Pietersma et al., 1997). Inhibitors of p38 MAP kinase such as the diaryl imidazoles (for example SB203580 or SB202190) were proven to be anti-inflammatory in animal models such as those for rheumatoid arthritis (Kumar et al., 2003). A major function of the p38 MAP kinase pathway is post-transcriptional control of inflammatory gene expression. Many of the mRNAs are unstable (or untranslatable) because of AU-rich elements in the 3'untranslated region. Signalling in the p38 pathway counteracts these and
stabilizes the mRNAs by preventing their otherwise rapid de-adenylation, as is the case for CXCL8 (Rot et al., 1996). Nevertheless, p38 MAP kinase phosphorylation, in response to CXCL10, does not induce the same response in endothelial cells, as we have demonstrated that there were no changes in permeability, CXCL8 or VCAM-1 expression. p38 MAP kinase activation has been implicated in other cell functions such as cell differentiation, in senescence, in apoptosis and in G1 and G2/M phases of the cell cycle, as reviewed by Zarubin and Han (2005). CXCL10 could play a role in some of these functions in endothelial cells, and this would need to be further investigated.

5.4.2. Effect of CXCL10 on the permeability of hCMEC/D3 monolayers

Permeability assays of hCMEC/D3 cells, grown on filters, to FD70 demonstrated no effect of CXCL10. IFN-γ has been shown to induce a decrease in TEER and increase in permeability of non-brain endothelial cell monolayers such as HUVEC (Huynh and Dorovini-Zis, 1993; Dewi et al., 2004) with a time lag suggesting that the IFN-γ effect is not direct but dependant on an additional inflammatory mediator affecting endothelial permeability. Some of these factors have been identified, such as NO or MMP (Lou et al., 1999; Wong et al., 2004). IFN-γ also induces the production of CXCL10, and both primary HBEC and hCMEC/D3 cells express high levels of CXCR3, the unique receptor for CXCL10. In this study, CXCL10 did not appear to have any effect on the permeability of hCMEC/D3 cells. CCL2, another chemokine, has been showed to increase the permeability of mouse brain microvascular endothelial cells rapidly within 2 h (Stamatovic et al., 2005). The increase in permeability is suggested to be due to the delocalisation of tight junction proteins from the membrane into the cytoplasm, and the formation of stress fibres (Stamatovic et al., 2003). After 20 h, CCL2 also decreases the expression of both occludin and ZO-1 in mouse brain endothelial cells (Song and Pachter, 2004). The increase in permeability of endothelial cell monolayers in response to CCL2 has been associated with its angiogenic properties. CXCL10 is an angiostatic chemokine, inhibiting proliferation of
HUVEC \textit{in vitro} (Luster \textit{et al}., 1995). However the angiostatic effect of CXCL10 was not observed by all (Angiolillo \textit{et al}., 1995)(personal communication, P. Hillyer). The angiogenic properties of chemokines such as CCL2 and CXCL8 include the capacity of the endothelial cells to detach from their actual location, proliferate and migrate into the tissue to form new vessels. CCL2 and CXCL8 regulate production of proteins that affect permeability, such as MMP-1 (Galvez \textit{et al}., 2005). Other chemokines such as CCL2, CCL3, and CX3CL1 were also shown to increase MMP-9 secretion by primary isolated rat brain microglia \textit{in vitro} (Cross and Woodroofe, 1999). In summary, CXCL10 alone does not appear to induce an increase in permeability of brain endothelial cell monolayers. This effect may be cell specific as ligand stimulation of CXCR3 in multiple myeloma cell lines increases MMP-2 and MMP-9 activity in the culture medium, but not in bone marrow plasma cells (Pellegrino \textit{et al}., 2004). It is possible that CXCL10 acts on the circulating leukocytes expressing CXCR3, which can, once activated by CXCL10 or any other ligand of CXCR3, induce the opening of the BBB. Indeed, it has been demonstrated that the binding of reactive leukocytes increases the permeability in non-brain endothelial cell monolayers (Damle and Doyle, 1990).

