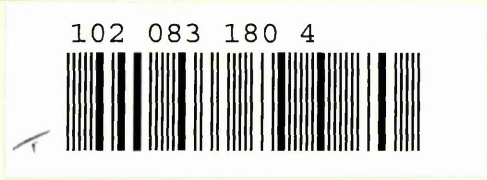

The Role of ADAMTS-1, -4 and -5 in Multiple Sclerosis

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Dedication

This thesis is dedicated

To

The soul of my father

Abstract

ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs)-1, -4 and -5 are secreted enzymes which are members of the glutamyl endopeptidases (GEPs) group of ADAMTSs. These enzymes break down chondroitin sulphate proteoglycans (CSPGs) which are key components of brain extracellular matrix (ECM). In multiple sclerosis (MS), CSPG breakdown by ADAMTSs may enable axonal regeneration or conversely it may lead to alterations of the ECM, allowing influx of inflammatory cells promoting tissue damage.

ADAMTS-1, -4 and -5 mRNA expression was studied by quantitative real-time PCR (qRT-PCR) using the Taqman method in SHSY-5Y and SK-N-DZ human neuroblastoma cells, undifferentiated or differentiated to a more neuronal phenotype using retinoic acid (RetA). Modulation by pro-inflammatory cytokines ((interleukin-1 IL-1) or tumour necrosis factor (TNF)), which are involved in the pathogenesis of MS, was also studied. As ADAMTS-1 was the most abundant ADAMTS in the neuronal cell lines, it was investigated at its protein level in both cell lines by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) with western blotting. Furthermore, the presence of ADAMTS-1 at its mRNA and protein levels was confirmed by the small interfering RNA (siRNA) technique in SHSY-5Y cells. Cryostat sections of normal and MS central nervous system (CNS) tissue white matter, obtained from the UK Multiple Sclerosis Tissue Bank, were used to determine the localisation of V0/V2 neoepitopes of versican, derived by ADAMTS cleavage, using immunohistochemistry.

SHSY-5Y and SK-N-DZ cells expressed mRNA for ADAMTS-1, -4 and -5. ADAMTS-1 expression was significantly increased on cellular differentiation with RetA in SHSY-5Y cells. Its expression was confirmed at the mRNA and protein level. IL-1β and TNF had no effect on ADAMTS mRNA expression in SHSY-5Y cells. However, ADAMTS-1 mRNA expression was upregulated by IL-1β in differentiated SK-N-DZ and there was also a significant increase in ADAMTS-4 mRNA expression with TNF treatment. ADAMTS-mediated versican breakdown, as determined immuohistochemically by versican (V0/V2) neoepitopes expression, was increased in MS brain tissue compared to normal brain tissue.

In conclusion, ADAMTS-1, -4 and -5 were constitutively expressed in SHSY-5Y and SK-N-DZ neuronal cells. Modulation by the cytokines tested was seen in the SK-N-DZ cells. From these *in vitro* studies, neuronal ADAMTSs in the CNS may have a potential role in MS pathogenesis. However further investigation is needed on primary neuronal cells and CNS to elucidate the role of neuronal ADAMTSs and their contribution in MS.

ADAMTSs do appear to be involved in increased proteolysis of versican at the known cleavage site in human brain tissue as indicated by (V0/V2) versican neoepitopes expression. Upregulation of versican (V0/V2) neoepitopes was observed in lesional MS sections on immunohistochemistry. These enzymes require further investigation, by immunohistochemical methods for co-localisation with versican (V0/V2) neoepitopes in MS, to determine which of the ADAMTSs generates these neoepitopes.

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I wish to express my gratitude to my supervisor, Dr. Rowena Bunning who was abundantly helpful and offered invaluable assistance, support and guidance. Deepest gratitude is also due to the members of the supervisory committee, Dr. Alison Cross, Dr. Gail Haddock and Dr. David Buttle without their knowledge, assistance and insightful criticisms this study would not have been successful.

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I am also extremely grateful to The UK Multiple Sclerosis Tissue Bank for providing me with human brain tissues, utilised in this study.

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Contents

List of Figures

List of Tables

Abbreviations

ADAM	A disintegrin and metalloproteinase
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
APCs	Antigen presenting cells
APP	Amyloid precursor protein
BBB	Blood brain barrier
BCA	Bicinchoninic acid
CAM	Cell adhesion molecule
cDNA	Complementary deoxyribonucleic acid
CESs	Cerebral endothelial cells
CNS	Central nervous system
CS	Chondroitin sulphate
CSPGs	Chondroitin sulphate proteoglycans
CT	Cycle threshold
DAB	Diaminobenzidine
DAMPs	Damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
dH_2O	Distilled water
DMEM	Dulbecco's modified eagle's medium
dNTPs	Deoxynucleotide triphosphates
dsRNA	Double-stranded RNA
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
EtBr	Ethidium bromide
F	Fluorophore
FRET	Forster Resonance Energy Transfer
GAGs	Glycosaminoglycans
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GEP	Glutamyl endopeptidase
GF	Growth factor
GHAP	Glial hyaluronate binding protein
H&E	Haematoxylin and eosin
HIFCS	Heat–inactivated foetal calf serum
HMGB	High-mobility group box
HRP	Horseradish peroxidase
HSP60	Heat shock protein 60

ICC	Immunocytochemistry
IHC	Immunohistochemistry
IL-1α	Interleukin-1 alpha
IL-1β	Intrleukin- 1 beta
IL-2	Interleukin-2
IL-6	Interleukin-6
INFγ	Interferon gamma
IOD	Integrated optical density
LDS	Lithium dodecyl sulphate
LPS	Lipopolysaccharide
LT	Lymphotoxin
M	Monoclonal
MAG	Myelin-associated glycoprotein
MBP	Myelin basic protein
MHC II	Major histocompatability complex class II
MMP	Matrix metalloproteinase
MOG	Myelin oligodendrocyte glycoprotein
MOPs	Morpholino propanesulfonic acid
MW	Molecular weight
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
NAWM	Normal appearing white matter
NC	Normal control
NF L	Neurofilament light
NFDM	Non-fat dried milk
Ng	Nanogram
O/N	Overnight
ORO	Oil red O
P	Polyclonal
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffer saline
PC	Proprotein convertase
PFA	Paraformaldehyde
PGs	Proteoglycans
PP	Primary progressive
PR	Progressive relaping
PRRs	Pattern recognition receptors
PTGS	Post-transcriptional gene silencing
Q	Quencher
qRT-PCR	Quantitative real time polymerase chain reaction
RARs	Retinoic acid receptors
RetA	Retinoic acid
RISC	Ribnucleic acid induced silencing complex

RNAi	Ribonucleic acid interference
RNAP-II	Ribonucleic acid polymerase II
RR	Relapse-remitting
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcription
SBB	Sudan Black B
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SFM	Serum free medium
siRNA	Small interfering RNA
SP	Secondary progressive
SVMPs	Snake venom metalloproteinases
TACE	TNF-alpha converting enzyme
TBE	Tris-boric acid-ethylenediaminetetraacetic acid
TE	Trypsin-ethylenediaminetetraacetic acid
TEP	Triethyl phosphate
TGF	Transforming growth factor
Th	T-helper cell
TIMP	Tissue inhibitor of metalloproteinase
TJ	Tight junctions
TLRs	Toll-like receptors
tMCAo	Transient middle cerebral artery occlusion
TNF	Tumour necrosis factor
TSP	Thrombospondin
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor

Chapter 1

Introduction

1.1 The Central Nervous System (CNS)

The CNS consists of the brain and spinal cord and is a complex well vascularised system that transmits electrical signals i.e. triggering neural functions of communication. From histological studies the nervous system can be divided into two major cell types: nerve cells (neurons) and supporting cells called glia. The function of neurons is electrical signalling and their function differs from glia. Glia do not participate directly in synaptic interactions and electrical signalling, they are supportive for neuronal cells. They also provide essential resources for repair of damaged CNS by promoting re-growth of damaged neurons and enable the CNS environment to be tightly regulated in order to permit uninterrupted and efficient neural chemistry (Purves *et al.*, 2008).

1.1.1 The Blood Brain Barrier (BBB)

The BBB separates vascular components from the brain parenchyma, impeding the entry of detrimental materials. In normal physiological conditions it maintains homeostasis, maintaining essential proteins and ionic balances and allowing the entrance of essential nutrients. It also prevents access by immune cells. However, disruption of the BBB in the CNS may lead to neurological damage. The potential source of damage comes from the immune system, with the entry of activated lymphocytes into the CNS. The immune system is equipped with numerous effector mechanisms and can greatly alter the homeostasis and function of the CNS. Autoimmunity and pathogenic infectious agents can all result in acute or chronic inflammation within the CNS which subsequently leads to demyelination and axonal loss as in multiple sclerosis (Purves *et al.*, 2008).

The BBB is formed by tight adherence of brain endothelial cells lining the cerebral micro-vessels. It is a selective barrier that limits or prevents blood-borne molecules larger than 0.6 kDa from moving into the brain and protects the brain from damage, toxins or other potentially damaging molecules. The brain capillary junctions are ~50–

100 times tighter than peripheral micro-vessels as a result of complex tight junctions (TJs) produced by the interaction of several transmembrane proteins. Transmembrane proteins occludins, are main contributors to the TJs along with claudins and the junctional adhesion molecules. Cytoplasmic proteins e.g. zonula occludens protein-1 and -2 link the transmembrane proteins to the actin cytoskeleton allowing paracellular transport to be modulated in response to different stimuli (Huber *et al.*, 2001, Ballabh *et al.*, 2004). Despite an estimated total surface area of between 10–20 m^2 of capillaries in the human brain, the TJs make the brain practically inaccessible for polar molecules unless they are transferred by transport pathways at the BBB that regulate the microenvironment of the brain (Pardridge *et al.*, 1990).

There are also adherens junctions, which stabilise cell–cell interactions in the junctional zone. Large molecules such as antibodies, lipoproteins, proteins and peptides can also be transferred to the central compartment by receptor-mediated transcytosis or non-specific adsorptive-mediated transcytosis. The receptors for insulin, low-density lipoprotein and iron transferrin are all involved in transcytosis.

The BBB contains a thick basal lamina and astrocytic end-feet surround the embedded endothelial cells in the basal lamina and provide biochemical support to these cells. The perivascular region contains microglia, synaptic terminals, neurons and smooth muscle cells as shown in Figure 1.1 (Abbott *et al.*, 2006). This region has been shown to contract and regulate the CNS vascular diameter and capillary blood flow, BBB endothelial cell differentiation/proliferation and phagocytosis (Rucker *et al.*, 2000).

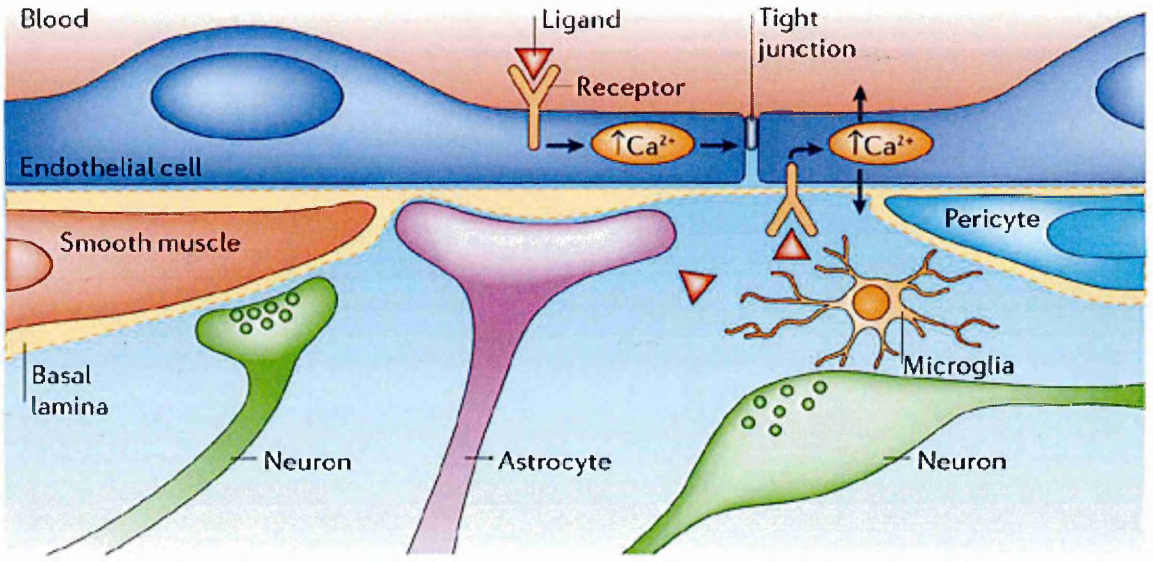


Figure 1.1: Cell to cell interactions within blood brain barrier. Different cell types are present in the blood capillary walls which communicate with each other. Pericytes are associated with endothelium within the endothelial basal lamina. The endfeet of astrocytic glial cells surround the endothelial cells in the basal lamina. In the perivascular space are found microglia, the synaptic terminals and nerve fibres, and smooth muscle cells. The endothelium can release substances to the blood or brain side after receptor activation as indicated by the arrows. Receptors can also increase the intracellular Ca^{2+} concentration (Reproduced with permission from Nature Reviews Neuroscience from Abbott *et al.*, 2007).

1.1.2 Neurons

A typical neuron has four distinct parts; cell body (soma), which is the main part and has all the necessary components of the cell, e.g. nucleus containing the DNA, endoplasmic reticulum (ER), ribosomes and mitochondria. The dendrites are the afferent thin structures of the neurons, frequently arranged around the neuronal cell body in the form of dendritic branches. They act to conduct electrochemical signals received from other neuronal cells to the cell body from which the dendrites project. At the other end of the soma a long unique extension, the axon 1μm in diameter and up to 1 m long in humans, conducts the signal away from the soma. The axons of many neurons have myelin wrapped around them to form the myelin sheath. This is formed by either of two types of glial cell Schwann cells which ensheath axons of peripheral neurons and oligodendrocytes which insulate those of CNS neurons. Along the myelinated axons, gaps in the sheath known as nodes of Ranvier occur at evenly-spaced intervals. The myelination enables efficient and rapid electrical impulse propagation. The demyelination of axons is what causes the multitude of neurological symptoms found in diseases such as MS (Siegel *et al.*, 2006).

In the human brain generally, there are three types of neurons depending on their functions. Motor neurons or multipolar neurons control muscle contractions by carrying messages from the CNS to the muscles or glands. Motor neurons include spinal motor neurons, pyramidal neurons and Purkinje cells. The second type are sensory neurons or bipolar neurons which carry signals from the body's sense receptors, as in the eye and ear to the CNS. The third type are the interneurons these neurons communicate with the spinal cord and with the skin or muscle which connect sensory and motor neurons e.g. dorsal root ganglia cells (Squire *et al.*, 2003).

1.1.3 Glial Cells

Another cell type in the CNS, are the glial cells and their name came from glia meaning "glue". They are known to provide physical and nutritional support for the neurons. There are different types of glial cells: Schwann cells, microglia, oligodendrocytes and astrocytes. These cell types have been studied extensively and been shown to have different functions in the CNS. Astrocytes, under normal conditions have been involved

in glutamate (as a neurotransmitter) uptake, whereby they detoxify it, converting it to glutamine (Farina *et al.*, 2007). There is also increasing evidence to suggest astrocytes have potential roles in immunity e.g. acting as antigen presenting cells to $CD4^+$ helper/inducer cells *in vitro* by expressing major histocompatibility complex class II (MHC II) antigens. Also they can synthesise and secrete cytokines e.g. IL-1, TNF and interferon gamma (INFγ). Microglia are macrophage-like cells resident within the CNS. Microglia are derived from bone marrow stem cells and early during development they occupy the CNS and remain quiescent as a resident macrophage population (Federoff, 1995). These cells can act as antigen presenting cells (APCs) and may have proinflammatory effector functions such as secreting cytokines following activation by inflammatory stimuli (Kreutzberg, 1996, Shrikant and Benveniste, 1996). Activated microglia have been shown to be present within demyelinating lesions and to phagocytose myelin debris and express MHC II, suggesting microglia may play an important role in the pathogenesis of MS (Loughlin *et al.*, 1993, Aloisi *et al.*, 2000).

1.1.4 The CNS Extracellular Matrix (ECM)

ECM is present in the intercellular spaces between neurons and glial cells to surround and support these cells (Figure 1.2) (Celio and Blumcke, 1994, Celio *et al.*, 1998). It is known that neurons and glial cells contribute to the secretion of ECM components. These are principally proteoglycans (PGs) which consist of a protein core to which glycosaminoglycans (GAGs) are bound. They may exist free or in aggregates bound to hyaluronic acid. Link proteins are involved in this binding (Neame and Barry, 1993). PGs containing the GAG chondroitin sulphate (chondroitin sulphate PGs (CSPGs)) predominate and include aggrecan, versican, neurocan, brevican and phosphocan. Aggrecan, versican, neurocan and brevican are also known as lecticans or hyalecticans as they contain an N-terminal hyaluronic acid binding domain and a C-terminal lectin domain (Iozzo, 1998, Yamaguchi, 2000). Through these domains, lecticans interact with carbohydrate and protein ligands in the extracellular matrix and act as linkers of these extracellular matrix molecules (Yamaguchi, 2000).

Structurally, CSPGs are highly anionic macromolecules, as a result of sulphate and carboxyl groups in their polysaccharide GAG side chains. The GAGs are key components of the brain ECM. They are involved in binding cations, e.g. potassium, calcium and water also regulating the movement of molecules through the matrix and cell adhesion and growth (Bellail *et al.*, 2004). Dermatan sulphate PGs are also present. In addition, the ECM contains tenascin-C and -R (Toole, 2000, Toole, 2004).

CNS ECM is deficient in collagen, fibronectin and laminin, the brain has a soft consistency when compared to cartilage. The ECM has an important role in physiological processes in the brain such as development (migration of neuronal and glial precursor cells), repair, proliferation and cell signalling (Bellail *et al.*, 2004). It has been reported that during maturation of the CNS, distinctive changes in the composition of the molecules of the ECM (lecticans, tenascins and link proteins in brain) occur (Milev *et al.*, 1998, Hirakawa *et al.*, 2000). In the developing ECM brain components are neurocan, the versican V1 splice variant, tenascin-C and link protein, while brevican, versican V2, aggrecan, tenascin-R and link proteins are characteristic components of the adult brain ECM (Figure 1.3) (Pesheva *et al.*, 1989, Yamaguchi, 1996, Schmalfeldt *et al.*, 1998). Also predominantly expressed in the mature nervous system are aggrecan, tenascin-N and brain link proteins (Hirakawa *et al.*, 2000, Bekku *et al.*, 2003, Neidhardt *et al.*, 2003). Brevican and tenascin-R are considered as specific CNS molecules. Similarly, neurocan and tenascin-C are representatives of juvenile brain ECM, both proteins decreasing significantly in brain after the first postnatal week (Rauch *et al.*, 1991, Dorries and Schachner, 1994).

The main role of CSPGs depends on their interactions with other ECM and neural adhesion molecules (Dow and Wang, 1998, Aspberg *et al.*, 1999). However, PG function in the injured adult CNS could differ from that in development and over-expression in injured CNS ECM may be inhibitory to neurite outgrowth and axonal regeneration (Nieto-Sampedro, 1999, Deller *et al.*, 2000, Schmalfeldt *et al.*, 2000, Levine *et al.*, 2001). PG expression is affected by a variety of stimuli, including cytokines (Jander *et al.*, 2000, Asher *et al.*, 2000). Lecticans can interact with other extracellular matrix components such as aggrecan and versican with fibulin family

members, brevican with sulphated glycolipids and neurocan with members of cellular adhesion molecules (Viapiano and Matthews, 2006).

In mammals, lectican family members, aggrecan, versican and brevican have protein cores with homologous G1 and G3 domains with GAG side chains attached in the central region. They are substrates for the ADAMTS with glutamyl endopeptidases (GEPs) activity which include ADAMTS-1, -4 and -5. These lecticans vary in their size, number of the chondroitin sulphates and ADAMTS cleavage sites as shown in Figure 1.4 (Zimmermann and Dours-Zimmermann, 2008).