Furthermore, CXCL10 is not able to induce significant transendothelial migration of unstimulated T lymphocytes in contrast to other chemokines such as CCL5 or CCL3 (Roth \textit{et al}., 1995). This would further suggest the lack of a direct effect of CXCL10 on the endothelial cells. Only FITC-dextran of a molecular size of 70kDa was tested but hantavirus infection of LMEC, which induces production of CXCL10, failed to increase the permeability of the LMEC monolayer to FITC-dextran of 3, 10, 40 and 70 kDa (Sundstrom \textit{et al}., 2001). If the response of hCMEC/D3 to CXCL10 is similar to LMEC, it is possible that CXCL10 has no effect on paracellular permeability. Nevertheless, the permeability of endothelial cells can be regulated at many different levels, such as the transcellular activity, which is not reflected using FITC-dextran, and could also be
modified with CXCL10. Before reaching a conclusion it would be important to investigate whether CXCL10 induces any changes in pinocytic activity.

5.4.3. Adhesion of THP1 cells to hCMEC/D3 monolayers in response to CXCL10 stimulation

The percentage of adhesion of THP1 cells to hCMEC/D3 monolayers at basal levels is 6% after 1 h and is increased three-fold by TNF-α treatment of the endothelial cells. In order to avoid a masking effect of the serum in the medium, endothelial and THP1 cells were in medium both serum and growth factor free medium at the time of the stimulation, and at the time of the experiment. This could explain the low percentage of adhesion obtained compared to others who obtained an adhesion of 28% of monocytes to HUVEC (Zhang and Issekutz, 2002) and 28% activated lymphocytes have been shown to adhere to HUVEC (Taub et al., 1993), also this is not directly comparable with the results of this study as monocytes were used, and activated lymphocytes could show different adhesion properties to hCMEC/D3 monolayer. Other studies have also reported a low 5% adhesion of another monocyte cell line, the U937, onto endothelial cells (Cavender et al., 1991). Serum is known to contain factors that promote the expression of adhesion molecules on endothelial cells (Lozada et al., 1995) but this effect is not apparent with heat-inactivated serum. Nevertheless, the absence of serum in the medium did not affect the response of the endothelial cells, as the adhesion of THP1 to TNF-α treated hCMEC/D3 increased compared to unstimulated monolayers, as has been previously reported (Che et al., 2002).

Another factor is the low expression of VCAM-1 on the brain endothelial cells compared to the other vascular endothelial cells (dos Santos et al., 1996), and also monocyte adhesion is dependent on VCAM-1. Indeed Floris et al. (2002) demonstrated that blocking VCAM-1 effectively blocked the adhesion of monocytes, and conversely, that the transfection of HUVEC with VCAM-1 could enhance the adhesion of monocytes by 100% (Gerszten et al., 1998). CXCL10 did not upregulate VCAM-1 expression on brain
endothelial cells which adds further evidence for the absence of a direct modulating effect of CXCL10 on the adhesive properties of brain endothelial cells.

Subcutaneous administration of human CXCL10 in immune deficient mice, and the intraperitoneal injection of human peripheral blood lymphocytes were used to demonstrate the recruitment of monocytes and lymphocytes in response to CXCL10 in vivo (Taub et al., 1996). There is an important physiological component, as the migration response towards chemokines differs in vitro and in vivo. T lymphocytes migrated towards CXCR3 ligands in vitro but showed little chemotaxis in response to CCR5 ligands, such as CCL5 (Stanford and Issekutz, 2003). In vivo, using subcutaneous chemokine injections, lymphocytes were recruited in large numbers in response to CXCL10 and CCL5 but to a lesser extent to CXCL9 and CXCL11 (Stanford and Issekutz, 2003). In addition, in vitro data showed that CXCL10 induced migration of monocytes and activated lymphocytes (Taub et al., 1993) and that anti-CXCL10 antibodies partly blocked the transendothelial chemotactic migration of IL-2 activated peripheral blood lymphocytes across an IFN-γ stimulated HUVEC monolayer (Salmaggi et al., 2002). In the present study, CXCL10 prestimulation of endothelial cells at different time points had no effect on the adhesion of THP1 on the hCMEC/D3. This suggests that the increased adhesion of monocytes to endothelial cells in the presence of CXCL10 originates from the activation of monocytes only. CXCL10 has been shown to induce T cell adhesion to purified ICAM-1 and VCAM-1 via an increase in the β-1 and β-2 integrins avidity (Lloyd et al., 1996), and in the case of migration assays this might be sufficient to increase leukocyte infiltration. Indeed, this increase in integrin avidity would mean an increase in ICAM-1 and VCAM-1 cross linking on the endothelial cells, which has been shown to result in cytoskeleton changes (Adamson et al., 1999), and an increase in permeability in the case of VCAM-1 cross-linking (van Buul et al., 2002), thereby facilitating the transmigration of the leukocytes across the barrier formed by the closely apposed brain endothelial cells.
In vivo, endothelial cells present the chemokines, bound onto the proteoglycans, to the passing leukocytes. There is no increase of adhesion of THP1 even after 3 h of CXCL10, which suggests that the chemokines are not retained at the surface of the endothelial cells in this period of time. This is in agreement with the results of Hillyer and Male (2005) showing that chemokines such as CCL5 and CCL3, bound to the surface of the endothelium, are removed within 150 min, and furthermore, that CXCL10 binding is low and only present on DMVEC and LMVEC, while non existent on HUVEC. The study by Luster et al. (1995) is the only report describing binding of CXCL10 on proteoglycans of endothelial cells. It would have been interesting to include an earlier time point to determine whether the bound CXCL10 on the surface of the endothelial cells would have had an effect on adhesion of THP1, and whether the bound CXCL10 would be sufficient to modify the adhesion of monocytic cells to the endothelium.

5.4.4. Regulation of chemokines production by CXCL10 in hCMEC/D3 cells

The leukocyte infiltrate in MS lesions is composed mainly of T cells and monocytes. No neutrophils are reported to be present. Moreover, cytokines up-regulated in MS, such as TNF-α or IL-1-α, trigger the production of CXCL8 in endothelial cells (Kaplanski et al., 1994; Kaplanski, 1995) (section 2.3.4). CXCL8 is a chemoattractant for neutrophils. Furthermore, there is a temporal variation in the type of infiltrating cells. The hypothesis tested here is that one chemokine such as CXCL10 which attracts activated lymphocytes (cells that are present in the early stage of the lesions) could in turn induce the secretion of other chemokines such as CCL2 or CCL5 in endothelial cells, both monocyte chemoattractants. In this part of the study, CXCL10 was investigated for its possible role as a modulator for the production of other chemokines. The regulatory effect of one chemokine on another has been previously demonstrated in murine astrocytes, since, when stimulated with CCL5, they can induce the production of CCL2 (Luo et al., 2002). CCL5
as well as CCL11, up-regulates the transcript expression of the CXCL8 analogue in mice, as well as CCL2, CCL3 and the cytokine TNF-α (Luo et al., 2002). There was no effect of CXCL10 on the production of CXCL8, CCL2 or CCL5 by hCMEC/D3 cells. It is possible that a regulatory loop exits between chemokines and cytokines. For instance, cells incubated in the presence of CCL3 showed enhanced IFN-γ production, (Karpus et al., 1997) and CCL2 enhanced production of IL-4 (Lukacs et al., 1997). In astrocytes a positive feedback seems to occur between CCL5 and TNF-α, which in turn up-regulates CCL2 expression. Indeed, when murine astrocytes are stimulated with CCL5, TNF-α is up-regulated after 12 h while CCL2 up-regulation occurs at 25 h (Luo et al., 2002). This effect in endothelial cells is unlikely to be via INF-γ, which is the principal stimulatory cytokine for CXCL10, as INF-γ is not expressed by endothelial cells under basal conditions or when stimulated with cytokines such as TNF-α or IL-1β (Nilsen et al., 1998). Nevertheless, in hCMEC/D3 cells, in contrast to HBEC, TNF-α also induced CXCL10 production. But in hCMEC/D3 cells, CXCL10 does not appear to have any feedback action on TNF-α expression in endothelial cells as CXCL8 and CCL2, which are up-regulated by TNF-α, were unchanged by CXCL10 stimulation at the time of 48 h. Also this would need to be confirmed by measuring TNF-α, as the release of the cytokine could be insufficient to induce CCL2 and CXCL8 release in the supernatant, or that the time point we should have looked at is different from 48 h.