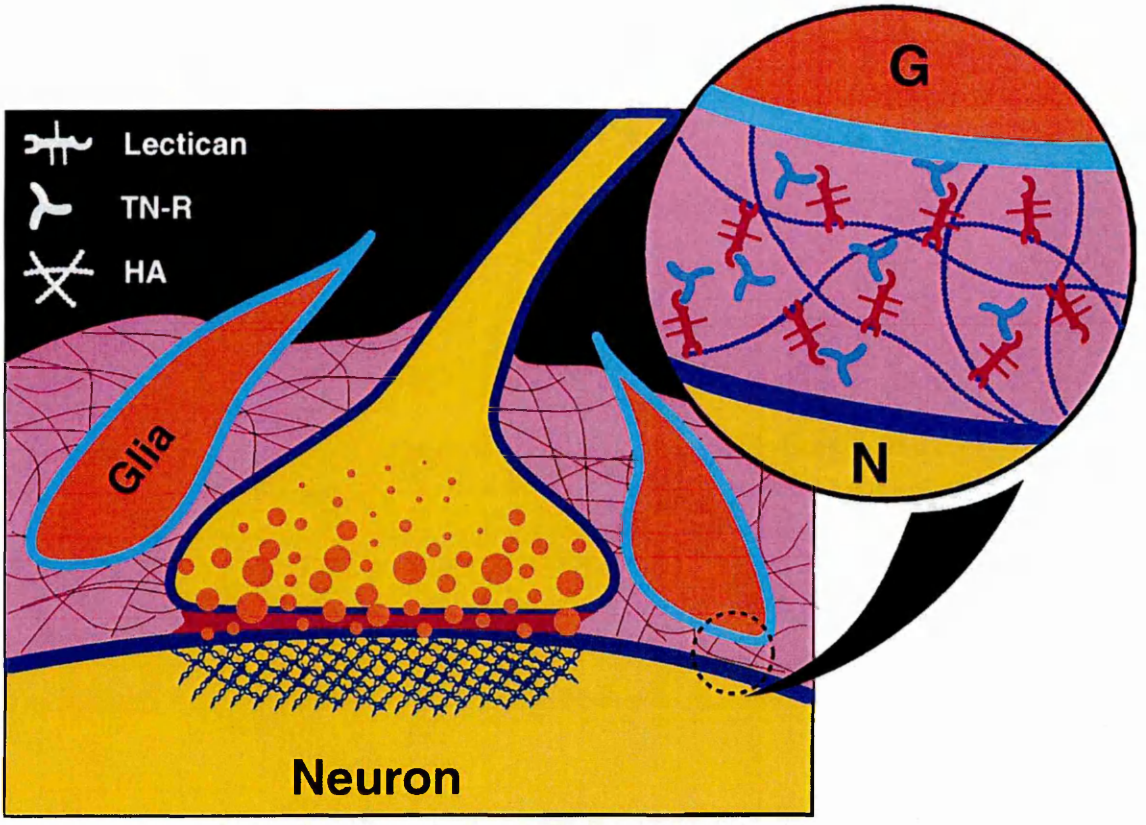


Figure 1.2: A schematic view of the ECM. Lecticans form complexes with hyaluronan (HA) and tenascin-R (TN-R) in the ECM and occupy the spaces between neuronal (N) and glial (G) cells (Reproduced with permission from Springer from Yamaguchi, 2000).

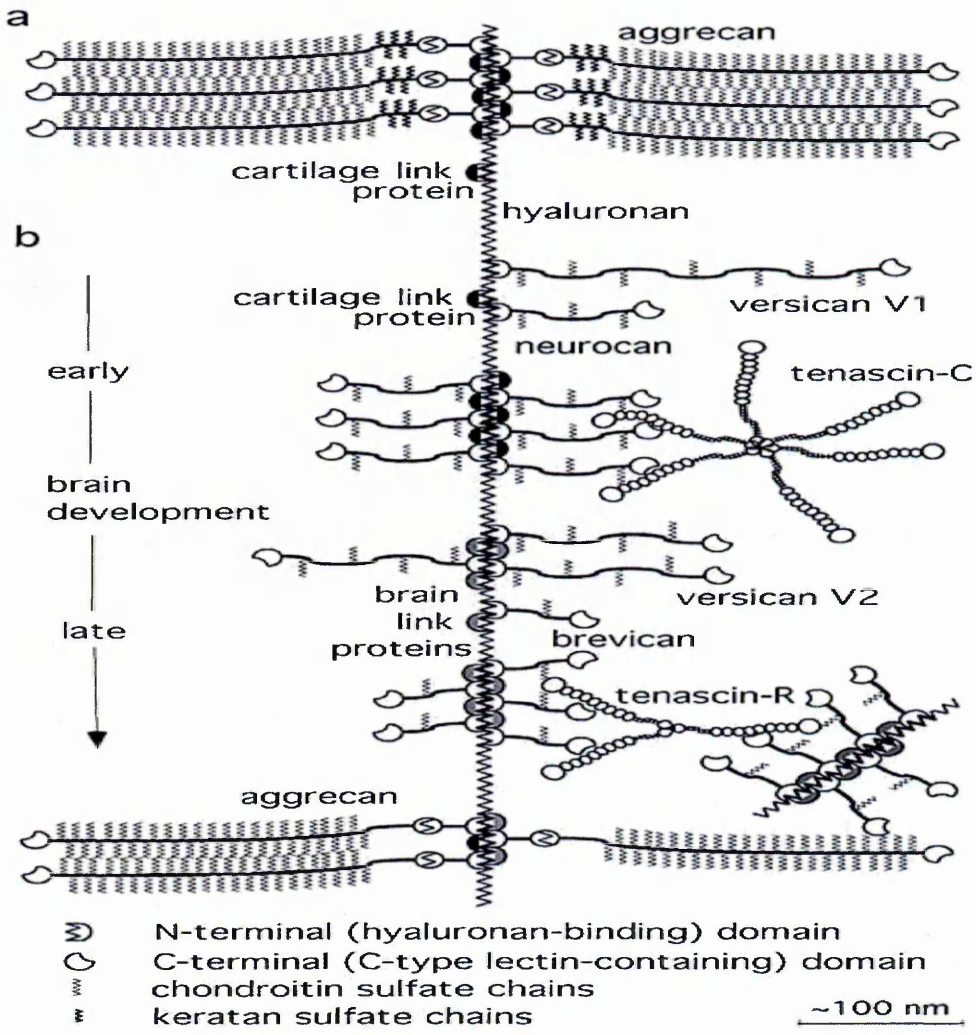


Figure 1.3: A schematic presentation of the lectican family members in CNS development and adult brain, and their binding to hyaluronan and tenascin complexes (Reproduced with permission from Springer from Rauch, 2004).

Furthermore, alternative splicing of versican results in transcripts that encode four variants: V0, V1, V2 and V3 (Zimmermann and Ruoslahti, 1989, Ito *et al.*, 1995, Zako *et al.*, 1995). All four isoforms have distinct amino and carboxy terminal globular domains (G1 and G3) (Figure 1.4). The G1 domain contains hyaluronan and link protein binding sites. The versican isoforms differ in numbers of the GAG chain binding sites with V0 containing both GAGα and GAGβ domains, V1 containing only GAGβ, V2 containing only GAGα, and the V3 isoform lacks the entire central domain including both GAG α and β domains: the G1 domain is directly followed by the G3 domain (Zako *et al.*, 1995). Lecticans, aggrecan and versican are expressed by a wide range of tissues (aggrecan by cartilage and CNS and versican by connective tissue, blood vessels, brain, kidney and cartilage). CNS-specific proteoglycans are brevican and neurocan (Viapiano and Matthews, 2006).

Immunohistochemical studies demonstrated alterations in the composition of the ECM in various types of MS plaques. In active and chronic active MS lesions that are characterised by a massive influx of inflammatory cells, a decreased immunoreactivity of chondroitin and dermatan sulphate proteoglycans was observed. In active lesions, white matter-associated proteoglycans accumulate in macrophages, suggesting that chondroitin and dermatan sulphate proteoglycans are phagocytosed together with myelin or myelin breakdown products. Accumulation of CSPGs around lesions contribute to their formation of a barrier to axonal growth (Sobel and Ahmed, 2001). Also, Back *et al*, (2005) observed accumulation of hyaluronan in MS lesions (Back *et al.*, 2005).

1.1.4.1 Previous Studies on CSPGs Proteolysis

Previous studies demonstrated that the matrix metalloproteinases (MMPs) were known to be responsible for the proteolytic cleavage of versican for example MMP-1, -2, -3, -7 and -9, have been shown to degrade versican (Perides *et al.*, 1995, Halpert *et al.*, 1996, Passi *et al.*, 1999). A subgroup of the ADAMTSs has GEPs activity and members of this subgroup have been shown to cleave CSPGs aggrecan, versican and brevican (see

also section 1.5.1). ADAMTS-1, -4 and -5 dependent degradation of aggrecan has been demonstrated (Abbaszade *et al.*, 1999, Kuno *et al.*, 2000, Tortorella *et al.*, 2000). ADAMTS-5 is a key enzyme in the breakdown of aggrecan in arthritic cartilage (Glasson *et al.*, 2005, Stanton *et al.*, 2005). ADAMTS-1, -4 and -9 have been reported to mediate versican proteolysis in different tissues including aorta and brain (Sandy *et al.*, 2001, Somerville *et al.*, 2003, Westling *et al.*, 2004). Versican can control several cellular processes such as adhesion, proliferation, apoptosis, migration and invasion via the highly negatively-charged chondroitin/dermatan sulfate side chains and by the interactions of the G1 and G3 domains with other proteins (LeBaron *et al.*, 1992, Wight, 2002).

ADAMTS-1 and ADAMTS-4 have been shown to cleave versican V1 in the GAG-β binding domain at Glu^{441}-Ala^{442} resulting in a 70 kDa fragment in human aorta (Sandy *et al.*, 2001). Furthermore, Westling *et al*, 2004 have demonstrated that versican V2 in human brain tissue can be cleaved by ADAMTS-4 at the Glu^{405}-Gln^{406} site within the GAG-α binding domain to produce a 64 kDa fragment (Westling *et al.*, 2004). Also it has been found that versican (V0/V1) proteolysis via ADAMTS leads to a 70 kDa fragment that is essential for the formation and differentiation of endocardial cushion mesenchyme (Kern *et al.*, 2007). Another study by McCulloch *et al*, (2009) suggests that ADAMTS-mediated proteolysis of versican is important in limb development and the generated fragment could regulate interdigital web regression (McCulloch *et al.*, 2009).

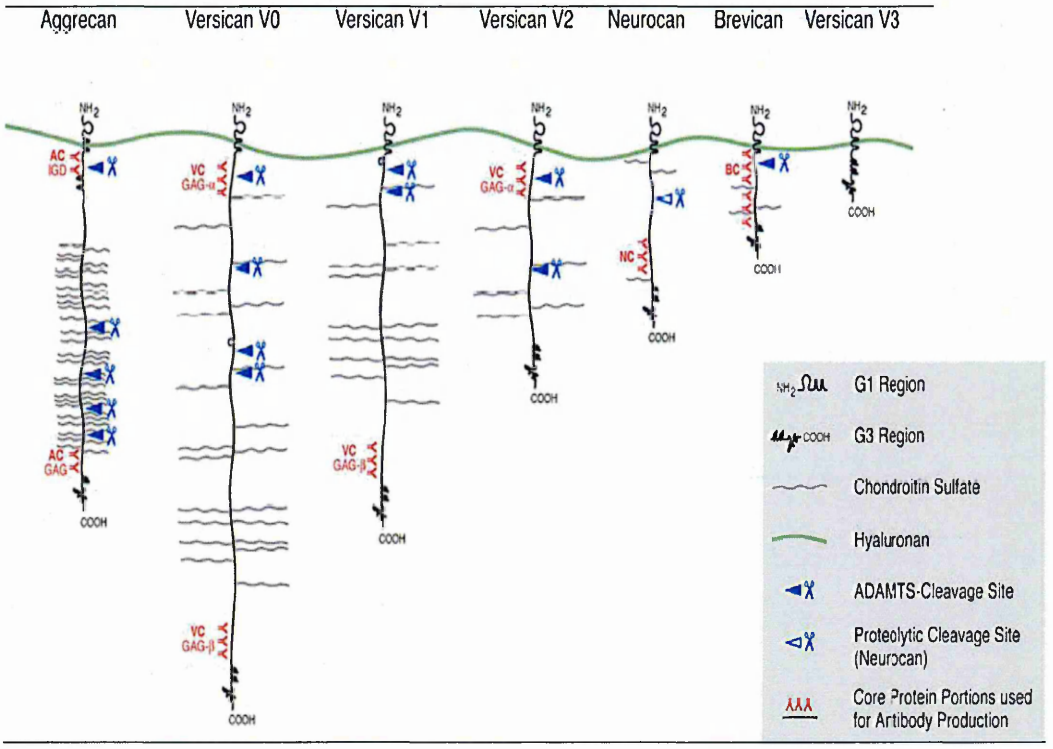


Figure 1.4: The basic four lectican family members in humans, aggrecan, brevican, versican and neurocan. These share the same features of large CSPGs with G1 and G3 domains with regions carrying most of the O- and N-linked oligosaccharides and all glycosaminoglycans in between (Reproduced with permission from Springer from Zimmermann and Dours-Zimmermann, 2008)

1.2 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic, inflammatory, demyelinating and neurological disorder which affects mainly the white matter of the CNS (Bebo *et al.*, 1999). MS is a complex disease and can have a personal, social and economic load on the society, which is estimated ~ £1 million per MS patient (Orton *et al.*, 2006).

1.2.1 Clinical course of MS

The majority of MS patients present with relapse-remitting (RR) symptoms, which starts with sporadic attacks and then periods of remission with partial or complete recovery. Symptoms may resolve completely, remission periods can last for months or years. Secondary progressive (SP) MS develops in more than half of RR patients. This will usually occur 15-20 years after the onset of the disease and involves fewer attacks and incomplete recovery. The primary progressive (PP) MS accounts for about 15% of MS cases in the UK and involves a progressive accumulation of disability without remission periods. Finally, the progressive relapsing (PR) disease is a very rare (< 1%) condition where symptoms steadily get worse from the beginning of the disease. Figure 1.5 shows the clinical course of MS types (Lublin and Reingold, 1996).

1.2.2 Epidemiology of MS

Epidemiological studies have linked viral infections with the aetiology of MS, including Epstein-Barr virus (EBV) antigen and Haemophilus influenza, both of which can lead to demyelinating disease (Olson *et al.*, 2001). MS is the most common neurological disorder among young adults, affecting approximately 2.5 million people worldwide. The onset of the disease usually occurs between the ages of 20-40 years old. It has a prevalence of 100-150 per 100,000 in the United Kingdom and an annual incidence of 7 per 100,000. As is the case with many other autoimmune diseases, MS occurs more in females than men. Overall MS affects twice as many women as men (MacDonald *et al.*, 2000).

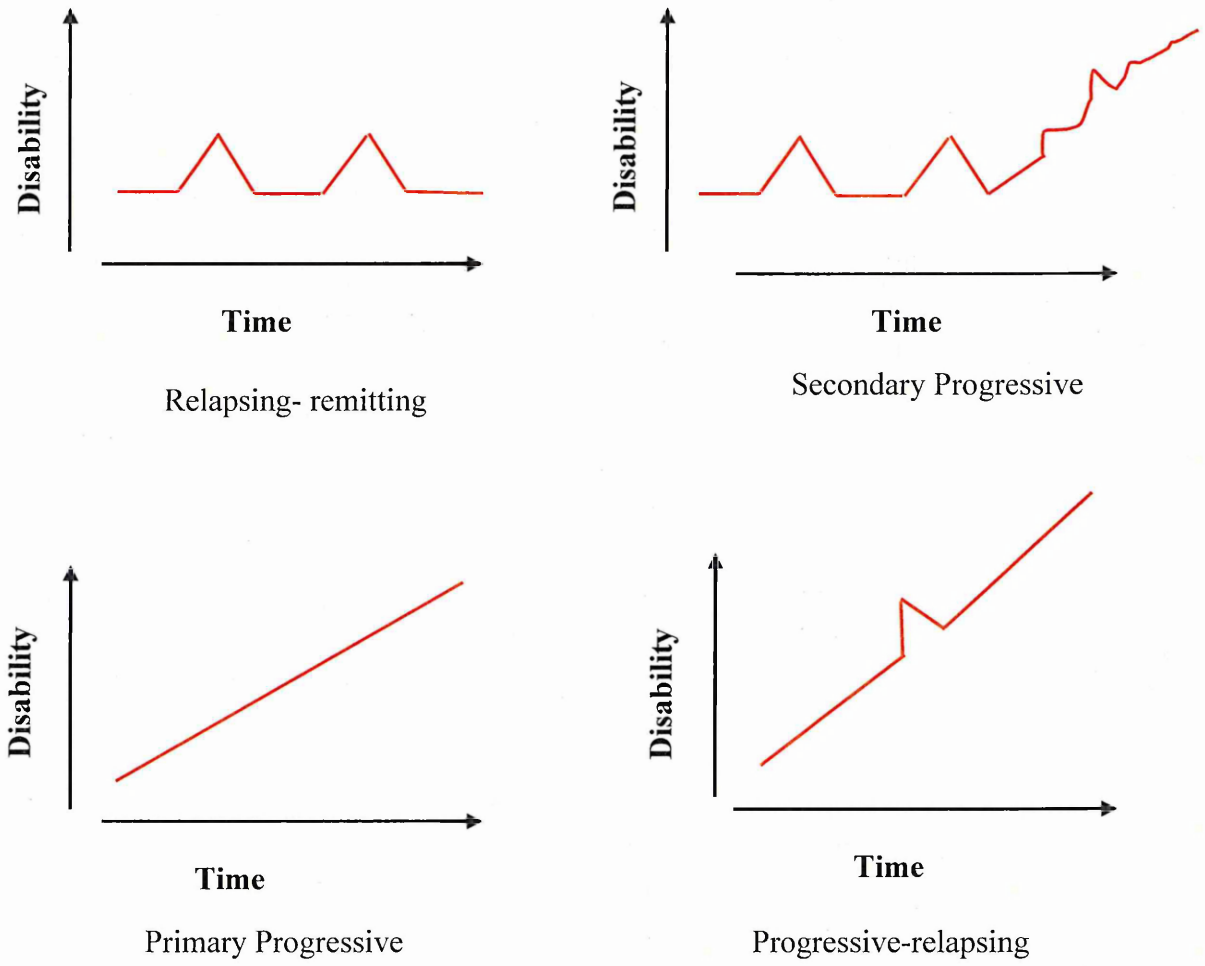


Figure 1.5: The clinical course of MS. MS has been classified according to the clinical course into different disease phases. 90% of patients show signs of relapsing-remitting disease, which starts with acute attacks involving worsening of neurologic functions followed by periods of remission (partial or complete recovery). Only 30% remain with this disease course. Approximately 60% of people with relapsing-remitting disease develop the secondary progressive MS phase where recovery from attacks is incomplete. 10% of patients with what is described as primary progressive MS show a steady increase in disability without distinct relapses or remissions. Progressive relapsing MS is a very rare condition, characterised by a steady progression of clinical neurological damage with relapses and remissions (Adapted and redrawn from Lublin, and Reingold, 1996).

There is a latitudinal gradient in prevalence, independent of genetic factors, with rates increasing moving south or north away from the equator. The prevalence of MS varies considerably, being highest in northern Europe, New Zealand, Australia, North America and Canada. Latitudinal gradients are even described within the UK, with the highest rates being observed in Scotland and Northern Ireland. Asia, Africa and South America that lie on the equator have low levels of MS (5-10 per 100,000) (Compston *et al.*, 1998). Migration studies demonstrate that those who emigrate from an area of low-prevalence to an area of high-prevalence remain at low risk if they move after 15 years of age (Alter *et al.*, 1966).

As the geographical distribution of MS prevalence is increased when approaching the poles, this highlighted the interest in low vitamin D levels as a risk factor for developing MS (Acheson and Bachrach, 1960). Recently, clinical observations and experimental work *in vitro* and in experimental autoimmune encephalomyelitis (EAE) animal models of MS has been reported, which showed that limited exposure to sunlight, which relates to vitamin D synthesis, has been associated consistently with an increased risk of developing MS (Ascherio *et al.*, 2010, Burton *et al.*, 2010, Solomon and Whitham, 2010). The prospect of a potential tool such as vitamin D supplements to prevent MS is tempting, yet challenging to investigate in an intervention study since it would require a huge population to measure any effect on MS incidence. However, vitamin D supplementation has not only been proposed to prevent MS, but also to attenuate disease activity of MS (Goldberg *et al.*, 1986).

1.2.3 Aetiology and Immunopathogensis of MS

Although MS was first described more than 150 years ago, the exact aetiology and the pathogenesis are still not fully understood. The incidence of MS in first degree relatives is 20 times higher than in the general population, suggesting the influence of genetic factors on the disease. Monozygotic twin studies show a concordance rate of 25%. Dizygotic twins show a concordance rate of less than 5%. The major causation is unknown but may depend on the cumulative effects of both genetic and environmental factors (Hafler *et al.*, 2007, Taylor, 2011). In this content, smoking (Pittas *et al.*, 2009)

and the degree of exposure to microorganisms in early life (hygiene hypothesis) (Ponsonby *et al.*, 2005) as well as personal UVR exposure (van der Mei *et al.*, 2003, Lucas *et al.*, 2011) are well-established environmental risk factors that significantly alter the risk of developing MS.

Treatment to reduce the severity of the disease has been improved substantially but there is not yet a cure. An immune reaction to a viral infection where viral proteins are similar to self proteins may cause a cross reaction to myelin self proteins, molecular mimicry. This may occur early in life, in the periphery, and may initiate an autoimmune disease process with a genetic susceptibility. Activated antigen-specific T cells and B cells cross the BBB and infiltrate the perivascular space (Hemmer *et al.*, 2006).