5.4.5. Conclusion

In this study, CXCL10 was demonstrated to play no major role in regulating some properties of the brain endothelial cells, such as in the permeability and adhesion properties. There is no inter-regulation of CXCL10 on CXCL8, CCL2 and CCL5 chemokines. CXCL10 stimulation of hCMEC/D3 cells induces the activation of specific signalling pathways suggesting that CXCR3 is a functional receptor. It is possible that the activation
of these pathways is correlated with its already known angiostatic properties on endothelium and it would have been interesting to investigate this hypothesis.
6. Chapter 6: General discussion
The aim of this study was to investigate the expression of chemokines and chemokine receptors on HBEC. Because of the limited number of endothelial cells obtained from brain tissue, a method of isolation for highly pure HBEC was set up and an immortalised human brain endothelial cell line was developed. After having established that CXCL10 was produced at high levels in response to cytokine stimulation and that its unique receptor, CXCR3, was expressed by HBEC, both in vitro and in vivo, the effect of CXCL10 on HBEC and its consequences on leukocyte infiltration was investigated.

A method to isolate purer primary rat brain endothelial cells (Perriere et al., 2005), relying on the expression of efflux pumps, was adapted to isolate HBEC. Using this method, sufficient HBEC were obtained, with a low number, if any, of other cell types, that under normal conditions outgrow HBEC, as a result of their lower proliferative rate. Nevertheless, the use of puromycin could contribute to a change in protein expression. For example, cells treated with puromycin showed an increase in P-gp expression (Perriere et al., 2005) which is already an artefact of the culture system, as P-gp expression on endothelial cells in vitro is lower than P-gp expression on freshly isolated vessels whilst multidrug resistance protein is increased in vitro (Seetharaman et al., 1998). This is an important factor to take into account when this method of isolation is used especially to study drug transport using an in vitro BBB model when compared to other isolation methods. The first investigations in this thesis were on cells obtained that had been untreated with puromycin and no differences were reported for chemokine production in response to cytokines, so in this study, a method of isolation using puromycin was appropriate. Another consequence of using puromycin is the lack of heterogeneity in the HBEC. Indeed, it has been shown that there are different phenotypes of endothelial cells within the same cell culture (Rupnick et al., 1988), some being a cobblestone shape and others were fusiform that could reflect the presence of cells from different vascular bed such as arteriolar or venular endothelial cells. These two types of cells could be found in our cell cultures with a predominance of the
fusiform shaped cells. When using a marker such as PECAM-1 to select the endothelial cells during the isolation procedure, the cells appeared more homogeneous (personal communication, A. Mabondzo). The significance of the appearance of different cell types in culture would need to be determined, in order to determine whether these cells are from different parts of the cerebral vasculature. HBEC heterogeneity could also explain the differences observed in CXCL8 secretion at basal levels.