Figure 1.6 B shows a schematic view of the immunopathology of the MS lesion. A number of immune and CNS cell types are involved in lesion development and repair. T cells, B cells and macrophages infiltrate the lesion. CD4+ T cells are located in the perivascular cuff. The antigen-specific T cells, guided by chemoattractants, infiltrate the lesion in CNS. These cells become reactivated by antigens in association with MHC II presented on dendritic cells, B cells, microglial cells and macrophages, and locally released cytokines and chemokines attracting macrophages to the lesions and their distribution throughout the lesion is reflected in the expression pattern of MHC molecules. Macrophages release proinflammatory cytokines (IL-6 and TNF) and toxic molecules (nitric oxide). MHC class I is expressed by all cells in the inflammatory surroundings of the CNS (Neumann *et al.*, 1995, Dandekar *et al.*, 2001). CD8+ T cells infiltrate the parenchyma and, as well as secreting inflammatory mediators, they directly attack cells expressing MHC class I such as neurons and oligodendrocytes (Hemmer *et al.*, 2006).

B cells are also present in the perivascular space, where they produce antibodies. These antibodies opsonise the antigen expressed on the surface of oligodendrocytes and neurons. Bound antibodies can initiate the complement cascade, or induce antibody-mediated phagocytosis by macrophages (Alter *et al.*, 2003). Astrocytes proliferate and induce gliosis at the edge of the lesion. Following the inflammatory damage, oligodendrocytes may proliferate and remyelination of axons in lesions. However the

remyelination is usually incomplete and loss may be irreversible. Overall, the extent of inflammation, neurodegeneration and remyelination is heterogeneous between patients (Hemmer *et al.*, 2006).

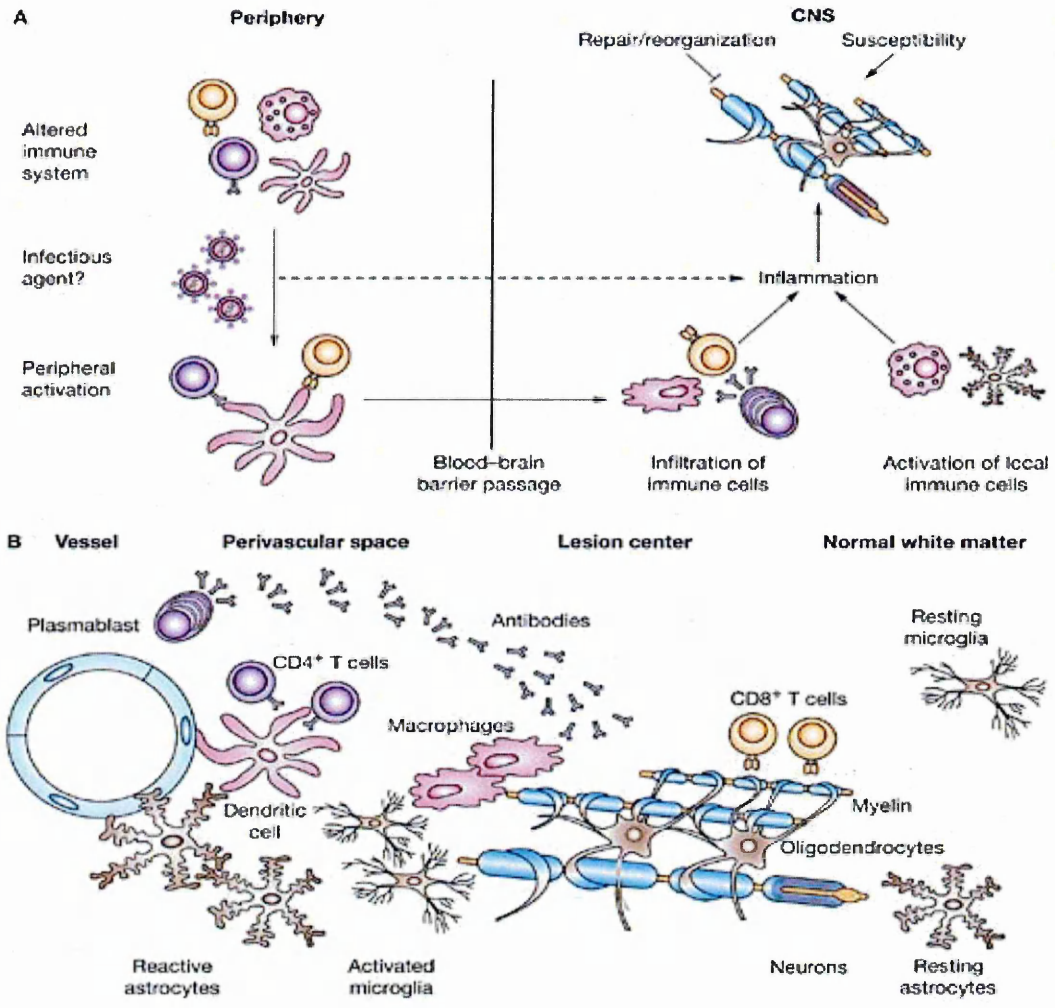
Studies in EAE mainly concentrated on the roles played by $CD4^+$ T cells in MS pathogenesis. It is now becoming clear that $CD8^+$ T cells also may play a significant role in the disease. It was observed that $CD8^+$ T cell numbers were significantly higher than those of $CD4^+$ T cells in MS lesions at all stages of MS (Lucchinetti *et al.*, 2000). Numbers of myelin-reactive $CD8^+$ T cells in the peripheral blood of people with MS were significantly higher than in individuals without MS (Crawford *et al.*, 2004). Another study by Skulina *et al*, (2004) showed the persistence of clonally expanded $CD8^+$ T cells in MS lesions (Skulina *et al.*, 2004).

The mechanism of the BBB breakdown in MS is uncertain but it is thought that the exposure of the endothelium to proinflammatory cytokines such as INFγ, IL-1β and TNF disturbs the BBB by disorganising cell-cell junctions and enhances leukocyte endothelial adhesion and migration and increases expression of MHC II (Minagar and Alexander, 2003).

Chemokines are composed of a large family of small proteins, which are involved in chemoattracting leukocytes into injured tissue (Asensio and Campbell, 1999). Chemokines and their receptors have been implicated in migration of mononuclear cells under physiological and pathological conditions (Murphy *et al.*, 2000, Zlotnik and Yoshie, 2000, Trebst and Ransohoff, 2001).

Figure 1.6: Immunopathogenesis in MS. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience (Hemmer *et al.*, 2006.

A) MS is believed to be initiated in genetically susceptible individuals when infected by infectious agents that contain protein sequences cross-reactive with self-myelin. APCs present the antigen and activate myelin-reactive cells in the periphery. These cells cross the blood brain barrier and enter the CNS. Within the CNS, myelin-reactive Th1-cells interact with microglia (localised APCs) presented antigens and secret inflammatory cytokines causing demyelination. B) A schematic view of the immunopathology of the MS lesion. Infiltration of the T cells, B cells and macrophages in the perivascular lesion. As a result, an inflammation cascade is initiated with the release of inflammatory mediators and proteases that damage oligodendrocyte-formed myelin sheaths.


A
Periphery
CNS
Repair/reorganization
Susceptibility
Altered immune system
Infectious agent?
Peripheral activation
Inflammation
Blood–brain barrier passage
Infiltration of immune cells
Activation of local immune cells
B
Vessel
Perivascular space
Lesion center
Normal white matter
Plasmablast
Antibodies
Resting microglia
CD4+ T cells
CD8+ T cells
Macrophages
Dendritic cell
Myelin
Oligodendrocytes
Reactive astrocytes
Activated microglia
Neurons
Resting astrocytes

It has been shown that the release of MMPs may facilitate the infiltration of activated immune cells into the CNS parenchyma by breaking down proteins, glycoproteins and PGs present in the basal lamina including type IV collagen and heparan sulphate proteoglycan of the BBB (Maeda and Sobel, 1996). Following migration into the CNS, activated T cells initiate an inflammatory process triggered by specific antigen. Immunological effector mechanisms include activation of microglia and infiltrating macrophages by T cell cytokines which may lead to ECM damage and demyelination. Recognition of myelin proteins e.g. myelin-associated glycoprotein (MAG), myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) as foreign leads to myelin damage (demyelination) and subsequently axonal damage and loss (Grigoriadis *et al.*, 2004). This damage causes a large variety of symptoms such as vision impairment, bladder or bowel dysfunction, motor symptoms (eg. weakness and spasticity), sensory symptoms (e.g. numbness and dysaethesia), tremor and ataxia and other symptoms such as fatigue and cognitive impairment.

Other factors which have also recently been associated with people with MS are the selective loss of immunologic self-tolerance of the myelin-reactive T cells. Although autoreactive T cells are present in both healthy individuals and patients with autoimmune diseases, autoreactive T cells found in patients with autoimmune disorders are more easily activated compared with those from normal subjects. Active suppression by $CD4^+CD25^+$ regulatory T cells has a significant role in immunologic tolerance *in vivo.* Their deletion causes spontaneous autoimmune diseases in mice (Sakaguchi, 2000). Human $CD4^+$ regulatory T cells expressing high levels of CD25 are suppressive *in vitro* and mimic the activity of murine $CD4^+$ $CD25^+$. Furthermore, immunisation of animals with suppressor T-cells can prevent the development of EAE, an animal model of MS (Suri-Payer *et al.*, 1998). People with MS show a significant reduction in the numbers and function of $CD4^+$ $CD25^{hi}$ regulatory T cells from the peripheral blood compared to non-MS individuals (Viglietta *et al.*, 2004).

1.2.3.1 Toll-like Receptors (TLRs) in MS Immunopathogenesis

Normally, when pathogens invade the CNS, the innate immune system provides a rapid but relatively nonspecific response to neutralise the infection via Toll-like receptors (TLRs). Due to this moderately nonspecific response, local tissue injury can occur (Lehnardt *et al.*, 2003). If infection persists, adaptive immunity, which involves T and B cell activation, can provide a delayed but more specific response that is typically less destructive to the host tissue. An exception to this theory is with autoimmune disease where adaptive immunity promotes extensive host tissue damage. In general, however, the immune system provides a response that is progressively more specific to pathogens and less destructive to host tissue. An innate immune response is also provoked in non-infectious CNS injury or disease, including neurodegenerative diseases (Zhang *et al.*, 2005, Boillee *et al.*, 2006, Yoshiyama *et al.*, 2007, Giunta *et al.*, 2008), stroke (Cao *et* al., 2007, Lehnardt *et al.*, 2007, Tang *et al.*, 2007), spinal cord trauma (Kigerl *et al.*, 2007), spinal nerve damage (Kim *et al.*, 2007) and tumour infiltration (Hussain *et al.*, 2006, Curtin *et al.*, 2009).

Innate immune activation in MS is indistinguishable from that associated with microbial exposure. The rapidity of resident immune cell activation is similar, for example occurring within 5 minute of spinal cord injury (Pineau and Lacroix, 2007) and within 8 minute of lipopolysaccharide (LPS) exposure (Clark *et al.*, 2006). The underlying stimulus is that injured tissue or cells release, secrete, or synthesise molecules associated with damage, that communicate the presence of injury to the innate immune system.

In addition to lymphocytes that control adaptive immune responses, dendritic cells and tissue macrophages that regulate innate immune responses also play a role in controlling MS disease pathogenesis. These cells express pattern recognition receptors (PRRs) including TLRs that recognize pathogen-associated molecular patterns (PAMPs) present on the surface of pathogens. Also, endogenous molecules created upon tissue injury, called damage-associated molecular patterns (DAMPs) are ligands for TLRs and signal the risk of either infection or injury to the organism. Following ligand binding to TLRs, innate immune cells produce proinflammatory cytokines and can serve as antigen

presenting cells (APCs) to T cells to recognize antigens (Takeda and Akira, 2005, Hacker *et al.*, 2006). These receptors represent a key molecular link between tissue injury, infection, and inflammation. Thus, TLRs play an important role in linking the innate to the adaptive immune response (Bell *et al.* 2005).

DAMPs include endogenous molecules released by activated cells and ECM molecules. These molecules which are generated upon tissue injury activate TLRs. One of the first endogenous TLR activators recognised was the heat shock protein 60 (HSP60), which was shown to induce cytokine synthesis and to induce an inflammatory response. TLR activators that have been associated with MS are HSPs and high-mobility group box (HMGB1) protein. HMGB1 protein, originally described as a DNA-binding protein that facilitates transcription, can also be released extracellularly during acute inflammatory responses. Exposure of neutrophils, monocytes, or macrophages to HMGB1 results in an enhanced expression of proinflammatory cytokines (Park *et al.*, 2004). Other TLR activators linked to MS are ECM molecules such as tenascin-C, versican and fragments of hyaluronan which may promote an inflammatory response (Piccinini and Midwood, 2010).

1.3 Cytokines in MS

It seems that immunoregulatory cytokines play a significant role in MS pathogenesis. Cytokines are derived principally from activated T-cells, activated in the lymphoid tissue. An excess of the proinflammatory cytokines leads to changes in the blood brain barrier which activated T-cells are then able to penetrate. The antigen presenting cells e.g. microglia and endothelial cells present the antigen to activated T- cells. $CD4^+$ T-helper (Th) cells differentiate into Th1 cells and Th2 cells depending on the cytokine milieu. These subsets differ from each other in their cytokine production and in their function. Th1 cells regulate cellular immunity e.g. viral immunity and delayed type hypersensitivity while Th2 cells control humoral immunity and have a role in fighting parasitic infections. Proinflammatory cytokines IL-1, TNF, IL-2 and INFγ produced and released are involved in the pathogenesis of many CNS diseases including MS (Merrill

et al., 1992). Th1 cells activate macrophages and microglia to produce proinflammatory cytokines (IL-1 and TNF) and toxic molecules such as nitric oxide.

Activated astrocytes, due to their ability to secrete cytokines, play a significant role in triggering and maintaining immune responses in the brain. IL-1 and TNF modulate apoptosis of CNS cells and lymphocyte differentiation and infiltration (Kim, 1996). The Th1 cytokines, lymphotoxin (LT), INFγ and IL-2 are upregulated in MS. In contrast, T-cells producing Th2 cytokines (IL-4, IL-5, IL-6 and IL-10) are non encephalitogenic and possess the capacity to induce resistance to EAE (Ramirez and Mason, 2000). An exception to this, reported by Lafaille *et al* (1997), was that MBP-specific Th2 cells have the capacity to cause EAE in immunodeficient mice (Lafaille *et al.*, 1997). Other findings, obtained with both EAE models and people with MS indicate the involvement of IL-17, produced by the Th17 cells another subset of T-helper cells, which in addition, toTh1 cells are thought to be critical in MS (Hofstetter *et al.*, 2009). In MS increased numbers of mononuclear cells have been shown to express IL-17 mRNA in the peripheral blood (Matusevicius *et al.*, 1999). Also these cells have been shown to migrate across the BBB (Kebir *et al.*, 2007). People with MS, have also been shown to have elevated IL-17 levels in their cerebrospinal fluid (Ishizu *et al.*, 2005) and IL-17 protein is restricted to active areas of MS lesions (Tzartos *et al.*, 2008).

1.3.1 The Interleukin-1 (IL-1) Family

IL-1 is a 17-kDa protein that is mostly produced by monocytes and macrophages but is also produced by endothelial cells, B cells, and activated T cells (Kennedy *et al.*, 1992, Bauer *et al.*, 1993). IL-1 family members are expressed at low concentrations within the normal CNS but their expression is rapidly up-regulated by various experimental brain insults e.g. ischaemia, trauma, hypoxia, EAE and neurotoxic or inflammatory stimuli. Interleukin-1 alpha (IL-1α) and IL-1β are members of the IL-1 family and bind to the same receptors (80 kDa cell surface receptor IL-1RI). A second, 68 kDa receptor (IL-1RII) also binds IL-1, but lacks an intracellular domain and does not initiate signal transduction. IL-1β is considered to be more important than IL-1α because it is able to modulate cerebral functions during systemic and localized inflammation (Gosselin and

Rivest, 2007). Pro-IL-1β must be cleaved by the enzyme caspase 1, to produce the active form and allow cellular release (Thornberry *et al.*, 1992). The third member of the IL-1 family is IL-1 receptor antagonist (IL-1ra). It blocks IL-1 action by binding to IL-1 receptors but does not induce any intracellular signal (Gibson *et al.*, 2004). In normal physiological conditions in the CNS, IL-1β is expressed at low concentrations, but it is upregulated in response to pathological CNS injuries (Giulian *et al.*, 1989).

Hauser *et al.*, (1990) investigated the cytokines IL-1 β, TNF, and IL-6 by specific radioimmunoassays in the cerebrospinal fluid (CSF) of people with MS and other neurologic diseases. There was an increase in IL-1β in people with active MS compared with other neurologic diseases (Hauser *et al.*, 1990). IL-1β also has been reported to be increased in the CSF of MS patients and EAE animals (Wang and Shuaib, 2002). Chronic expression of IL-1 in rat brain results in extensive demyelinating lesions, mimicking MS and IL-ra decreases disease progression in EAE.

1.3.2. Tumour Necrosis Factor (TNF)

Tumor necrosis factor (TNF) is a protein produced by monocytes, macrophages and a wide variety of other cell types in response to other cytokines. In contrast, lymphotoxin (LT) is a 25-kDa glycoprotein but it is lymphokine cytokine. Both TNF and LT are homologous (28%) in their amino acid sequences and formerly these were known as TNFα and TNFβ respectively. As they have common cell surface receptors, it is thought that all cellular responses mediated through TNF are also mediated by LT (Chaturvedi *et al.*, 1994).

TNF can be synthesised in the CNS by microglia, astrocytes, and some populations of neurons (Lieberman *et al.*, 1989, Morganti-Kossman *et al.*, 1997, Chung *et al.*, 2005). TNF has been suggested as an important mediator of MS pathogenesis (Wajant *et al.*, 2003, Shen and Pervaiz, 2006, Wallach *et al.*, 1999). It is synthesized as a trimeric type II transmembrane protein precursor and cleaved by TNF alpha converting enzyme (TACE; ADAM-17) to a 51 kDa (3x17 kDa), soluble circulating protein. Both the transmembrane and soluble forms of TNF are biologically active (MacEwan, 2002).

TNF binds to two receptors (TNFRI [p55] and TNFRII [p75]), which initiates intracellular signalling (Viviani *et al.*, 2004). The resultant gene expression modulation can contribute to inflammation and hence TNF is described as a pro-inflammatory cytokine. A study done by Hauser *et al.* (1990) shown that TNF was found more frequently in active MS than in other neurologic diseases. Gregersen *et al*, (2000) demonstrated that TNF was localised to microglial cells in mice post-ischaemia (Gregersen *et al.*, 2000).

TNF is expressed by macrophages, astrocytes, microglia and endothelial cells in chronic and active MS. Furthermore, MS studies demonstrate that there is a correlation between TNF concentration and severity of the disease (Matusevicius *et al.*, 1996).

1.4 ADAM-17

The ADAMs (a disintegrin and metalloproteinases) are a family of transmembrane and secreted proteins with important roles in regulating cell phenotype via their effects on cell adhesion, migration, proteolysis and signalling. The ADAMs belong to the M12B adamalysin protease subfamily in the MEROPS classification (Rawlings *et al.*, 2008), which contains the closely related snake venom reprolysins and the ADAMTSs. ADAMs are multi-domain proteins with a pro-domain, metalloprotease, disintegrin, cysteine-rich, epidermal growth factor (EGF)-like, transmembrane and cytoplasmic tail domains. The functional ADAM metalloproteinases are involved in "ectodomain shedding " of growth factors, cytokines, receptors and adhesion molecules. Proteins can be cleaved and thereby released (shed) from the plasma membrane by these proteases or sheddases. TACE or ADAM-17 is a well known sheddase which is involved in releasing soluble TNF from its membrane-bound precursor (pro-TNF) (Moss *et al.*, 1997, Black *et al.*, 1997).

ADAM-17 is responsible for the cleavage of a wide variety of substrates involved in inflammation including transforming growth factor-alpha (Pro-TGFα), p75 (Peschon *et al.*, 1998) and p55 TNF receptors (Reddy *et al.*, 2000), the chemokine (fractalkine) (Garton *et al.*, 2001) and amyloid precursor protein (Garton *et al.*, 2001). The release of TNF from different cells occurs in response to injury or infection and plays an important role in the adaptive immune response to these conditions. However, TNF can also be produced in excess and can cause tissue damage. Generation of soluble TNF by ADAM-17 cleavage has been found to have a pathogenic role in CNS inflammatory responses involving MS (Kieseier *et al.*, 2003, Plumb *et al.*, 2006).

1.4.1 ADAM-17 and the CNS

Within the normal CNS, ADAM-17 expression has been observed in astrocytes and endothelial cells using double indirect immunofluorescence and confocal microscopy (Goddard *et al.*, 2001). Plumb *et al* (2006) observed expression of ADAM-17 in activated macrophage/microglia and parenchymal astrocytes in MS white matter (Plumb *et al.*, 2005, Plumb *et al.*, 2006). Its expression is upregulated in active MS lesions and in EAE. Expression levels correlated with disease activity indicating that it may be involved in disease pathogenesis (Plumb *et al.*, 2005, Plumb *et al.*, 2006). ADAM-17 expression by astrocytes and activated macrophage/microglia may therefore allow shedding of TNF producing soluble proinflammatory TNF. ADAM-17 cleavage of TNF has several proinflammatory effects including increased endothelial cell attachment of inflammatory cells via upregulated adhesion molecules, chemotaxis, migration and BBB disruption (Dobbie *et al.*, 1999). Alternatively, it may induce a beneficial immune response by causing apoptosis of auto-reactive T cells as seen in experiments on the MS animal model EAE (Probert and Akassoglou, 2001, Weishaupt *et al.*, 2004).

1.5 ADAMTSs

ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs) are a group of Zn-dependent, secreted, multidomain enzymes in family M12 of Clan MA

belonging to the same subfamily (B) of metzincin proteins as ADAMs, the adamalysins according to MEROPS database (Rawlings *et al.*, 2010). ADAMTS-1 was first described by Kuno *et al*, (1997) identified as a novel murine complementary DNA (cDNA) expressed in a cachexigenic adenocarcinoma cell line. There are 19 known ADAMTSs identified in humans, numbered 1-20 (ADAMTS-5 protein is the same as ADAMTS-11) (Figure 1.7) and their known functions include cleavage of the ECM proteoglycans (aggrecan, versican and brevican), collagen processing, organogenesis, anti-angiogenesis and blood coagulation homoeostasis.

1.5.1 ADAMTS Subgroups

ADAMTS proteins can be divided into seven divisions, according to their structural characteristics and activities (targeted substrates) (Jones and Riley, 2005). The members of ADAMTS-1, -4, -5, -8, -9 and -15 forms a subgroup that is able to cleave the major cartilage proteoglycan aggrecan from which they derived the name aggrecanases. They also have a more general name, which is hyalectanases due to their ability to cleave substrates of the hyalectan (lectican) family of proteoglycans, including aggrecan, versican and brevican (Gao *et al.*, 2002). They have GEP activity, cleaving peptide bonds at the carboxyl end of the glutamate residue. The GON-ADAMTSs are ADAMTS-9 and -20. ADAMTS-20 shares its modular arrangement with that of the long isoform of ADAMTS-9, having 14 C-terminal thrombospondin (TSP)-repeats and a gon domain (Somerville *et al.*, 2003, Llamazares *et al.*, 2003). Moreover, though ADAMTS-9 is classified as a GON-ADAMTS with ADAMTS-20, it too is able to cleave aggrecan at the Glu1771–Ala1772 bond and versican at the Glu441–Ala442 (Somerville *et al.*, 2003).

Another, well-defined, subgroup contains ADAMTS-2, -3 and -14 which are procollagen N-proteinases. ADAMTS-13 is in a category on its own and is involved in the blood coagulation hemostasis as the von Willebrand factor (vWF) cleaving protease. The remaining ADAMTS members form a subgroup called 'others', whose members are further divided into four pairs (ADAMTS-18 and -16, ADAMTS-19 and -17, ADAMTS-12 and -7, and ADAMTS-10 and -6) based on their structural characteristics.

1.5.2. The Domain Structure of the ADAMTS Proteins

The main difference between ADAMs and ADAMTS is that ADAMs are transmembrane proteins while the ADAMTSs are secreted proteins, which bind to the ECM. ADAMTSs consist of prodomain, metalloprotease and disintegrin domains, but lack the ADAMs' transmembrane domain. The thrombospondin motif(s), present on the C-terminal side of the disintegrin domain of ADAMTS also distinguishes them from ADAMs (Figure 1.8) and are thought to function, with the disintegrin domain, in binding to the ECM.

The molecular structure of the ADAMTS proteins can be subcategorized into domains. The ADAMTSs are synthesized as inactive zymogens. From the N- to the C-terminus, they each consist of: a signal peptide, a prodomain, a metalloproteinase catalytic domain, a disintegrin-like domain, a central thrombospondin (TSP)-repeat, a cysteine-rich domain, a spacer domain and a variable number of C-terminal thrombospondin (TSP)-repeats, which range from 14 C-terminal repeats in the case of ADAMTS-20 to none in the case of ADAMTS-4 (Stocker *et al.*, 1995, Kaushal and Shah, 2000).

1.5.2.1 The Signal Peptide and Prodomain

ADAMTS proteins are initially synthesized as pre-proenzymes with a signal peptide and prodomain, which is generally to maintain enzyme latency, accurate protein folding and secretion (Milla *et al.*, 1999, Cao *et al.*, 2000). Firstly, the ADAMTSs undergo an N-terminal processing to remove the signal peptide on the endoplasmic reticulum membrane. Removal of the prodomain has also thought to occur in the trans Golgi. This process in the ADAMTSs is catalysed by furin, also called proprotein convertase (PC), at the furin recognition site between the prodomain and the catalytic motifs. Furin is a calcium-dependent serine protease that is known to cleave precursor proteins. Furin has been shown to interact with the pro-form of ADAMTS-4 and to co-localize within the trans-Golgi network (Wang *et al.*, 2004). Removal of the prodomain has also been reported for ADAMTS-1 (Kuno *et al.*, 1999).

The prodomain of human and mouse ADAMTS-5 contains three potential furin recognition sites (Hurskainen *et al.*, 1999) within a multi basic sequence ^{258}RRRRR^{262} at the activation site. Although the predicted activating cleavage site at R^{262}-^{263}S has been detected in an *in vitro* expression system (Zeng *et al.*, 2006), cleavage at this site has not been confirmed *in vivo*. Whether proprotein convertases other than furin can activate ADAMTS-5 and whether removal of the prodomain is required for its secretion has yet to be resolved (Wang *et al.*, 2004).

1.5.2.2 The Metalloproteinase Domain (Catalytic Domain)

All ADAMTSs have a Zn-binding peptidase consensus HE**XX**H, catalytic domain which contains a zinc-binding active site similar to that in the ADAMs, which distinguishes the ADAMs and ADAMTSs from other metalloproteinases (reviewed in Porter *et al.*, 2005).

1.5.2.3 The Disintegrin-like Domain

This domain in ADAMs and ADAMTSs has some primary sequence similarity (25 to 45%) to that of the snake venom disintegrins. The snake venom disintegrins have an integrin recognition sequence RGD (Arg-Gly-Asp) and bind to integrins. ADAMTSs lack this RGD sequence and do not appear to interact with integrins (Perutelli, 1995).

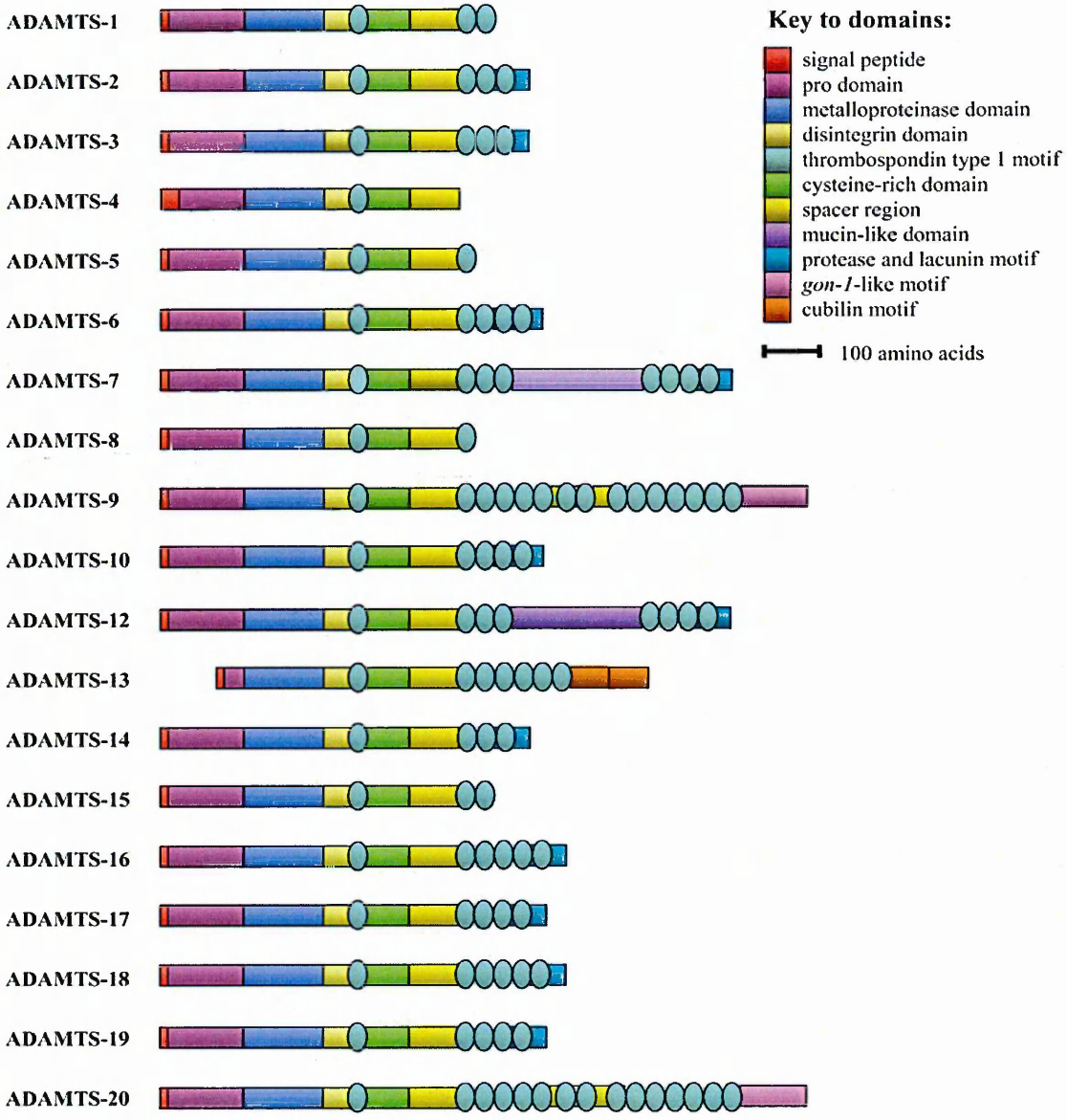


Figure 1.7: Domain structure of ADAMTS proteins Reproduced with permission, from S. Porter, I.M. Clark, L. Kevorkian and D.R. Edwards, (2005), The ADAMTS metalloproteinases, (386), (15-27).© the Biochemical Society.

1.5.2.4 The Cysteine-rich Domain (CRD)

This domain is a cysteine rich sequence containing ten cysteine residues. Little is known of the function of this domain. However, the expression of various domain-deletion constructs of murine ADAMTS-1 showed that the CRD-spacer sequence is a functional ECM-binding domain (Kuno and Matsushima, 1998).

1.5.2.5 The Spacer Domain

There are no common structural features in this region and it is variable in length. ADAMTSs may undergo an extracellular C-terminal processing in this domain, which may have a significant effect on enzyme activity, localization and substrate specificity. ADAMTS-4 undergoes a C-terminal truncation to generate two isoforms with a distinct reduction in affinity of binding to their substrate (Flannery *et al.*, 2002). Efficient aggrecanase activity requires the presence of GAGs attached to the aggrecan core protein. Flannery *et al*, (2002) demonstrated that full-length ADAMTS-4 (~ 68 kDa) undergoes autocatalytic C-terminal truncation to generate two distinct isoforms (~ 53 kDa and 40 kDa), which showed a clear reduction in affinity of binding to sulphated GAGs. ADAMTS-4 without the spacer region was 53 kDa (Gendron *et al.*, 2007).The C-terminal spacer domains also affect binding of full-length ADAMTS-4 to sulphated GAGs (Flannery *et al.*, 2002).

Kashiwagi *et al*, (2004) found the full-length ADAMTS-4 of 70 kDa was the most effective aggrecanase, but shows less activity against the Glu373-Ala374 bond, the site originally characterised as the target for aggrecanase activity. However, it cleaved the Glu1480-Gly1481 bond in the chondroitin sulphate-rich region of aggrecan. There were considerable changes in substrate specificity upon deletion of the spacer domain. ADAMTS-4 with the C-terminal spacer domain deletion cleaved more effectively both the Glu373-Ala374 and Glu1480-Gly1481 bonds. Processed forms (53 kDa and 40 kDa) cleaved non-PG substrates, decorin and fibromodulin, in addition to the chondroitin sulphate-rich region of aggrecan (Kashiwagi *et al.*, 2004).

1.5.2.6 The Thrombospondin (TSP) - Repeats

The major difference between ADAMTSs, MMPs and ADAMs is the ability of ADAMTSs to bind to the ECM (Kuno and Matsushima, 1998, Somerville *et al.*, 2003, Kashiwagi *et al.*, 2004). They possess a well conserved thrombospondin (TSP)-repeat, homologous to the type I repeat of thrombospondins 1 and 2. The anti-angiogenic activity of ADAMTS-1 and -8 is thought to be mediated through their TSP-repeats (Porter *et al.*, 2005). It has been found that ADAMTS-4 cannot cleave GAG-free aggrecan. Kuno and Matsushima, (1998) demonstrated that ECM binding was mediated through the central and C-terminal TSP-repeats and the spacer region, and that sulphated GAGs were probably binding sites (Kuno and Matsushima, 1998).

With the exception of ADAMTS-4, which has no C-terminal TSP-repeats, all ADAMTSs have between 1 and 14 TSP-repeats, C-terminal to the spacer region (Figure 1.7). C-terminal TSP-repeats are more variable in sequence than the central TSP-repeats. For murine ADAMTS-1 it was demonstrated that C-terminal TSP-repeats had a significant role in binding to heparin (Kuno and Matsushima, 1998). The TSP-repeats of the C-terminus are arranged in groups separated by a short linked sequence between the groups such as in ADAMTS-9 and ADAMTS-20 or a mucin-like domain as in ADAMTS-7 and ADAMTS-12 (Somerville *et al.,* 2004). Other types of domains may be C-terminal to the TSP-repeats groups; ADAMTS-9 and -20 contain gon-1 domains, containing ten conserved cysteine residues (Somerville *et al.*, 2003). ADAMTS-6, -7, -10, -12, -16, -17, -18 and -19 contain a protease and lacunin domain with six conserved cysteine residues (Nardi *et al.*, 1999). Additional C-terminal domains are present in ADAMTS-13 which contains two cubilin domains, (Zheng *et al.*, 2001) (Figure 1.7).

1.5.3 ADAMTS-1, -4 and -5 and Brain ECM Breakdown

A subgroup of ADAMTSs, ADAMTS-1, -4, -5, -8, -9 and -15 are GEPs, cleaving peptide bonds at the carboxyl end of the glutamate residues.

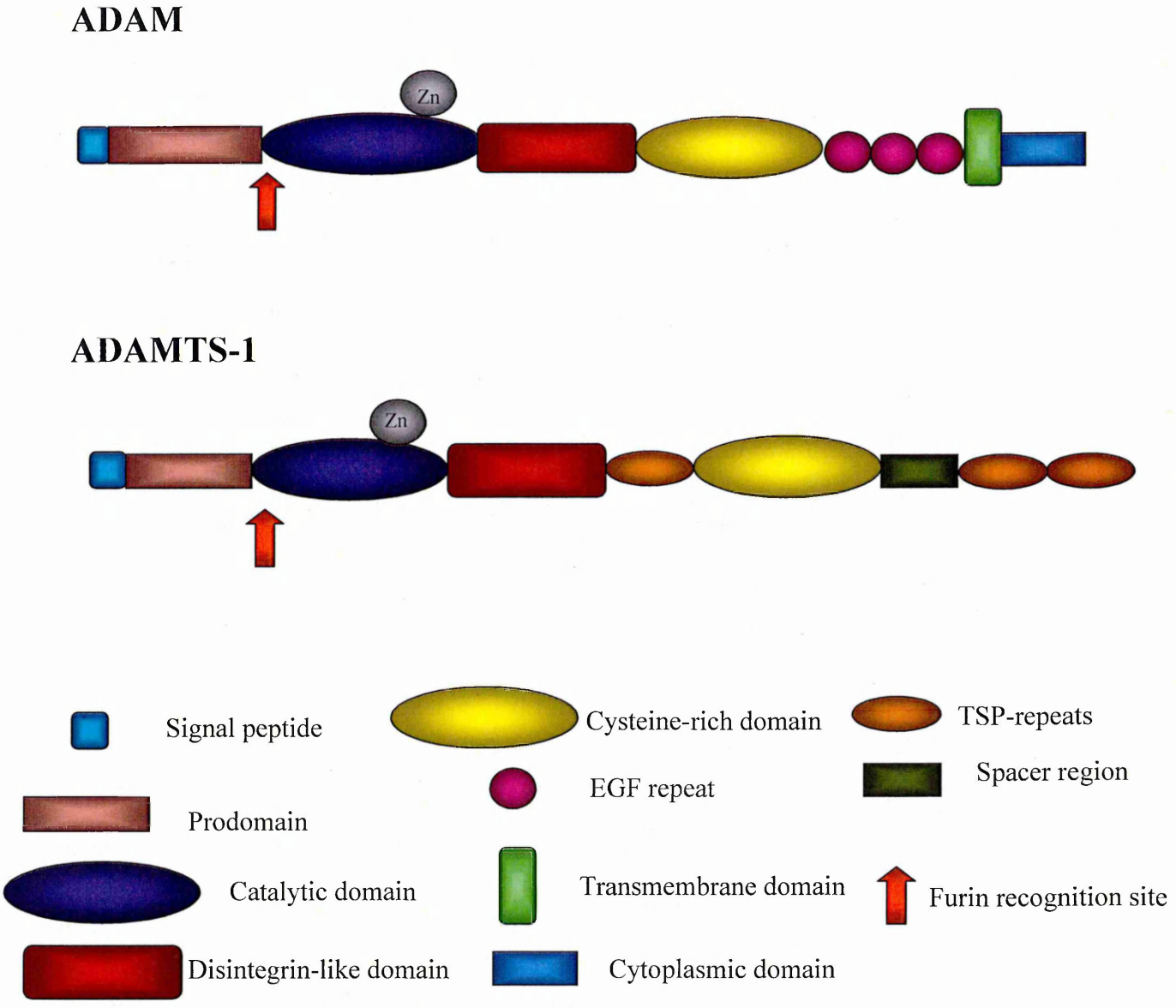


Figure 1.8: Schematic representation of the domain structure of ADAMs and ADAMTSs. A typical ADAM and ADAMTS-1 are similar in structure but most ADAMs have a transmembrane domain that incorporates them into the cell membrane, which ADAMTSs lack and instead have central TSP-repeats with variable numbers at the C-teminus. Most ADAMs and ADAMTS zymogens are processed and converted into a mature form at a furin recognition site by furin proprotein convertase enzymes (red arrow).

They also have been demonstrated to cleave the major cartilage proteoglycan aggrecan, and have been termed 'aggrecanases' (Porter *et al.*, 2005). ADAMTS-1, -4 and -5 also cleave other lectican family members and have other functions as shown in Table 1.1. ADAMTS-1 can cleave both aggrecan and versican. Kuno *et al*, (2000) showed that ADAMTS-1 cleaves aggrecan *in vitro* and that the spacer region is necessary for this. N-terminal sequence analysis of the cleavage product revealed that the chondroitin sulphate attachment domain of aggrecan was cleaved (Kuno *et al.*, 2000).

ADAMTS-4 and -5 are the most extensively studied of the aggrecanases. Previous studies have shown that both enzymes can cleave aggrecan (Ilic *et al.*, 2000) but ADAMTS-4 can also cleave brevican and versican. The main aggrecanase cleavage site of ADAMTS-4 and -5 is Glu373-Ala374 but there are also four other sites in the GAG attachment regions at Glu1480–Gly1481, Glu1667–Gly1668, Glu1771–Ala1772 and Glu1871–Leu1872 (Sugimoto *et al.*, 1999, Tortorella *et al.*, 2000) (Figure 1.9). In addition, ADAMTS-5 exhibited an additional site of cleavage in the region spanning residues Gly1481 and Glu1667, representing a unique cleavage of ADAMTS-5 (Tortorella *et al.*, 2002). Nakamura *et al*, (2000) demonstrated that ADAMTS-4 cleaves brevican at only one site Glu395-Ser396. Furthermore, Nakada *et al* (2005) showed that glioblastoma cells transfected with ADAMTS-4 and -5 produced brevican cleavage products, whereas ADAMTS-1 and un-transfected cells displayed no cleavage (Nakada *et al.*, 2005).