Four chemokines, CCL2, CCL5, CXCL8 and CXCL10, and their receptors were studied for their implication in MS pathology. These chemokines were all expressed by primary HBEC when stimulated with TNF-α, with the exception of CXCL10 which was induced by IFN-γ. The specificity of CXCL10 induction by IFN-γ and not TNF-α confirmed the absence or the extremely low level of contaminating astrocytes in the culture as astrocytes produce CXCL10 in response to TNF-α (Oh et al., 1999). Another interesting finding with respect to chemokine production is their regulation by cytokines. TGF-β is an anti-inflammatory cytokine, but could up-regulate certain chemokines when used in combination with IFN-γ or TNF-α. IFN-γ, generally referred to as a pro-inflammatory cytokine, decreases the level of CXCL8 release by the endothelial cells in response to TNF-α. The role of TNF-α in MS is controversial. While it has been established that in EAE, the animal model used for MS, TNF-α suppression is beneficial (Glabinski et al., 2004), this has not been the case for MS (Skurkovich et al., 2001). The lack of neutrophil infiltration in MS, despite the increase of CXCL8 in the CSF of MS patients, also suggests that there is a means of down-regulating the expression of a CXCL8 chemotactic gradient that could be induced by TNF-α. The presence of IFN-γ could explain this phenomenon. IFN-γ does not play an important role in EAE (Ferber et al., 1996), but anti-IFN-γ has a greater effect than anti-TNF-α in treating MS (Skurkovich et al., 2001). However,
complete suppression of IFN-γ could lead to an infiltration of neutrophils as is the case in
knock-out mice or IFN-γ used for EAE model (Tran et al., 2000b).

Immortalisation of HBEC by transduction of a highly efficient lentiviral vector system
containing hTERT and the SV40 large T antigen, resulted in a stable human brain
endothelial cell line, named hCMEC/D3. This cell line was characterized for its properties
of HBEC, as well as for the expression of chemokines and chemokine receptors. Overall,
whether from primary human brain or from the hCMEC/D3, brain endothelial cells
produced significant amounts of the chemokines which are involved in MS in response to
cytokines, also up-regulated in MS. It is interesting that the first cell type which comes into
contact with leukocytes itself produces such high quantities of chemokines, as the classical
view is that CNS resident cells, such as astrocytes and microglia produce chemokines that
are transported across the endothelial cells to be presented on the luminal side to
leukocytes. The role of chemokines, secreted from endothelial cells, would be interesting
to study. They could participate in early recruitment of certain types of leukocytes. For
instance, T cells are present early on in the lesion in MS (Lucchinetti et al., 2000) which
could be related to the high level of CXCL10 released by the endothelium. More striking is
the case where neutrophils can be recruited within 3 h into the brain and this correlates
with the rapid release of CCL2 and CCL3 (Kim et al., 1995), again highly produced by the
endothelium. Endothelial cells could also contribute to the gradient of chemokines to
attract leukocytes into the brain, as a part of its release is from the basolateral side.
Alternatively, the chemokines released by HBEC could prevent the formation of a gradient
promoting the entry of leukocytes into the brain. Indeed, HBEC grown on filters released
more chemokines on the apical side than on the baso-lateral side. The non-bound
chemokines so released by the endothelium could bind to the leukocyte chemokine
receptors leading the internalisation of the receptor, and hence the disensitivation of the
response of the leukocyte to the chemotactic gradient.
Amongst the four chemokines investigated for their production, CXCL8 and CXCL10 were released in higher quantities compared to CCL2 and CCL5. Interestingly, the receptor for CXCL10, CXCR3, and the receptors for CXCL8, CXCR1 and CXCR2 were highly expressed by the brain endothelial cells. On the other hand, CCL2 and CCL5 release into the culture supernatant of endothelial cells, in response to cytokines, was smaller when compared to the two CXCR chemokines, as were the expression level of the receptors for these two chemokines, either expressed at a very low level, or absent for CCL2. The correlation between chemokine production and chemokine receptor expression could indicate the possibility of an autocrine action of the chemokines on the endothelial cells or it could participate in the regulatory mechanism of chemokine production. Chemokine receptors are quickly internalised upon chemokine binding. This mechanism maintains the gradient of chemokines and limits the action of the chemokines. It is in general the DARC receptor that is responsible for this function, but CXCL10 does not bind to this receptor. The organism must hence have developed other mechanisms to degrade chemokines such as CXCL10. A known mechanism of chemokine degradation and regulation are by the MMPs (Van den Steen et al., 2003 a,b). Another possibility is that the receptor present on the endothelial cells could fulfill this function. The high presence of the receptor in the cytoplasm in situ could suggest that this could be the case, as a high turnover of the receptor would be observed if the chemokines were present (Venkatesan et al., 2003). Furthermore, association of these chemokines with endothelial cells has also been observed in vivo (personal communication, P. Rezaie). Moreover, primary data suggests that the internalisation of CXCL10 by non-brain endothelial cells is not CXCR3-mediated (personal communication, D. Male).