Brain proteoglycans aggrecan, brevican, neurocan, phosphocan, appican and versican components are important in normal homeostasis of the brain (Bandtlow and Zimmermann, 2000, Novak and Kaye, 2000). Alterations in synthesis or breakdown of the ECM may contribute to disease processes. In MS, ECM and BBB breakdown enables inflammatory mediators to migrate to sites of destruction i.e. myelin. Also, it is thought that ADAMTS-1, -4 and -5 mediated degradation of CSPGs may increase access of inflammatory cells that release proinflamatory cytokines TNF and IL-1 (Figure 1.10) promoting axonal damage. Alternatively, ADAMTS-1, -4 and -5 may be beneficial in MS by breaking down CSPGs which accumulate at the edge of MS lesion and which normally have an inhibitory effect on axonal outgrowth and prevent axonal regeneration.

Table 1.1: ADAMTS-1, -4 and -5 functions

ADAMTS	Other names	Functions
ADAMTS-1	METH-1/ Aggrecanase-3	Inflammatory response, anti-angiogenic activity, organ morphogenesis, aggrecan and versican cleavage.
ADAMTS-4	Aggrecanase-1	Aggrecan, brevican, versican cleavage also cleaves fibromodulin, decorin, carboxymethylated transferrin and anti-angiogenic activity
ADAMTS-5	Aggrecanase-2/ ADAMTS11	Aggrecan cleavage

(Porter *et al.*, 2005, Hsu *et al.*, 2012).

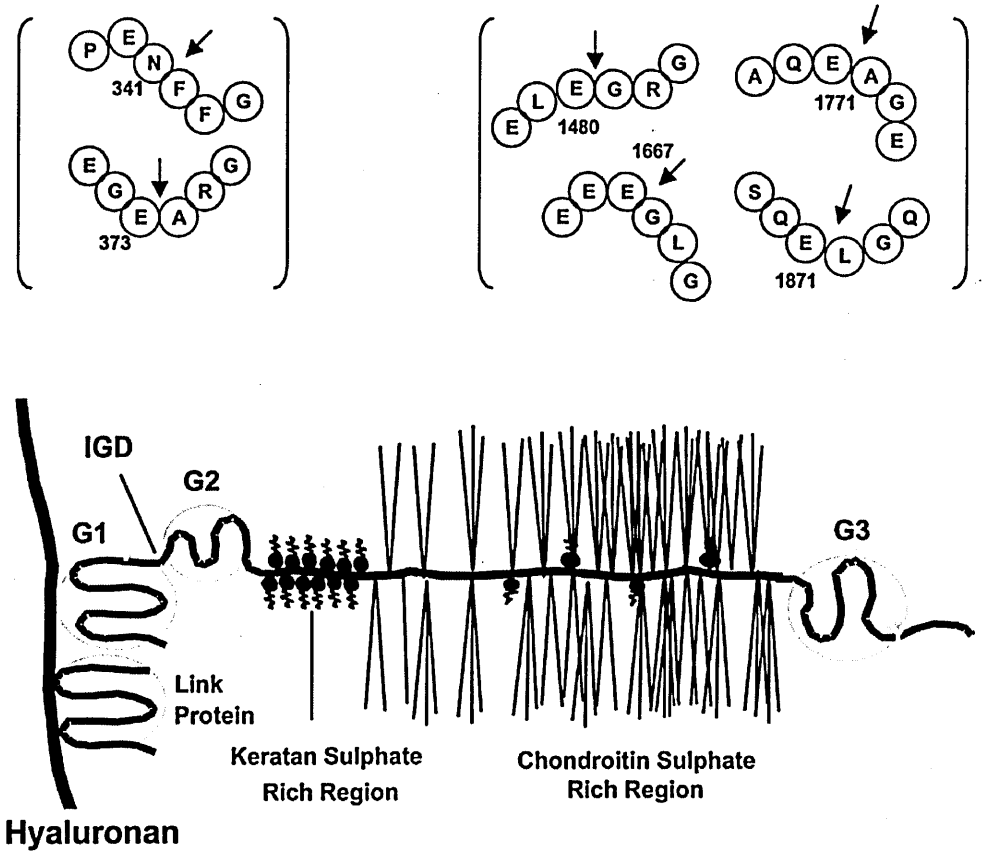


Figure 1.9: Aggrecan cleavage sites. The cleavage sites are located within the G1/G2 interglobular (IGD) domain. The Asn341–Phe342 is the main MMP cleavage site, whereas the Glu373–Ala374 is cleaved by aggrecanases. There are also four cleavage sites within the chondroitin sulphate-rich region of aggrecan for ADAMTS-4 and ADAMTS-5, arrows indicate cleavage sites (Reproduced by permission from Biochem J. from Porter *et al.*, 2005).

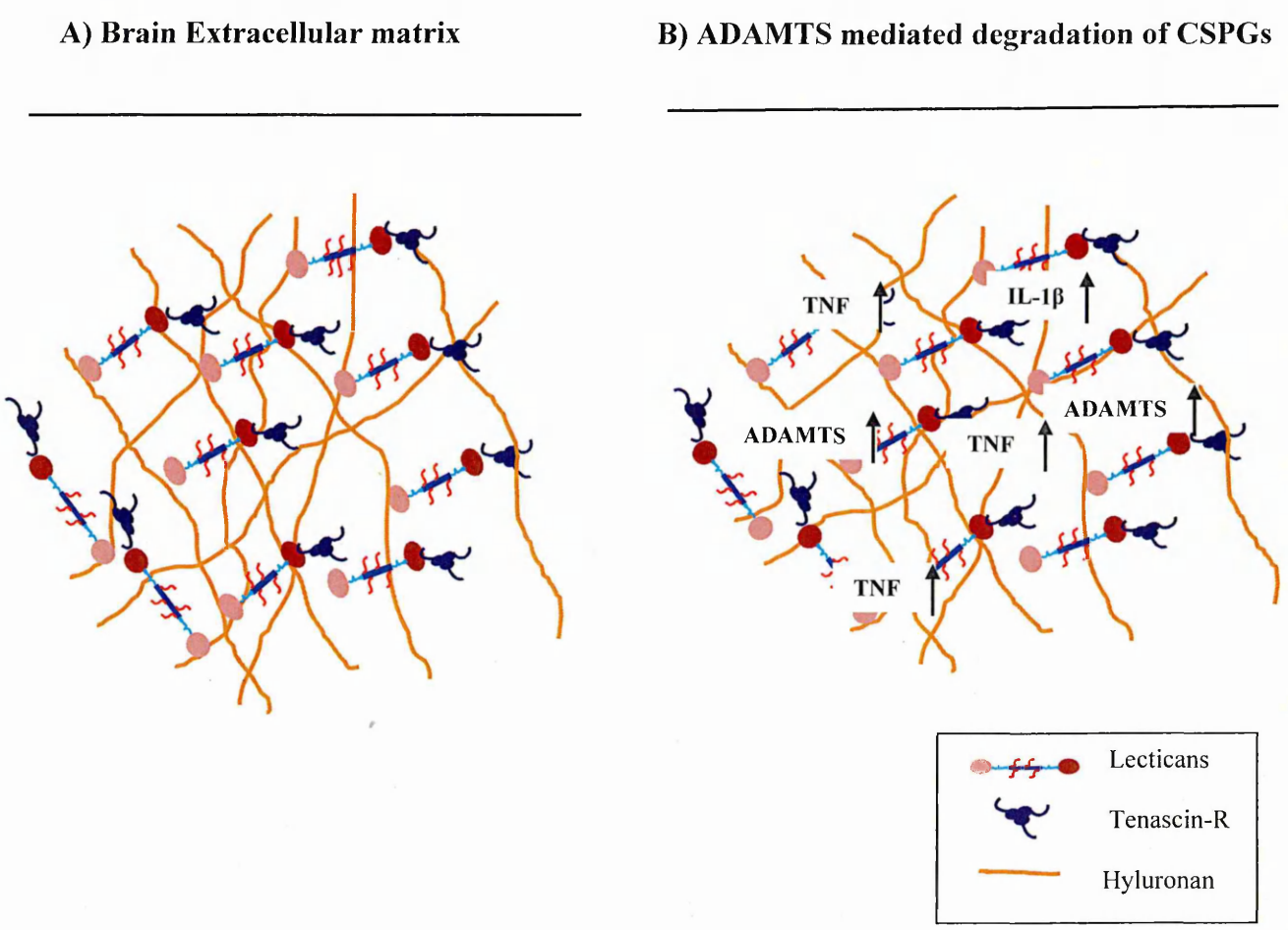


Figure 1.10: A schematic representation of the brain ECM in normal and degraded ECM. It is suggested that lecticans play a central role in assembling a well organised ECM in the adult brain. Lecticans bind the hyaluronan and tenascin-R to form a complex (A). It is thought when degradation of lecticans by ADAMTSs occurs after injury or in MS the ECM is disrupted, the matrix becomes loosened. (B) Such a loose matrix may provide constructive surroundings for cell migration and the infiltration of inflammatory cells and the secretion of cytokines such as IL-1β and TNF and may contribute to the disease (Adapted and redrawn from Viapiano and Matthew, 2006).

1.5.4 Studies of ADAMTS-1, -4 and -5 in MS

Previous studies have shown that MMPs contribute in CNS inflammatory disorders (Yong *et al.*, 1998, Yong, 1999). However, more recent studies have shown that other peptidases such as ADAMTSs are expressed by CNS tissue and modulated in inflammatory CNS disorders (Cross *et al.*, 2006b, Haddock *et al.*, 2006). MS is an inflammatory demyelinating disorder of the CNS and is thought to involve cytokines and proteases. Only a limited number of studies have demonstrated that ADAMTS-1, -4 and -5 are expressed in the CNS. These ADAMTSs are thought to have a significant role in the CNS during the disease process due to their ability to cleave CSPGs, which are essential molecules in the CNS ECM.

Previous studies, performed in our laboratory, demonstrated that in primary human astrocytes *in vitro*, TNF can upregulate ADAMTS-4 expression at both the mRNA and protein level (Cross *et al.*, 2006a). In EAE, differential changes of ADAMTS-1, -4, -5 and TIMP-3 were identified during different stages of CNS inflammation which may contribute to ECM degradation in disease progression (Cross *et al.*, 2006b). In addition, ADAMTS-1, -4 and -5 (mRNA and protein) were expressed in post mortem MS brain tissue. However, immunohistochemical studies demonstrated that ADAMTS-4 protein was increased in post mortem MS brain tissue and it was associated predominantly with astrocytes (Haddock *et al.*, 2006). ADAMTS-4 immunoreactivity was co-localised with brevican in rat brain as shown in Figure 1.11 (Haddock *et al.*, 2007).

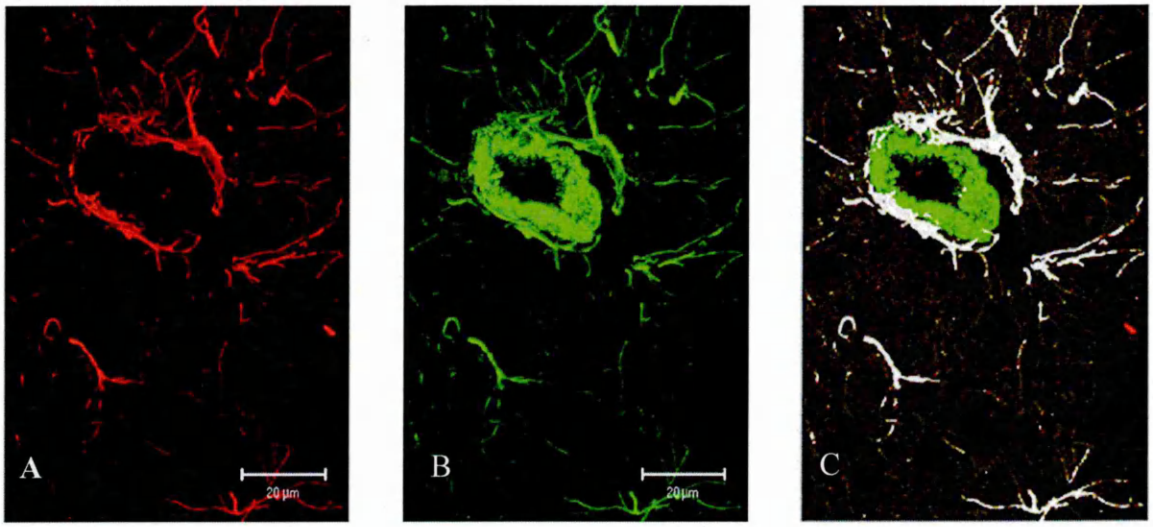


Figure 1.11: Immunohistochemical confocal microscope image of rat CNS tissue following transient middle cerebral artery occlusion (tMCAo). (A) ADAMTS-4, (B) brevican and (C) co-localisation is represented in white in the merged image (Haddock *et al.*, 2007).

1.6 Tissue Inhibitors of Metalloproteinases (TIMPs)

Metalloproteinase activity is regulated by a group of physiological inhibitors (TIMP-1, -2, -3 and -4), the tissue inhibitors of metalloproteinases (TIMPs) (Amour *et al.*, 1998, Yu *et al.*, 2000, Borland *et al.*, 1999). TIMPs are proteins of between 21 and 34 kDa, with twelve conserved cysteine residues. The proteins are folded into two domains, with all the TIMPs containing a conserved binding site for proteinases, in the domain responsible for the inhibitory activity, the N-terminal domain (Lambert *et al*, 2004). TIMP-3 has been shown to be the only member of the TIMP family able to effectively inhibit the actions of ADAM-17 (Amour *et al.*, 1998). It is also known as the main inhibitor of ADAMTSs, although TIMP-1 and TIMP-2 are inhibitory at higher concentrations (Hashimoto *et al.,* 2001). TIMP-3 is the only TIMP known to bind to the ECM (via GAGs) (Yu *et al.*, 2000), suggesting it has the potential to inhibit ADAMTSs in cartilage or brain.

Hashimoto et al (2001) suggests that TIMP-3 can inhibit aggrecanase activity of ADAMTS-4 *in vivo* (Hashimoto *et al.*, 2001). While Kashiwagi *et al* (2001) demonstrated that TIMP-3 blocked ADAMTS-4 and -5 cleavage of aggrecan *in vitro*. In contrast, TIMP-1 and TIMP-2 had no effect on aggrecanase activity of both ADAMTSs. 35% inhibition of ADAMTS-4 was shown with TIMP-4 in high concentrations but there was no inhibition of ADAMTS-5 (Kashiwagi *et al.*, 2001).

1.7 The Aims and Objectives of this Study

Hypothesis

- Neuronal ADAMTS-1, -4 and -5 expression is modulated in response to proinflammatory cytokines in MS.
- Increased ADAMTS activity in the brain causes breakdown of ECM components in MS

Aim

The main aim of this project was to elucidate a role for neuronal ADAMTS-1, -4 and -5 in the pathogenesis of multiple sclerosis. Their presence has been shown previously in MS and normal CNS tissue but their production by neuronal cell lines has received little attention and no studies have looked at neoepitopes versican (V0/V2) produced by these ADAMTSs in the CNS as a result of their specific GEP activity. This study may indicate the role of these enzymes in MS. To achieve this aim, the objectives were:

- *In vitro* investigations of the ADAMTS-1, -4, and -5 gene expression and their modulation by cytokines IL-1 and TNF. These cytokines have been implicated in the pathogenesis of MS, as it is highly likely ADAMTSs are induced by cytokines during the pathogenesis of the disease. This was achieved by using the human neuroblastoma cell lines SHSY-5Y and SK-N-DZ with and without retinoic acid differentiation as neuronal models.

- Studies of the expression ADAMTS-1 at the protein level. ADAMTS-1 was the most highly expressed ADAMTS at the mRNA level. ADAMTS-1 has been previously shown to be increased at the mRNA level in rat hypoglossal motor neurons following nerve injury (Sasaki *et al.*, 2001). Knockdown of ADAMTS-1 and peptide blocking experiments were performed to confirm the identity of ADAMTS-1 on western blotting and verify the specificity of the antibody used.

- Studies of CSPG breakdown in normal and MS CNS post mortem tissue by ADAMTS. This was investigated via a study of neoepitopes of ECM component versican, as a potential measure of ADAMTS activity in normal and MS CNS tissue

using immunohistochemistry. The accumulation of CSPGs around lesions forms a barrier which inhibits neurite outgrowth and prevents axonal regeneration (Sobel and Ahmed, 2001). Breakdown of CSPGs by ADAMTSs may enable axonal outgrowth and repair. Conversely, it may increase the access of inflammatory cells and the secretion of cytokines such as IL-1β and TNF and may promote axonal damage.

Chapter 2

Materials and Methods

2.1 Materials

Materials used in this study and their details i.e. catalogue number, supplier and the methodology used are shown in Table 2.1.

2.2 Cell Culture

2.2.1. Human neuroblastoma SHSY-5Y and SK-N-DZ cell lines

All cell culture procedures were carried out under sterile conditions in a HERA safe class II laminar flow cabinet. Two human neuroblastoma cell lines (SHSY-5Y cell line and SK-N-DZ) were used in this study and their details are shown in Table 2.2. These cell lines have an adherent mode of growth with neuronal cell morphology. Cells were maintained at 37°C in 5% CO_2/95% air in a Hera Cell incubator (Heraeus Instruments, Kandro Laboratories Products, and Germany). Complete media was changed every 3 days until they became 80-90% confluent.

When cells were 80-90% confluent, media were discarded and cells rinsed in 5-10 ml phosphate buffer saline (PBS (GIBCO®) without Ca^{2+} and Mg^{2+}). 1.5 or 3 ml of trypsin-ethylenediaminetetraacetic acid (EDTA) (0.5% trypsin and 0.53 mM EDTA (GIBCO® (TE)) was added to a culture flask (25 cm^2 or 75 cm^2 respectively) to detach the cells from the substratum. The flask was gently rocked back and forth to ensure all the cells were covered with the TE. The cells were incubated at 37°C for 2 minutes or until the cells began to detach. The flask was removed from the incubator and the cells were monitored under the microscope to ensure the majority of cells were detached. They were removed from the flask into a sterile falcon tube and the flask was rinsed with a volume of media equal to that of the TE used and these were pooled.