hCMEC/D3 will prove to be a useful cell line and a more appropriate model to study the BBB in vitro compared to HUVEC, than most studies used in the past. hCMEC/D3 has the
advantage of retaining a number of phenotype characteristics intrinsic to HBEC, such as the expression of tight junction proteins and a low permeability to macromolecules above 4 kDa, as well as the ability to respond to BBB-promoting factors such as ACM and high intracellular cAMP levels. Nevertheless, it does not represent a perfect BBB model as not all of the junctional proteins are located at the cell-cell junction in cells forming a confluent monolayer as they are in primary HBEC. Furthermore, the chemokine expression pattern is overall similar, but presents small differences. Indeed, CXCL10, which is not induced by TNF-α in primary HBEC, is released by hCMEC/D3, when the cells are stimulated in the same conditions. This is interesting as most peripheral endothelia release CXCL10 in response to TNF-α (Hillyer et al., 2003). It suggests a regulation in response to TNF-α in primary HBEC, which is different in hCMEC/D3 cells. This could suggest a negative regulation in response to TNF-α stimulation in the primary HBEC, and hCMEC/D3 cells as a result of immortalisation, have lost this characteristic or a positive regulatory mechanism acquired by immortalisation. They could provide a useful tool to study the fine regulation that differentiates brain endothelial cells from peripheral endothelial cells in their chemokine production. CCL2 and CXCL8, which were released by primary HBEC in vitro at basal levels, were found only to be expressed by hCMEC/D3 at extremely low level, outside the range of a physiologically significant amount. The pattern of expression was similar between primary HBEC and hCMEC/D3 for CCR1-5 and CXCR1-3 but the absolute level of expression could not be compare because the technique of immunocytochemistry used was not quantitative.

Chemokines are important in directing the leukocytes toward the site of inflammation infiltrating across the vascular bed into the tissue. In the case of brain inflammation, the immune cells have to cross the barrier which is formed by the brain endothelium. One of the aims of this study was to determine whether chemokines could exert an effect on the BBB to facilitate the infiltration of the leukocytes into the brain, in addition to their effect
on leukocytes. CXCL10, amongst the chemokines produced by the brain endothelium was selected, as it has been consistently found to be up-regulated in the CSF and in MS lesions (Simpson et al., 2000b; Mahad et al., 2002). No effect of CXCL10 could be detected on either the permeability or the adhesive properties of the brain endothelial cells in vitro. As in vivo injection of CXCL10 is sufficient to induce infiltration of leukocytes, this could suggest that the role of endothelial cells is secondary to the infiltration of leukocytes and that activation of leukocytes is sufficient for the transmigration of the cells. Alternatively it could suggest that there is a complex response to CXCL10 in vivo. Other cells could respond to CXCL10, releasing factors that would trigger the activation of the endothelium. In vivo, there are also a number of chemokines present such as CCL2 and CCL5, which could have synergistic effects whereas stimulation with one chemokine alone would not be sufficient. The relevance of an effect of CXCL10 on the BBB in vivo could be questionable as it is generally concomitant with upregulation of IFN-γ. IFN-γ may itself lead to the opening of the BBB (Annunziata et al., 2002) and to endothelial activation such as the up-regulation of its adhesive properties (Annunziata et al., 2002).