Table 2.1: Materials utilised in this study

No.	Materials	Catalogue number	Supplier	Methodology
1	Acetone 2.5 litre	A/0560/17	Fisher Scientific, UK	IHC
2	Actin antibody produced in rabbit (IgG fraction)	A5060	Sigma-Aldrich, UK	WB
3	ADAMTS-1(L-16), antibody	sc-31080	Santa Cruz Biotechnology	WB/IHC
4	ADAMTS-1(L-16), blocking peptide	sc-31080-P	Santa Cruz Biotechnology	WB/IHC
5	ADAMTS-1carboxyterminal end (human) rabbit polyclonal	ab39194	Abcam limited, UK	WB
6	ADAMTS-1carboxyterminal end (human) rabbit polyclonal	RP1-ADAMTS1	Triple Point Biologics, Inc	WB
7	Agarose multipupose	Bio-41026	Bioline	RNA gel electrophoresis
8	Alexa Fluor 568 goat anti-mouse (red) 2mg/ml (0.5 ml)	A11004	Invitrogen, UK	IHC
9	Alexa Fluor 568 goat anti-rabbit (red) 2mg/ml (0.5 ml)	A11011	Invitrogen, UK	IHC
10	Analytical balance	aB104-S	Mettler-Toledo Ltd	Weighing materials
11	Azo Wipe®, Bactericidal wipes	Hyg-231-001T	Fisher Scientific, UK	Bench disinfection before and after experiments
12	Bicinchonic Acid kit for protein determination	BCA1-1KT	Sigma-Aldrich	Protein estimation
13	Cell Dewar	-	Forma Scientific,USA	Cell storage for cell culture
14	Chondroitinase ABC from Proteus vulgaris	C3667	Sigma-Aldrich	IHC
15	Choroform, 4 X 25 mL	C2432	Sigma-Aldrich	RNA extraction
16	Clear-view snap-cap microtubes, size 0.6 ml, assorted colors, pack of 1000	T2566	Sigma-Aldrich	cDNA synthesis / Real Time RT-PCR
17	Coverslip glasses 22 X 22 mm	FB58633	Fisher Scientific, UK	Weighing materials

Table 2.1 (continued): Materials utilised in this study

No.	Materials	Catalogue Number	Supplier	Methodology
18	Coverslip glasses 22 X 50 mm	FB58661	Fisher Scientific, UK	IHC
19	Cryo M-bed	CO28	TAAB laboratories Equipments, UK	IHC
20	Cryo spray freezer spray 175g	KNA-0173-00A	Cell Path Ltd	Cryo-sectioning IHC
21	Dimethyl sulfoxide (DMSO) 100 ml	D4540	Sigma-Aldrich, UK	Cell preservation
22	Disposable centrifuge tube, sterile, polypropylene, 15 ml, 50/bag	05-539-12	Fisher Scientific, UK	Cell culture
23	Disposable centrifuge tube, sterile, polypropylene, 50 ml, 50/bag	05-539-8	Fisher Scientific, UK	Cell cuture
24	dNTP 4X 100 µmol	Bio39026	Invitrogen, UK	cDNA synthesis
25	DPX mountant for histology	44581	Sigma-Aldrich, UK	Histology
26	Dry block heating system	QBD2	Grant Instrument (Cambridge) Ltd	cDNA synthesis, WB
27	Dulbecco's modified eagle medium DMEM + GlutaMAX 1X	31965-023	Invitrogen, UK	Cell culture
28	Dulbecco's phosphate buffer saline (D-PBS) 10X GIBCO®500 ml	14200-067	Invitrogen, UK	Cell culture
29	Dulbecco's phosphate buffer saline (D-PBS) 1X GIBCO® 500 ml	14190-094	Invitrogen, UK	Cell culture
30	ECL Plus western blotting detection reagents ECL Plus western blotting detection reagents	RPN 2132	GE Healthcare LTD, UK	WB
31	Eosin Y solution 500 ml	HT110116	Sigma-Aldrich, UK	IHC
32	Eppendorf LoBind microcentifuge tubes DNA/RNA, volume 1.5 ml, pack of 250	Z666548	Sigma-Aldrich	RNA extraction
33	Ethanol for molecular biology, 500 ml	E7023	Sigma-Aldrich	RNA/ Protein extractions
34	Ethanol, 2.5 litre	M/4450/17	Fisher Scientific, UK	ICC

Table 2.1 (continued): Materials utilised in this study

No.	Materials	Catalogue number	Supplier	Methodology
35	Filter paper	FAB-OFF-330W	Fisher Scientific, UK	Stain filtration
36	Filter tips (101-1000µL), sterile	S1122-1830	Starlab (UK) Ltd	RNA/Protein extraction, estimation and qRT-PCR
37	Filter tips (1-10µL), sterile	S1121-3810	Starlab (UK) Ltd	RNA/Protein extraction, estimation and qRT-PCR
38	Filter tips (1-200µL), sterile	S1120-8810	Starlab (UK) Ltd	RNA/Protein extraction, estimation and qRT-PCR
39	Filter tips (1-20µL), sterile	S1120-3810	Starlab (UK) Ltd	RNA/Protein extraction, estimation and qRT-PCR
40	Foetal bovine serum	10106-169	Invitrogen, UK	Cell culture
41	GAPDH antibody [6C5] 100µg	AB8245	Abcam limited, UK	IHC
42	Glass racks	E98	Raymond A lamb limited	IHC
43	Glial Fibrillary Acid Protein (GFAP)	MAB3402	Millipore, Ireland	IHC
44	Glycerol gelatine	GG1	Sigma-Aldrich, UK	Histology
45	Goat anti- rabbit IgG HRP	A9169	Sigma-Aldrich, UK	WB
46	Guanidine hydrochloride 100g	G3272	Sigma-Aldrich, UK	Protein estimation
47	Harris hematoxylin solution 1 Litre	HHS32	Sigma-Aldrich, UK	IHC
48	HERA cell incubator	-	Heraeus Instruments, Germany	Cell culture
49	HERA safe class II laminar flow cabinet	-	Heraeus Instruments, Germany	Cell culture
50	Hoechst 33342	B2261	Sigma-Aldrich, UK	siRNA knockdown
51	Hybond blotting paper (20x20cm)	RPN6101M	GE Healthcare LTD, UK	WB
52	Hybond-C extra (20cm x3m)	RPN203E	GE Healthcare LTD, UK	WB

Table 2.1 (continued): Materials utilised in this study

No.	Materials	Catalogue number	Supplier	Methodology
53	ImmEdge hydrophobic barrier pen (2 pen set)	H-4000	Vectors Laboratories UK Ltd	IHC
54	Immuno slide staining tray (24 slides)	E103.3	Raymond A lamb limited	IHC
55	Lab-Tek II (8-well chamber slide with cover)	TKT-210-916Y	Fisher Scientific, UK	ICC
56	Large glass troughs with glass lid	E105	Raymond A lamb limited	ICC/IHC
57	Light microscope with Xli camera and XLiCAP image software	N/A	Leica Microsystems, UK Ltd	Cell culture
58	Marvel original dried skimmed milk powder	N/A	N/A	WB
59	Methanol 2.5 litre	M/3950/17	Fisher Scientific, UK	WB
60	Micro Amp Optical 384-well reaction plate with bar code (50 plates /package)	4309849	Applied Biosystem, UK	qRT-PCR
61	Micro Amp Optical 96-well reaction plate with bar code	4314320	Applied Biosystem, UK	qRT-PCR
62	Micro Amp Optical Adhesive films (100) PCR compatible, DNA/ RNA/ RNase free	4311971	Applied Biosystem, UK	qRT-PCR
63	Millex syringe-driven filter unit	SLGP033RS	Millipore, Ireland	Liquid sterilization
64	Mini transfer blot (Electrophoretic transfer cell)	170-3930	Bio-Rad, Hertfordshire, UK	WB
65	MOG monoclonal (clone z12) Kind gift from Dr Stephen McQuaid Queens University Belfast	-	-	IHC
66	Monoclonal mouse anti- human von Willebrand Factor clone F8/86	M 0616	Dako/UK	IHC
67	Nalgene ® 2 ml Cryo 1°C freezing container	CRY-120-010T	Fisher Scientific, UK	Cell culture
68	Nalgene ® 2 ml cryowareTM Cryogenic vials 25/bag	5000-0020	Fisher Scientific, UK	Cell culture
69	Neubauer-Improved Haemocytometer chamber	MNK-510-020J	Fisher Scientific, UK	Cell count
70	Neurofilament light (NF L) antibody (KA2) neuronal marker 68 kDa	ab7255	Abcam limited, UK	ICC/IHC

Table 2.1 (continued): Materials utilised in this study

No.	Materials	Catalogue number	Supplier	Methodology
71	Nitrile medium, powder-free, disposable gloves	SAR-265-050G	Fisher Scientific, UK	General purposes
72	Normal donkey serum	sc-2044	Santa Cruz Biotechnology	IHC
73	Novex X cell II mini cell electrophoresis	EI9001	Invitrogen, UK	Electrophoresis
74	Nuclease free water 1 litre	W4502	Sigma- Aldrich, UK	RNA extraction
75	NunclonTM delta surface cell culture flasks, T25	156367	Fisher Scientific, UK	Cell culture
76	NunclonTM delta surface cell culture flasks, T75	156499	Fisher Scientific, UK	Cell culture
77	NunclonTM delta surface cell culture plates with lid, 24-well	142475	Fisher Scientific, UK	Cell culture/cytokine treatment
78	NunclonTMdelta surface cell culture plates with lid, 6-well	140675	Fisher Scientific, UK	Cell culture
79	Nupage 10% Bis-Tris gel 1.0 mm x12 well box	NP0301	Invitrogen, UK	WB
80	NuPage anti oxidant	NP0005	Invitrogen, UK	WB
81	NuPage LDS sample buffer (4X) 10ml	NP0007	Invitrogen, UK	WB
82	Nupage MOPs SDS running buffer (20X)	NP0001	Invitrogen, UK	WB
83	NuPage sample reducing agent	NP0009	Invitrogen, UK	WB
84	NuPage transfer buffer	NP0006-1	Invitrogen, UK	WB
85	Oil Red O (ORO) 25g	625	Sigma-Aldrich, UK	IHC
86	Olympus BX60 fluorescence microscope with cool snap colour digital camera	-	Olympus Media Cybernetics, Silver Spring, USA	ICC/IHC
87	Olympus BX51 fluorescence microscope with ColorView III digital colour camera	-	-	IHC
88	ADAMTS1 On-TARGET plus SMART pool (5 nmol)	L-005761-00-0005	Perbio Science UK Ltd	siRNA knockdown
89	On- TARGETplus GAPDH control pool (5 nmol)	D-001830-10-05	Perbio Science UK Ltd	siRNA knockdown
90	On- TARGETplus non-targeting pool (5 nmol)	D-0018310- 10-05	Perbio Science UK Ltd	siRNA knockdown

Table 2.1 (continued): Materials utilised in this study

No.	Materials	Catalogue number	Supplier	Methodology
91	Paraformaldehyde (PFA) powder 95%	158127	Sigma-Aldrich, UK	ICC/IHC
92	Pechiney plastic packaging parafilm M laboratory wrapping film	13-374-10	Fisher Scientific, UK	Covering mixtures
93	Penicillin/Streptomycin Solution liquid [(5000 units/ml)]	5070-063	Invitrogen, UK	Cell culture
94	Pipette Tips, 1000 µL blue	FB31611	Fisher Scientific, UK	Multi- purposes
95	Pipette Tips, 200 µL yellow	FB34531	Fisher Scientific, UK	Multi-purposes
96	Propanol for molecular biology, 4x25ml	I9516	Sigma-Aldrich, UK	RNA/Protein extraction
97	Propidium iodide Solution (1.0 mg/ml in H_2O)	P4864	Sigma-Aldrich, UK	ICC
98	Protease inhibitor cocktail for general use	P2714	Sigma-Aldrich, UK	Deglycosylation (WB)
99	Rabbit anti-mouse IgG (H+L)-fluorescein iothiocyanate (FITC)	61-6511	Invitrogen, UK	ICC
100	Random primers 9 units	48190-011	Invitrogen, UK	cDNA synthesis
101	Recombinant human Interferon gamma,20µg	300-02	PeproTech, UK	Cytokine treatment
102	Recombinant human Interleukin-1 beta, size B, 50µg	200-01B	PeproTech, UK	Cytokine treatment
103	Recombinant human Tumor Necrosis Factor alpha, size B, 50µg	300-01A	PeproTech, UK	Cytokine treatment
104	Restore™ Plus western stripping buffer 500ml	46430	Thermo Scientific, UK	WB
105	Retinoic >98% (HPLC) powder	R2625	Sigma- Aldrich, UK	Retinoic acid cell differentiation
106	RNA polymerase II gene expression assay	Hs00992801_ml	Applied Biosystem, UK	qRT-PCR
107	RNaseOUT, 5000 units (Recombinant Ribonuclease Inhibitor)	10777-019	Invitrogen, UK	cDNA synthesis
108	SeeBluePlus2 molecular weight marker	LC5925	Invitrogen, UK	WB

Table 2.1 (continued): Materials utilised in this study

No.	Materials	Catalogue Number	Supplier	Methodology
109	Shandon MB Dyna sharp microtome blade (50 blades 34°/ 80 mm)	3050836	Thermo Electron Corporation	Cyro-sectioning (IHC)
110	SHSY-5Y cell line	94030304	ECACC, UK	Cell culture
111	siGLO red transfection indicator (5 nmol)	D-001600-01-05	Perbio Science, UK Ltd	ADAMTS-1 knockdown
112	SIGMAFAST 3, 3' Diaminobenzidine (DAB) tablets	D4418	Sigma- Aldrich, UK	Western blotting
113	SK-N-DZ cell line	94092305	ECACC, UK	Cell culture
114	Sodium acetate trihydrate	S-7670	Sigma-Aldrich, UK	IHC (deglycosylation)
115	Sodium chloride 1 kg	S7653	Sigma-Aldrich, UK	Western blotting
116	Sodium dodecyl sulphate (SDS) powder	L-3771	Sigma-Aldrich, UK	Protein estimation
117	Sovall Benchtop centrifuge	RT7 PLUS	Kendro Laboratory Products, Newtown, USA	Cell culture
118	Sudan Black B stain (25g)	199664	Sigma-Aldrich, UK	IHC
119	Superscript II reverse transcriptase 10,000 units	18064-022	Invitrogen, UK	cDNA synthesis
120	Taqman gene expression assay (ADAMTS-4)	Hs00192708_ml	Applied Biosystem, UK	qRT-PCR
121	Taqman gene expression assay (ADAM-17)	Hs00234224_ml	Applied Biosystem, UK	qRT-PCR
122	Taqman gene expression assay (ADAMTS-1)	Hs00199608_ml	Applied Biosystem, UK	qRT-PCR
123	Taqman gene expression assay (ADAMTS-5)	Hs000199841_ml	Applied Biosystem, UK	qRT-PCR
124	Taqman gene expression assay (GAPDH)	Hs99999905_ml	Applied Biosystem, UK	qRT-PCR
125	Taqman gene expression assay (TIMP-3)	Hs00165949_ml	Applied Biosystem, UK	qRT-PCR

Table 2.1 (continued): Materials utilised in this study

No.	Materials	Catalogue number	Supplier	Methodology
126	Tri-reagent	T9424	Sigma-Aldrich, UK	RNA Extraction
127	Tris-hydroxymethyl-methlamine	T/3710/60	Fisher Scientific, UK	WB
128	Trypsin- EDTA 0.05% 1X GIBCO®	25300-062	Invitrogen, UK	Cell culture
129	Tween 20	P1379	Sigma-Aldrich, UK	Western blotting
130	Ultra violet product (UVP) bioImaging systems, Epichemi II Darkroom, with Labworks image acquisition and analysis software	-	Bio-Rad, Hertfordshire, UK	Western blotting
131	Vectashield mount medium with DAPI	H-1200	Vectors Laboratories, UK Ltd	IHC
132	Versican V0/V2 Neo polyclonal antibody	PA3-119	Perbio Science , UK Ltd	IHC
133	Wallac Victor^2 1420 multi label counter with Wallac 1420 manager V 3.0	-	Perkin Elmer, USA	Protein estimation
134	Weigh boats, large	FB50337	Fisher Scientific, UK	Weighing materials
135	Weigh boats, medium	FB50335	Fisher Scientific, UK	Weighing materials
136	Weigh boats, small	FB50333	Fisher Scientific, UK	Weighing materials
137	Xylene	534056	Sigma-Aldrich, UK	Histology

Cells were centrifuged in a Sorvall RT7 plus centrifuge (Kendro Laboratory Products, Newtown, USA), at 200 x *g* for 10 minutes and the supernatant discarded. The cell pellet was resuspended in 1 ml of complete culture media and mixed gently by pipetting up and down. Cells were counted using a haemocytometer and the number of cells in the central and four corner squares of the middle square (25 small squares) of the counting area were counted. Then the number of cells per ml was calculated using the following equation:

Cell per ml = Total cell count in 5 squares x 5 x 10^4.

The counted cells were further diluted to the densities required for experimental procedures.

2.2.2. Cryopreservation of Neuroblastoma Cells

Harvested cells were resuspended and mixed gently in HIFCS by pipetting and prior to making aliquots. A cryopreservative, dimethyl sulfoxide (DMSO, Sigma Aldrich, and Poole, UK) was added to a final concentration 10%, which prevents ice crystal formation in the cells when freezing them. 1 × 10^6 cells/ml were put in cryovials, which were then placed in a Cryo 1°C freezing container (Nalgene, Hereford, UK) containing isopropanol (-80°C overnight) in order to gradually lower the temperature by 1°C/minute for maximal cell preservation. Cryovials were stored in a Dewar (Forma Scientific Inc., Ohio) containing liquid nitrogen. When cryovials of cells were removed from the liquid nitrogen storage for culture they were immediately taken to be thawed by placing the vials in a 37°C water bath. 1 ml of pre-warmed complete medium was added to the cells, mixed and then they were quickly transferred to cell culture flasks (75 cm^2) containing 15 ml pre-warmed complete culture media. The flasks were gently agitated to distribute the cells evenly and then incubated at 37°C in 5% CO_2/95% air until they became 80-90% confluent.

Table 2.2: The details of neuroblastoma cell lines and media composition used in this study

Cell Line / Cat. No.	Supplier	Origin	Media Composition
SHSY-5Y/ 94030304	European Cell Culture Collection (ECACC)	Bone marrow of female	-Dulbecco's Modified Eagle's Medium (DMEM) containing glutamax -10% v/v heat–inactivated foetal calf serum (HIFCS) - 1% v/v penicillin (100 U/ml) and streptomycin (100 µg/ml).
SK-N-DZ/ 94092305	European Cell Culture Collection (ECACC)	Bone marrow of a female	-Dulbecco's Modified Eagle's Medium (DMEM) containing glutamax - 10% v/v heat–inactivated foetal calf serum (HIFCS) - 1% v/v penicillin (100 U/ml) and streptomycin (100 µg/ml) 1% non essential amino acids

2.3 Characterisation of SHSY-5Y and SK-N-DZ Neuroblastoma Cell Lines with a Neuronal Marker Neurofilament L (NF L)

Neurofilaments are 10 nm intermediate filament proteins found specifically in neurons, which are the main structural elements of neuronal axons and dendrites. They are composed predominantly of three major proteins called neurofilaments L, M and H; named according to the apparent molecular size of the mammalian subunits on Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). The light or lowest, NF L, runs at 68-70 kDa, the medium or middle NF M runs at 145-160 kDa and heaviest or highest (NF H) runs at 200-220 kDa. NF L was used as a neuronal marker to characterise both neuroblastoma cell lines (SHSY-5Y and SK-N-DZ) in this study, which is the light or low molecular weight polypeptide with about 68 kDa on SDS-PAGE gels according to the manufacturer's instructions.

Cells were plated out into 8-well chamber slides at a density of 5×10^4 cells /500 µL media/well) and were allowed to adhere for 24 hours. Immunocytochemistry (ICC) was performed as follows: slides were fixed in 4% (w/v) paraformaldehyde (PFA) for 15 minutes. After 2 x 5 minutes washes with PBS, the cells were placed in ice-cold methanol for 4 minutes followed by ice-cold acetone for 2 minutes. Slides were then blocked with blocking buffer (10% normal rabbit serum of the animal in which the secondary antibody was produced), (Sigma-Aldrich, UK) in PBS for 30 minutes to prevent non-specific binding of the secondary antibody. Primary antibody against NF L (mouse monoclonal antibody, Abcam) was diluted (1:500) in PBS (according to manufacturer's instructions). PBS without primary antibody was used as a negative control. Incubation with antibody was performed overnight at 4°C in a humidified chamber. Slides were then washed for 3 x 5 minutes in PBS. The secondary antibody was a rabbit anti-mouse IgG (H+L)-fluorescein isothiocyanate (FITC) (Invitrogen, UK) 1:50 diluted with PBS containing 10% normal rabbit serum, according to manufacturer's suggestions. The secondary antibody was applied for 2 hours at room temperature before washing 3 x 5 minutes in PBS. The nuclei of the cells were counterstained with propidium iodide (Sigma-Aldrich, UK) by diluting the 1 mg/ml stock solution (1:5000) in PBS. 300 µl was added to each well to cover the cells and slides were incubated, for

15 minutes at room temperature in the dark to stain the nuclei orange to red. Then cells were washed 2 x 5 minutes each with PBS to remove unbound dye. Chambers were removed at this step and slides were mounted in citifluor glycerol solution. Cells were viewed and immunofluorescent images were captured using an Olympus BX60 fluorescence microscope with Cool Snap Pro an integrated solution colour digital camera (Olympus Media Cybernetics, Silver Spring, USA). Images were analysed by LabWorks Image Acquisition and Analysis Software.

2.4 Treatment with Cytokines

Cells were passaged into 24-well plates at a density of 1×10^5/well in 1 ml medium and allowed to adhere for 24 hour in a cell incubator. Then the media was aspirated from the wells and cells were washed with PBS to remove any serum that could interfere with the experiment. Cells were then treated with either IL-1β or TNF (recombinant cytokines; Peprotech, UK) in triplicate at 0, 1, 10 or 100 ng/ml for 24 hours in 1 ml serum-free media. Media were removed then 333 µl of Tri-reagent (Sigma-Aldrich, UK) was added per well for RNA and protein extractions. Each experiment was repeated 3 times.

2.5 SHSY-5Y and SK-N- DZ Cell Differentiation with Retinoic Acid

Retinoic acid (RetA), vitamin A can control the expression of genes in the CNS (Clagett-Dame *et al.*, 2005). RetA treatment has been shown to increase extension of neurite outgrowths producing a more neuronal phenotype comparable to *in vivo* neurones (Encinas *et al.*, 2000). Both SHSY-5Y and SK-ND-Z cells were treated for 7 days with RetA (Sigma Aldrich, UK). A 10^{-2} M RetA stock solution was prepared in 100% molecular grade ethanol. Cells were cultured, as described in section 2.2.1 and were seeded in 24 well plates at 1×10^5 cells/well density for the experimental treatments. RetA was added 24 hours after plating at a final concentration of 10^{-5} M in DMEM with 10% HIFCS. After 7 days in the presence of RetA, cells were treated with cytokines in serum free medium for a 24 hour incubation period at 37°C and 5% CO2. These neuroblastoma cell lines were examined for cellular differentiation under the contrast light microscope (Leica Microsystems, Wetzlar, Germany) x100 or x200 and

images were captured with the USB 2.0 camera/XLi-Cap software (XL Imaging Ltd. Swansea, UK).

2.6 RNA and Protein Extraction

2.6.1 Sample Preparation

In accordance with the manufacturer's protocol (Sigma-Aldrich, UK), RNA and protein were extracted from SHSY-5Y or SK-N-DZ cells by lysis in Tri-reagent. For 24 well plates, treatments were carried out in triplicate and 333 µl of Tri-reagent added to each well. For 25 cm^2 culture flasks of cells, 2 ml of Tri-reagent was added to lyse the cells followed by repeated pipetting. Extracts were placed in microcentrifuge tubes (1 ml in each) and stored at -20°C.

2.6.2 RNA Extraction

Samples were left in Tri-reagent for 5 minutes at room temperature to dissociate nucleoprotein complexes. Upon addition of 0.2 ml chloroform/ml Tri-reagent and centrifugation at 12,000 x *g* for 15 minutes at 4°C, the mixture separated into three phases: a lower pink organic phase containing the protein, a white interphase containing DNA and upper clear phase containing RNA. At this step the interphase and the organic phase were stored at -20°C for later protein isolation. RNA was isolated by transferring the upper aqueous phase into a clean microcentrifuge tube and 0.5 ml of isopropanol was added to each tube and centrifuged at 12,000 x *g* for 10 minutes. The RNA was precipitated in pellet form on the side/bottom of the tube. The microcentrifuge tubes were placed in the centrifuge, with the top hinges facing the outside of the rotor to enable the location of the (usually) tiny pellet.

The supernatant was carefully removed and discarded then 1 ml of 75% ethanol added to each pellet. Tubes were vortexed and centrifuged at 8000 x *g* for 5 minutes. The supernatant was discarded and the RNA pellets were briefly air dried for 5-10 minutes and resuspended in nuclease free water (20-30 µl) depending on the size of the pellet. The RNA samples were stored at −20 °C prior to use.

2.6.3 Protein Extraction

Following lysis of cells with Tri-reagent and subsequent removal of the RNA and DNA containing phases, the pink layer containing the protein was divided into two 1.5 ml microcentrifuge tubes. 750 µL of isopropanol was added to each tube, mixed and left to stand for 10 minutes at room temperature. Tubes were centrifuged at 12,000 x *g* for 10 minutes at 4°C and the supernatant was discarded. 1 ml of 0.3M guanidine hydrochloride in 95% ethanol was added to each tube and allowed to stand for 20 minutes and then centrifuged 8,000 x *g* for 5 minutes. The 1 ml of 0.3M guanidine hydrochloride in 95% ethanol and wash steps were repeated two more times. Then the pellet was finally washed in 1 ml of 100% ethanol, vortexed and allowed to stand for 20 minutes at room temperature. This was followed by centrifugation at 8,000 x *g* for 5 minutes and the supernatant was discarded. Protein samples were resuspended in 100 µL 1% SDS and stored at -20°C until used.

2.7 Agarose Gel Electrophoresis of RNA

The quality of intact RNA was determined by the presence of two bands representing 18S rRNA and 28S rRNA subunits on 1% agarose gel after electrophoresis. The basic principle of this method is that nucleic acids are separated by applying an electric current to move the negatively charged molecules through an agarose matrix. Agarose (1%) was dissolved in Tris-boric acid-EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) by heating prior to addition of 5 µL ethidium bromide (EtBr). EtBr fluoresces under UV light when bound to DNA/RNA and allows their visualisation. RNA samples (1 µL) were mixed with 4 µL H_2O and 1 µL of 6 x loading buffer (40% sucrose, 0.01% bromophenol blue, 1x TBE) prior to loading in agarose wells. Electrophoresis was carried out at 100 volts for 30 minutes. Images were captured by UVP Bio-imaging System, USA and analysed with LabWorks Image Acquisition and Analysis Software (UVP, USA) and RNA/DNA gel programme.

2.8 Bicinchoninic Acid (BCA) Protein Assay

The bicinchoninic acid (BCA) (Sigma-Aldrich, UK) protein assay was performed to determine the loading concentration of protein/well for the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The principle of this test is that protein reduces Cu^{2+} ions to Cu^{+} and interacts with the BCA reagent forming a violet-coloured product. Protein concentrations were calculated by plotting a standard curve in Microsoft® Office Excel 2007 of known protein concentrations of bovine serum albumin (BSA) as following 4, 2, 1, 0.8, 0.6, 0.4, 0.2 and 0.1 mg/ml and the unknown samples were read off. The volumes (µl) of unknown protein samples for loading were calculated by the following equation: amount required for loading (6 µg) / the concentration of the unknown sample then multiplied by 2 as the sample was already diluted 1:1 with sample buffer. Dilutions for the standard curve were prepared using 20% BSA stock in 1% sodium dodecyl sulphate (SDS) diluent to yield the concentrations of the previously mentioned standards. Protein samples (in duplicates), standards (0-4 mg/ml) and blank (20 µL) (in triplicate) were added to 96-well plates. BCA reagent was prepared by adding 0.5 ml 4% [w/v] copper [II] sulphate ($CuSO_4$) to 24.5 ml BCA reagent (Sigma-Aldrich, UK). 200 µL was added to each well containing protein samples and incubated for 30 minutes at room temperature. Then absorbance at 570 nm was measured using a Wallac Victor^2 plate reader (PerkinElmer, USA).

2.9 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

2.9.1 Introduction

PCR technology was first developed in 1983 to amplify minute amounts of DNA. It uses the ability of a thermo-stable DNA polymerase enzyme (Taq polymerase) to extend a pair of oligonucleotide primers. The primers are designed to be complementary to each of the DNA fragments to be amplified during repeated cycles of denaturation (~95°C), primer annealing (~55°C) and primer extension (~72°C).

qRT-PCR was developed by Higuchi et al (1992) and has become a common technique, where a RNA strand is reverse transcribed into its DNA complement (cDNA) using the

enzyme reverse transcriptase, and the resulting cDNA is amplified using PCR (Orlando *et al.*, 1998, Bustin, 2000). Benefits of this procedure over conventional methods its sensitivity and accurate quantification. To achieve this, however, appropriate normalisation strategies are required to control for experimental error introduced during the multistage process required.

qRT-PCR has the same basic principles as the classical PCR but the monitoring of accumulating amplicon has been made possible by using fluorescent molecules to label probes. The change in signal which occurs when these labels directly interact with or hybridise to the amplicon is proportional to the amount of amplicon present during each cycle (Wong & Medrano, 2005).

2.9.2 The Amplification Plot

Amplification plots consist of two phases; an exponential phase and a plateau phase (cycle 28–40) Figure 2.1. The amount of product (amplicon) is doubling in each cycle of PCR, resulting in an exponential increase in fluorescence throughout the amplification reaction. However, as the reaction progresses the reactants are being consumed and the reaction slows and at an endpoint the reaction stops (plateau phase). qRT-PCR allows accurate and precise quantification of product during the exponential phase. Therefore, results are more sensitive than conventional PCR, in which products are measured during the plateau phase, where differences in target levels are not proportional.

2.9.3 Cycle Threshold (C_T)

A number of cycles of the qRT-PCR show a background level prior to significant accumulation of the target (cycle 1–18) in Figure 2.1. The cycle number at which the fluorescence generated crosses the threshold known as the cycle threshold (C_T) and is taken into account and used for qRT-PCR results analysis. The C_T values or the number of amplification cycles depends on the amount of target, the larger the amount of

template in the reaction the fewer amplification cycles to form a significant fluorescent signal. In contrast a small amount of the target will require more amplification cycles for the fluorescent signal to generate a detectable fluorescence signal. Thus, the reaction will have a high, or late, C_T.

Relative mRNA levels of the genes in this study were determined using the cycle threshold (C_T) and the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001), where $\Delta\Delta C_T$ is calculated using the following formulae: C_T (target gene) − C_T (housekeeping gene e.g. RNA Polymerase-II) = ΔC_T, and ΔC_T (treated sample) − ΔC_T (control sample) =$\Delta\Delta C_T$.

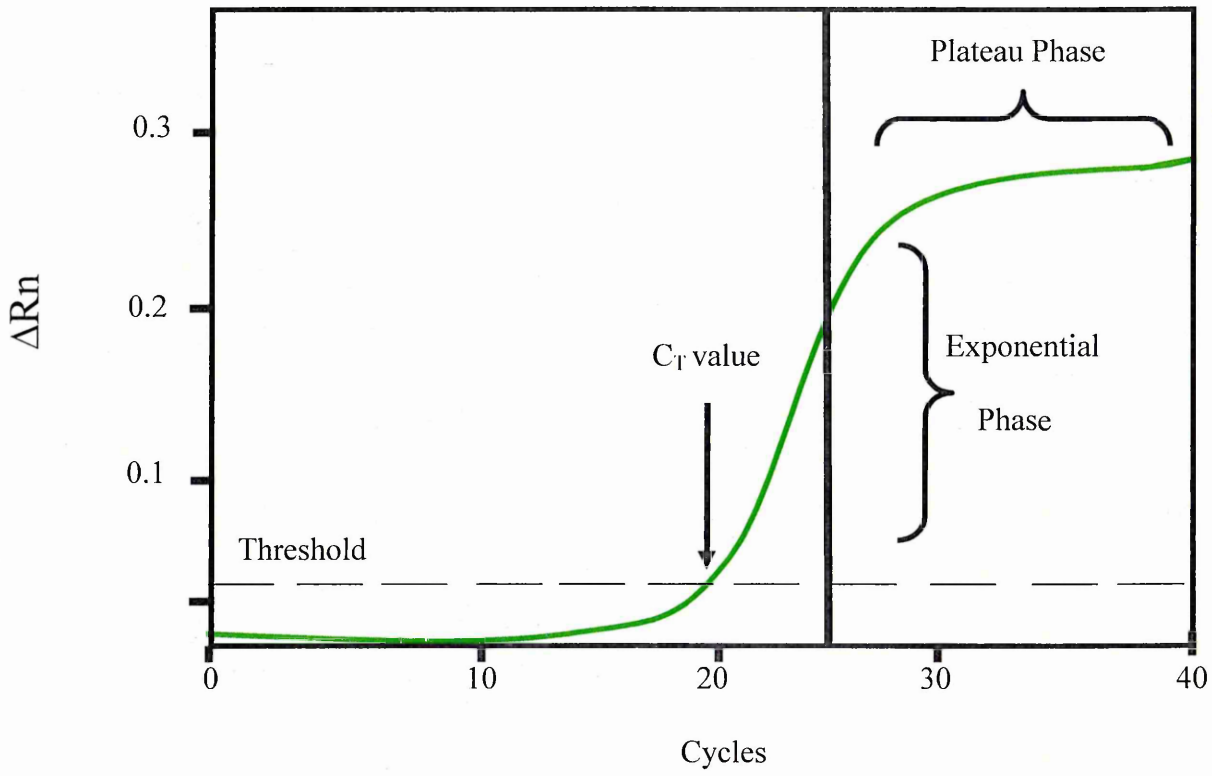


Figure 2.1: qRT-PCR amplification plot. This plot shows the exponential and plateau phases of qRT-PCR amplification. The PCR cycle number is shown on the x-axis, and the change in fluorescence from the amplification, which is proportional to the amount of the amplified product, is shown on the y-axis.

2.9.4 TaqMan Probes

In this study the relative qRT-PCR technique was used with primers and TaqMan probes. TaqMan probes are also called double-dye oligonucleotide or dual labeled probes and are the most widely used type of probes. The principle of the TaqMan probe action is based on the 5'-3' nuclease activity of Taq polymerase to cleave a dual-labeled probe during hybridization to the complementary target sequence and fluorophore-base detection (Figure 2.2). The TaqMan probe consists of single stranded probe sequence that is complementary to one strand of the template DNA. A fluorophore called 6-carboxy fluorescein (FAM) is attached to the 5' end and the quencher, tetramethylrhodamine (TAMRA) is attached to other 3' end. While the probe is intact and is attached or unattached to the template DNA and prior to the polymerase actions, the quencher (Q) (TAMRA) reduces the fluorescence from the fluorophore (F).When the Taq polymerase extends from the primer it displace the 5' end of the probe, which is then degraded by the 5'-3' exonuclease activity of the Taq polymerase. The end result of this process is to separate the fluorophore from the quencher. This leads to an increase in the fluorescence.

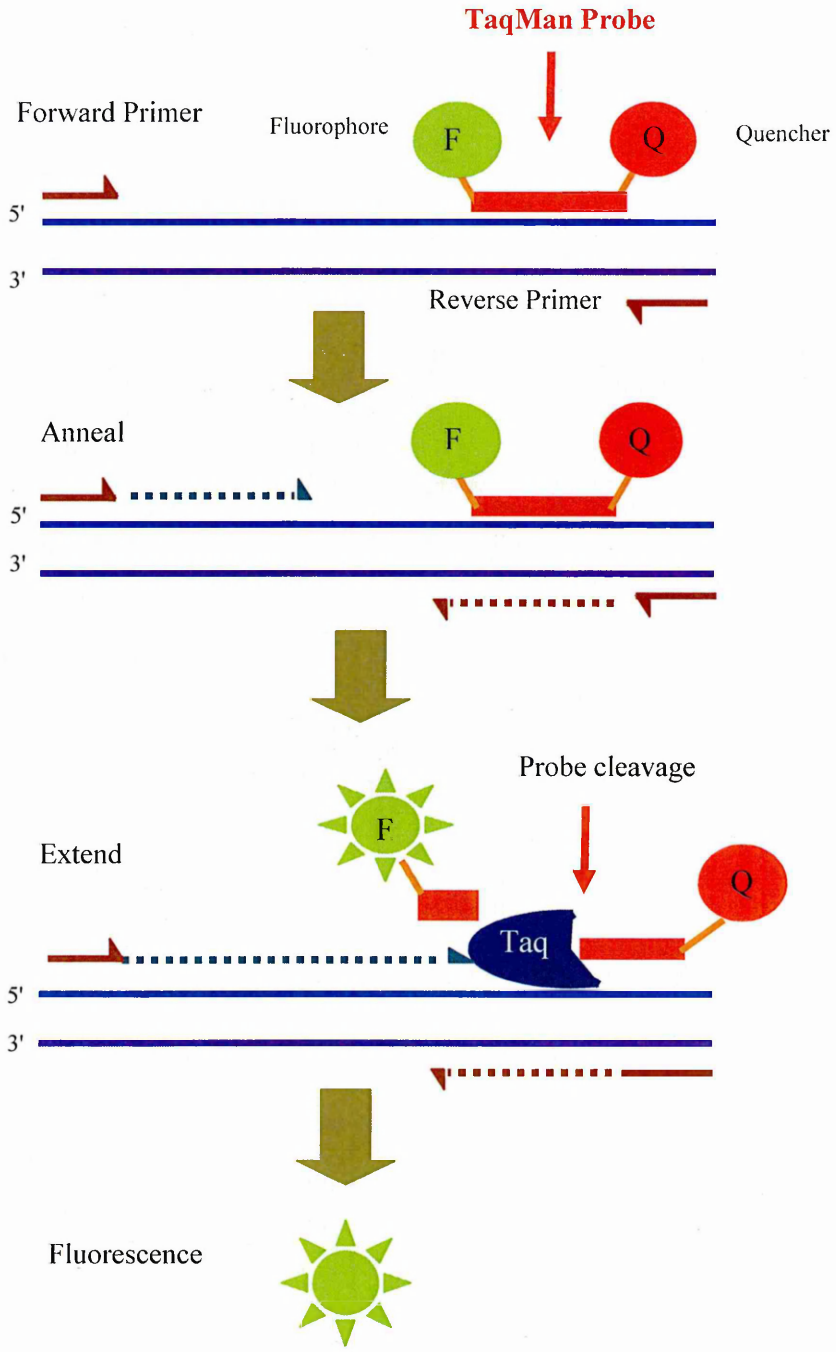


Figure 2.2 TaqMan Probes Action Adapted and redrawn from Walter H. Koch Nature Reviews Drug Discovery **3**, 749-761 (2004)

2.9.5 cDNA Synthesis and qRT-PCR

RNA, extracted as described in section 2.6.2, was reverse transcribed using the SuperScript™ II Reverse Transcriptase (RT). All reagents were from Invitrogen, unless otherwise specified. Filter tips (Starlab, UK) and clear-view snap-cap microtubes, size 0.6 ml were used throughout the preparation of cDNA. The master mix for each RNA consisted of 4 µl 5x first strand buffer, 2 µl dithiothrietol (DTT), 0.5 µl RNAseOut ribonuclease inhibitor, 0.5 µL random primers and 1 µl deoxynucleotide triphosphates (dNTPs), which carry the bases adenine (dATP), cytosine (dCTP), guanine (dGTP) and thymine (dTTP) (Bioline, London, UK) and 9 µl nuclease-free water. 1 µl RNA was mixed with mastermix and 1 µl RT was added to all samples in an RNase-free PCR tube on ice. The primers in the mix bind to the RNA randomly, and the RT inserts dNTPs into the gaps to complete the cDNA strand, which represents all the transcribed RNA. Negative controls were performed for each experiment by using samples set up as above but with either no RT or without RNA. The reaction mix was placed in a heating block set at 42°C for 1 hour and followed by 95°C for 5 minutes to inactivate the RT enzyme. Synthesised cDNA was cooled to 4°C and then stored at -20°C until required.

Synthesised cDNA is used as a template for qRT-PCR and was diluted 1:1 in nuclease-free water. All PCR experiments were prepared on ice. Amplification was carried out in an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Warrington, UK) at The University of Sheffield (Genetics Core Facilities, the School of Medicine and Biomedical Science) and Sheffield Hallam University, the Biomedical Research Centre (BMRC) with the following thermo-cycler programme: 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C, then 1 minute at 60°C.

Primers and FAM labelled probes were used for determination of ADAMTS-1, -4, -5, ADAM-17 and TIMP-3, (Applied Biosystems, Warrington, UK) assay numbers: Hs00199608_m1, Hs00192708_m1 and Hs00199841_ml Hs00234224_ml and Hs00165949_ml respectively). Expression of ADAM-17, TIMP-3 and ADAMTS-1, -4, -5 was normalised against expression of the housekeeping gene RNA polymerase-II

(assay number: Hs00992801_ml). According to the manufacturer's information primer efficiencies of the targets and reference genes were approximately 100%. These were by Applied Biosystems Assays-On-Demand, gene-specific TaqMan® probes (TaqMan® Gene Expression Assays, Applied Biosystems). All information that could be provided from Applied Biosystems is shown in Table 2.3.

2.9.6 Housekeeping Genes for qRT-PCR Validation

Variations due to different amounts of RNA can be excluded by normalization to one housekeeping gene. However, it is important to make sure that the house keeping gene is expressed at constant level throughout the experiment and between the samples. Housekeeping genes that were used in the SHSY-5Y neuroblastoma cell line qRT-PCR experiments were optimised from the following three genes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cyclophilin-A and RNA polymerase-II (RNAP-II), which were analysed for stability as normalisers for qRT-PCR. RNAP-II showed the least variation among the housekeeping genes used in this study, therefore this gene was used in subsequent experiments.

Table 2.3: The details of gene expression assays used in this study

* Gene expression assays	Interrogated sequence Ref sequence	Exon bondary	Amplicon length (bp)
ADAMTS-1	NM_006988.3	1-2	68
ADAMTS-4	NM_005099.4	4-5	63
ADAMTS-5	NM_007038.3	3-4	65
ADAM-17	NM_003183.4	1-2	111
TIMP-3	NM_000362.4	1-2	59

* Sequences: Assays-on-Demand (Applied Biosystems)

2.10 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

2.10.1 General Principle

SDS-PAGE is a commonly used technique for separating proteins in an electric field on the basis of molecular weight. Proteins migrate through a polyacrylamide gel as a support medium. The common electrophoresis technique cannot be used to measure the molecular weight of the biological molecules in biological samples because the mobility of the molecules in the matrix is influenced by both charge and size. The SDS gel electrophoresis, samples are treated with SDS so that proteins have an even negative charge. The molecular weight (MW) of proteins may be estimated if they are subjected to electrophoresis in the presence of the detergent SDS and a reducing agent. SDS disrupts the secondary, tertiary and quaternary structure of the protein and coats it with a negative charge uniformly. Negatively charged SDS coated proteins migrate in the gel relative to their size only and not to their charge. A marker containing proteins of known molecular weights is run in parallel to enable determination of molecular weights of proteins in the test sample.

Proteins from polyacrylamide gels can be transferred electrophoretically to nitrocellulose membranes by western blotting. The presence of the target protein within a sample can be detected on the blot using a specific primary antibody raised against the target antigen. Then it will form an antigen-primary antibody complex. Enzyme-labelled secondary antibodies can then bind to the primary antibodies prior to the detection of the antigen-antibody complex with an appropriate enzyme substrate as shown in Figure 2.3.

2.10.2 SDS-PAGE and Western Blotting Procedure to Determine ADAMTS-1 Protein Expression in SHSY-5Y

All reagents and materials were from Invitrogen, Paisley, UK, unless otherwise mentioned. The extracted proteins from the SHSY-5Y cells (100 µl) were denatured to their primary structures by dilution in 50 µl (4x concentrate) NuPAGE lithium dodecyl sulphate (LDS) sample buffer (pH 8.4), which allows maximal activity of the reducing agent and 20 µl (5x concentrate) reducing agent dithiothreitol (DTT) and 30 µl H_20. The denaturation process involves the breaking of S-S bonds by DTT. The process occurs most efficiently at 60°C for 30 minute as described in Cross *et al*, (2006a).