6.1 Future studies

As part of future work, the function of CXCR3 on HBEC still remains to be elucidated. CXCR3 is known as an angiostatic chemokine although this needs to be confirmed on hCMEC/D3 cells. In order to determine whether CXCL10 can act on brain endothelial cells to promote leukocyte infiltration, it would be interesting to investigate whether intrathecal injections of CXCL10 in a dose similar to that released by the HBEC (around 200ng/ml) into the brain of rat or mice could trigger the infiltration of the leukocytes into the brain. This experiment could answer the question of whether the brain environment could suppress leukocyte infiltration that has been observed in peripheral tissue in response to CXCL10 injection. Furthermore, investigating markers for inflammatory activation (adhesion molecules or MHC class I) or the opening of the BBB (trypan blue or junction
protein expression) would allow investigation as to whether CXCL10 could act directly on the endothelium to promote leukocytes infiltration. It could be argued that leukocytes could get activated in response to CXCL10 and in return activate the endothelium in a chain of events, so that the effect of CXCL10 on endothelial cells would be secondary to the leukocyte activation by CXCL10. Severe combined immunodeficient mice would provide a useful control to ensure that CXCL10 exerts a direct effect on the endothelial cells. If intrathecal injection of CXCL10 into these mice induces the same changes on the endothelium in severe combined immunodeficient mice as in normal mice, this could confirm that CXCL10 acts via CXCR3 on the endothelial cells to promote leukocyte infiltration. By contrast, if there is a difference in the level of activation of the endothelium, that would demonstrate that the activation of the endothelium in response to CXCL10 is secondary to leukocyte activation.

The importance and role of the chemokines released by different cells within the CNS would also be an important and interesting question to answer. Recent advances in mice engineering have allowed production of mice in which a specific gene could either be blocked or induced within one cell type using a specific marker such as GFAP for astrocytes. Using a brain endothelial marker and blocking the production of CXCL10 in an EAE model could allow determination of whether chemokines released from the endothelial cells promote or inhibit leukocyte extravasation into the brain. This could also be important for designing future therapy targeting chemokine production, as when the chemokines from CNS cells such as astrocytes or microglia determine the migration of leukocytes, new drugs should then be able to cross the BBB.

Opening of the BBB and increased chemokine production is a characteristic feature of EAE and MS. cAMP can act on both these characteristics feature by decreasing the permeability of the endothelium \textit{in vivo} and by decreasing the release of CXCL10, one of
the chemokines that is a major chemoattractant for activated T lymphocytes. The effect of an inhibitor of phophodiesterase, preventing the degradation of cAMP, such as RO 20-1724, in EAE could be interesting to study as a therapeutic agent.

6.2 Final conclusion

The aim of this study was to identify chemokines and chemokine receptors expressed by HBEC in vitro, and correlate those with the chemokines and chemokine receptors found to be up-regulated in MS. CXCL8 and CXCL10 were highly produced by HBEC when stimulated by cytokines. Interestingly, CXCR1 and CXCR3, the receptor for CXCL8 and CXCL10 respectively, were both found to be highly expressed by HBEC in vitro, and the expression of these receptors were confirm in situ by immunogold and electron microscopy. Also there were no changes in CXCR1 level of expression in the lesions when compared to NAWM and a tendency to decrease for CXCR3. In order to determine whether CXCR3 activation on HBEC could promote leukocyte migration by an increase in permeability or a change in adhesion molecules, hCMEC/D3 cells, an immortalized human brain endothelial cell line, were stimulated by CXCL10. In vitro stimulation of CXCR3 by CXCL10 did not appear to have an effect or the permeability of an endothelial cell monolayer, nor on the adhesion property of the endothelium to monocytes, suggesting that CXCL10 plays little role at the level of the BBB in leukocyte extravasation. However, further work needs to be undertaken.


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