SDS-PAGE was performed in Novex Minicell tanks with pre-cast gels, 10% NuPAGE Novex Bis-Tris gels (pH 7.0) used in this study, 1 mm thick with 10 wells, in the presence of NuPAGE 1x 3-(N-Morpholino) propanesulfonic acid (MOPS) SDS running buffer (pH 7.7). Protein was loaded per well and gels run for 1 hour at 100 volt (300 mA) as described (Cross *et al.*, 2006a) in detecting ADAMTS-1. Protein samples were run concurrently with 7 µL SeeBlue Plus2 pre-stained marker which contained protein standards of known molecular weight. Protein was transferred to Hybond-C nitrocellulose membranes (GE Healthcare, UK) at 150 volt for 1 hour in the presence of 1X NuPAGE transfer buffer with 10% methanol and 0.1% NuPAGE antioxidant. Following transfer, blots were immediately placed in blocking solution, 5 % non-fat dried milk (NFDM) in Tris-buffered saline (TBS; 20mM Tris, pH 7.4/0.9% NaCl; containing 0.05% Tween 20 (Sigma-Aldrich, UK) (TBST).

2.10.2.1 Optimisation of Abcam and Triple Point Antibodies to Detect ADAMTS-1 Protein by Western Blotting in Human Neuroblastoma Cell Lines

Western blot optimisation for ADAMTS-1 was carried out using various polyclonal antibodies (see Table 2.4) raised against the C-terminus of the human protein to confirm the identity of ADAMTS-1. Briefly, protein was separated by electrophoresis on 10%

NuPAGE precast gels (Invitrogen, Paisley, UK) under reducing conditions. The proteins in the gel were electroblotted onto a nitrocellulose membrane (Hybond-C, Amersham, UK) at 150 volts for 1 hour. Blots were blocked with NFDM in Tris-buffered saline (20 mM Tris, 0.9% [w/v] NaCl, pH 7.4) (TBS) (Sigma-Aldrich, UK) containing 0.05% Tween 20 (Sigma-Aldrich, UK) (TBST) for 90 minutes prior to 3 x 10 minute washes with TBST. Blocking the membrane prevents non-specific background binding of the primary and/or secondary antibodies to the membrane (which has a high capacity at binding proteins and therefore antibodies). The ADAMTS-1 primary antibodies (Abcam, Triple Point Biologics) were added at a 1:100 and 1:500 dilutions in TBST (see Table 2.4). The blots were incubated overnight at 4°C and unbound antibody was washed off with TBST (3 x 10 minute washes) prior to incubation with secondary antibody [goat anti-rabbit IgG with horseradish peroxidase (HRP) conjugate (1:80,000 in 0.05% NFDM in TBST)] (see Table 2.4) for 2 hours with gentle shaking. Further 3 x 5 minute washes in TBST were followed by 3 x 5 minute washes in TBS to remove excess Tween 20. Blots were developed with the enhanced chemiluminescence (ECL) PlusWestern Blotting detection reagent chemiluminescence substrate (GE Healthcare, UK) and diaminobenzidine (DAB) detection methods in accordance with the manufacturer's protocol. Detection of actin using an anti-actin antibody (Sigma-Aldrich, UK) was included as a loading control on blots. Details of the control, primary and secondary antibodies mentioned are shown in Table 2.4.

2.10.2.2 Optimisation of Santa Cruz Biotechnology Antibody to Detect ADAMTS-1 Protein by Western Blotting in Human Neuroblastoma Cell Lines

Protein samples were extracted from both SHSY-5Y and SK-N-DZ neuroblastoma cell lines (section 2.6.3) and protein determined (section 2.8) in order to load 15 µg/well in precast gels, which was according to the supplier's recommendations. The western blotting protocol was as previously described in section 2.10.2. A few modifications to the procedure were made to optimise the ADAMTS-1 Santa Cruz Biotechnology (sc-31080) goat polyclonal antibody. Its epitope is about 15-25 amino acids long and maps

within the last 50 C-terminal amino acids of the human ADAMTS-1 protein. Details of the primary and secondary antibodies are shown in Table 2.4. Briefly blots were blocked with 5% NFDM in TBST for 1.5 hour prior to 3 x 10 minute washes with TBST. The primary antibody was added (1:500) in TBST and incubated overnight at 4°C. The blots were then washed 3 x 10 minutes in TBST and incubated with secondary antibody donkey anti-goat IgG with HRP conjugate (Santa Cruz Biotechnology), 1:50,000 in 2.5% NFDM TBST on an orbital shaker at room temperature for 2 hours. This was followed by 2 x 5 minute washes in TBST with the excess Tween 20 removed by a further 2 x 5 minute washes in TBS with gentle agitation. Recombinant human ADAMTS-1 (rhADAMTS-1) was used as positive control.

The detection method was with the ECL Plus Western Blotting Detection Reagent chemiluminescence substrate (GE Healthcare, UK). Images were captured and analysed using a UVP bioimaging system (Biorad, Hertfordshire, UK).

2.10.2.3 Antibody Detection (Enhanced Chemiluminescence (ECL) and SIGMAFAST 3, 3' Diaminobenzidine (DAB)) Tablets

ECL detection reagent (Amersham Biosciences, UK) was prepared by mixing 2 ml reagent solution A with 50 µl reagent solution B. The excess washing buffer was drained from membranes and blots were placed, protein side up, on a plastic wallet spread on the bench. 2 ml ECL reagent was applied directly to the blot and incubated for 5 minute at room temperature. Excess detection reagent was drained off by holding the membrane gently in forceps and touching the edge against a tissue. In a similar manner the DAB detection method was performed with DAB SIGMA*FAST* tablets (Sigma-Aldrich, UK), which were developed for use in immune-blotting as a precipitating substrate for the localization of peroxidase activity. Tablets were dissolved in 15 ml deionized water to produce a buffered solution containing DAB and urea hydrogen peroxide. The membranes that the substrates were added to were transferred to the inside of new clear plastic wallets and placed protein side up. HRP-conjugated antibodies were detected by using a UVP bioimaging system (Biorad, Hertfordshire, UK). The camera detects the chemiluminescence emanating from the membrane,

transforming the signal into a digital image for rapid analysis UVP bioimaging software. The programme was setup for each 15 minutes developing time. i.e. 3 images were captured every 5 minutes for each blot to obtain the best exposure time. A diagram illustrating protein detection on western blots is shown in Figure 2.3. Semi-quantitative analysis of protein expression by measuring the integrated optical density (IOD) of each band (densitometry) used Labworks4 software. Normalization was performed by using this equation:

Relative IOD = IOD of protein of interest / IOD of internal control protein.

2.10.2.4 Measurement of the Molecular Weight

In this study, measuring the MW was done by running a set of standard proteins of known MW to one well in the gel. These are called molecular weight markers, which come pre-stained in a range of colours (e.g. rainbow markers). SeeBlue Plus2 pre-stained markers (Invitrogen, Paisley, UK) was used. The relative migration from the top in centimetres of each of the marker proteins was plotted against $\log_{10}$ MW to form a standard curve. The MW of unknown protein could then be calculated from migration of that protein.

2.10.2.5 Stripping for Re-probing Western Blots

Stripping was performed to eliminate the primary and secondary antibodies from the membrane (Restore Plus western blot). Membranes were rinsed with TBST and the stripping buffer was added on the blots for 15 minutes on orbital shaker, and then washed for 10 minutes in TBST. The advantage of re-probing a single membrane for more than one protein is saving samples, materials, time and it is easier to compare immunoreactivity on the blots as the loading is identical.

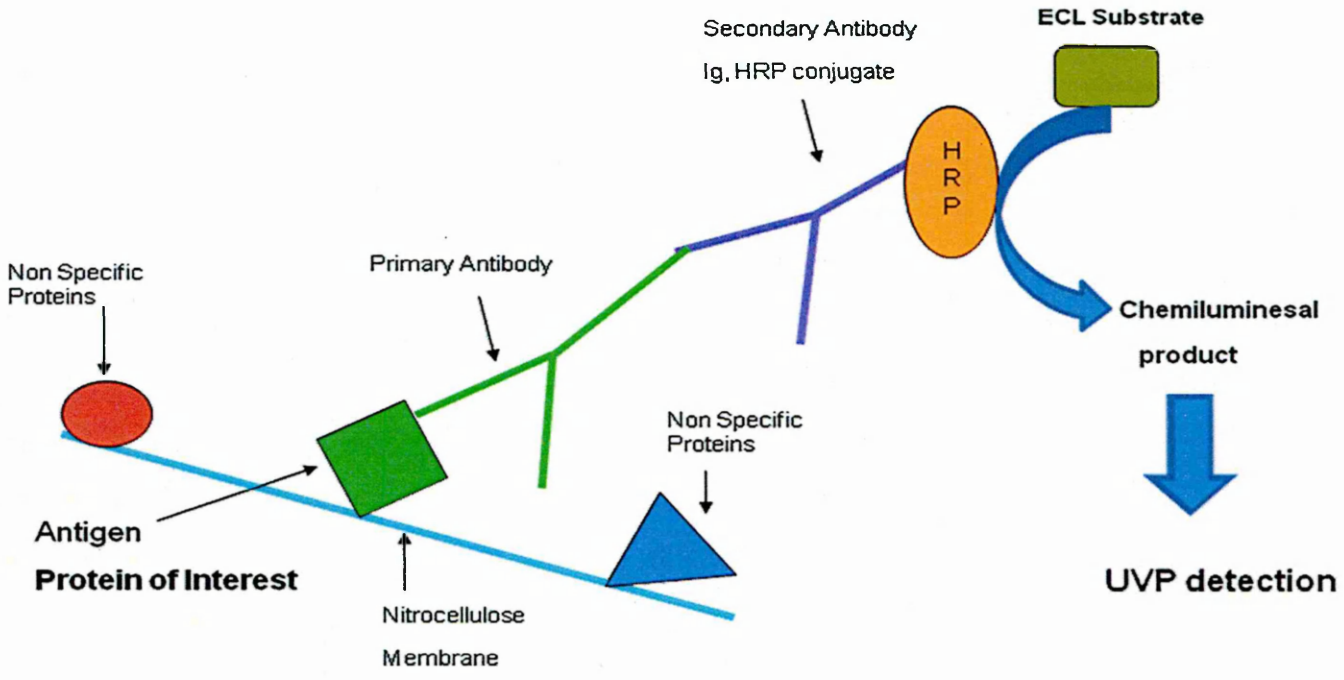


Figure 2.3: Protein detection on western blots

Table 2.4: Details of the primary and secondary antibodies used in western blotting for detecting ADAMTS-1

Primary Antibodies	Species raised in & isotype	Obtained from (Primary antibodies)	Dilution	Secondary Antibodies /Dilutions /Supplier
ADAMTS-1	Rabbit IgG	Abcam ab39194	1:100 in TBST	Goat anti-rabbit IgG HRP conjugate 1:80,000 in TBST with 0.05% milk, Abcam
ADAMTS-1	Rabbit IgG	Triple Point Biologics, Inc RP1-ADAMTS1	1:500 in TBST	Goat anti-rabbit IgG HRP conjugate 1:80,000 in TBST with 0.05% milk Abcam
ADAMTS-1	Goat IgG	Santa Cruz Biotechnology sc-31080	1:500 in TBST	Donkey anti-goat IgG HRP conjugate 1:50,000 in TBST with 2.5% milk / Santa Cruz Biotechnology
* β actin	Rabbit IgG	Sigma-Aldrich A5060	1:1,000 in 5% blocking buffer	Goat anti-rabbit IgG HRP 1:80,000 in TBST with 5% milk Sigma-Aldrich (A9169)
GAPDH	Mouse IgG1	Abcam ab8245	1:5,000 in blocking buffer	Rabbit anti-mouse 1:1,000 in TBST with 5% milk/Abcam

* Working dilutions previously determined and described in Haddock *et al.*, 2006

2.11 Small Interfering RNA (siRNA) Mediated Gene Silencing for ADAMTS-1 Knockdown in SHSY-5Y Cells

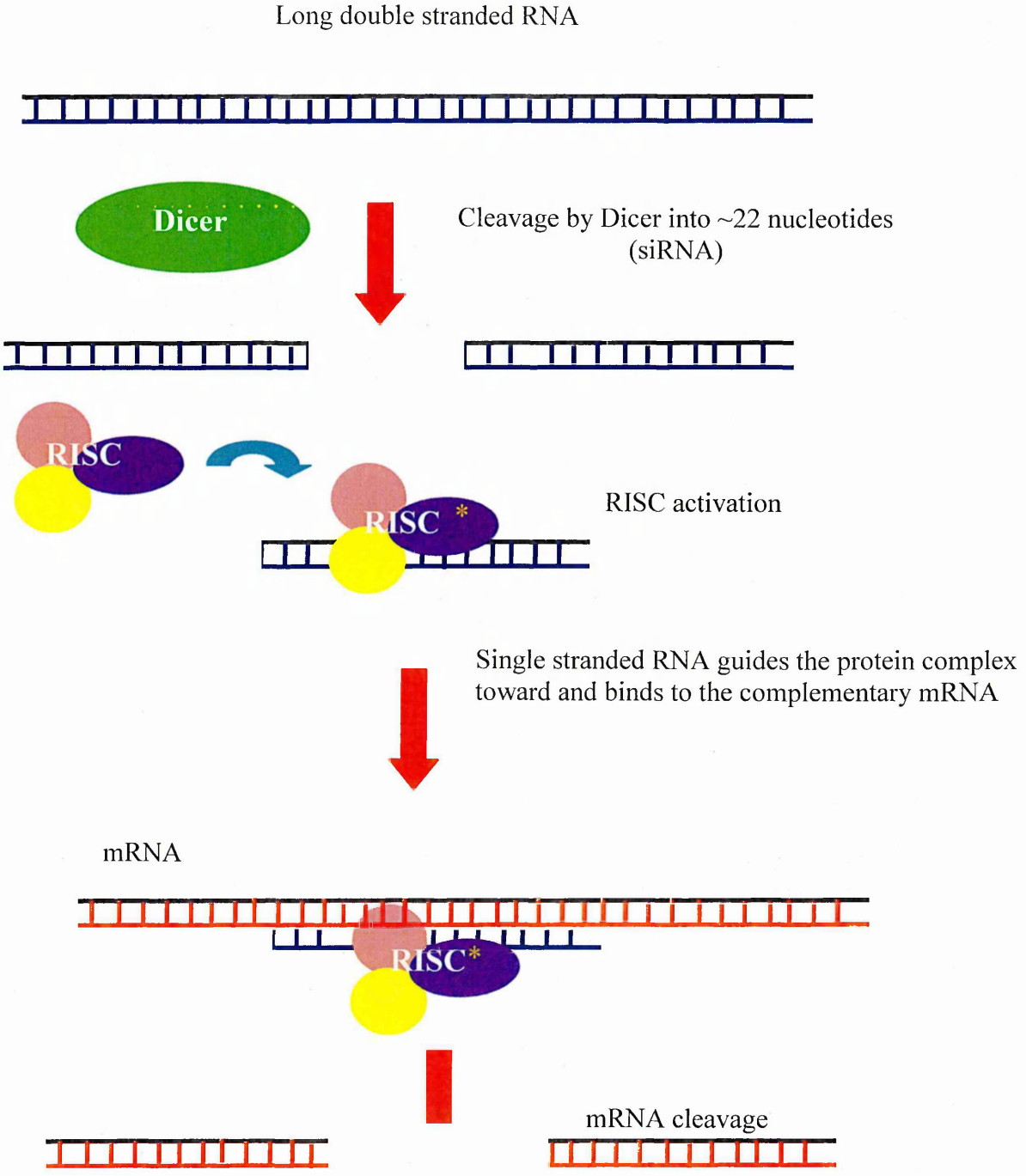
2.11.1 General Principle

RNA interference (RNAi) was first discovered by Hamilton and Baulcombe (1999) as part of post-transcriptional gene silencing (PTGS) in plants. It is a regulatory mechanism of most eukaryotic cells that uses small double stranded RNA (dsRNA) molecules in cellular anti-viral mechanisms and post-transcriptional regulation (Hutvagner and Zamore, 2002, Hannon, 2002, Zamore, 2001, McManus *et al.*, 2002). RNAi silencing is initiated when dsRNA is processed into small interfering RNA (siRNA) between 19-25 base pairs in length with a characteristic 2 nt 3' overhang that allows them to be recognized by an RNase-III enzyme called Dicer. These siRNAs are subsequently incorporated into RNA-induced silencing complexes (RISC) which leads to the degradation of the target mRNA (Figure 2.4). SHSY-5Y cells were transfected with siRNA pre-designed for ADAMTS1 On-TARGET plus SMART pool (Perbio Science, UK) to knockdown human ADAMTS-1 at its mRNA and therefore protein levels. The procedure was performed according to the manufacturer's instructions.

2.11.2 Cell Plating

SHSY-5Y cells were seeded into a 24-well plate at a density of 5 x 10^4 cells per well (mRNA determination) in 500 µl complete culture media without antibiotics. For the protein determination a 6-well plate at 2 x 10^5 cells in 2000 µl/well in complete culture media free of antibiotics was set up. Cells were allowed to adhere for 24 hours in an incubator. The media were then aspirated and cells were washed twice with PBS before adding siRNA and transfection reagents.

Figure 2.4: Principle of siRNA method. This technique is based on the RNA interference pathway, which is triggered by double-stranded RNA (dsRNA) molecules that share sequence-specific homology for target mRNA. The appearance of these dsRNAs induces a cytoplasmic RNAase III-like protein called Dicer and RNA induced silencing complex (RISC). The Dicer digests the long dsRNA into siRNA duplexes (19-25 base pairs). The siRNAs are incorporated into RISC. RISC is then activated and binds the complementary mRNA. Upon identifying the specific region of complementarity, RISC mediates the mRNA cleavage and consequently gene inhibition.


Long double stranded RNA
Dicer
Cleavage by Dicer into ~22 nucleotides
(siRNA)
RISC
RISC *
RISC activation
Single stranded RNA guides the protein complex
toward and binds to the complementary mRNA
mRNA
RISC*
mRNA cleavage

2.11.3 Transfection

ADAMTS-1 gene knockdown was performed by the use of lipid-mediated siRNA transfection (Perbio Science, UK Ltd) of SHSY-5Y cells which required optimisation.

2.11.3.1 Optimisation of Transfection

The first step of the optimisation of the siRNA knockdown was determining the most efficient transfection reagent for the SHSY-5Y cells, because this cell line had not been optimised for DharmaFECT transfection reagents. Four DharmaFECT transfection reagents, 1, 2, 3 and 4, were tested for their efficiency for siRNA delivery into the SHSY-5Y cells and the most efficient was selected.

siGLO transfection indicator (red) is a fluorescent oligonucleotide, suitable for delivery into mammalian cells by lipid mediated transfection and localises to the nucleus. This was used to determine the optimal siRNA transfection conditions. Cells were plated out in chamber slides at a density of 2 x 10^4 cells per well. After 24 hours they were transfected with siGLO and ADAMTS-1 siRNA using each of the four DharmaFECT transfection reagents. ADAMTS-1 siRNA (12.5 µl) was added to siGLO (12.5 µl), mixed and diluted 1:1 in SFM (25 µl) in 4 tubes (tube1). 1.5 µl of each transfection reagent 1, 2, 3 and 4 separately was mixed with 48.5 µl SFM in another tube (tube 2). Tubes were incubated at room temperature for 5 minutes. Each diluted transfection reagent tube 2 was mixed with tube 1 to form the transfection medium and incubated for 20 minutes at room temperature. Cells were washed 1X with PBS, then the transfection medium was added. 400 µl of antibiotic free media was added to each well to make a total volume of medium of 500 µl. After 24 hours of transfection, the optimum time for signal development according to the manufacturer's recommendation, cells were washed once with PBS. Then they were fixed with 4% PFA, which has no effect on the signal and the nuclei were counterstained with Hoechst 33342 (Sigma-Aldrich, UK) (blue stain). 10 µl aliquots (10 mg/ml) of Hoechst 33342 were stored at -20°C and diluted 1:1000 in dH_2O (10 µg/ml) for final use.

2.11.3.2 Transfection of SHSY-5Y Cells

Cells were transfected in triplicate wells, with siRNA targeting GAPDH as positive control (ON-TARGETplus GAPDH control pool), siRNA for the target gene (ON-TARGETplus ADAMTS-1 SMART pool), negative control or ON-TARGETplus non-targeting pool a scrambled siRNA sequence, used to determine any non specific effects. Untreated control samples containing culture medium, without antibiotics and foetal calf serum were used to compare or measure the variations in the experiment.

Transfection of samples was performed in triplicate wells. In a 24 well plate, for each siRNA reaction 25 µl siRNA solution (2 µM siRNA solution), which was prepared in 1X siRNA buffer (100 mM KCl, 30 mM HEPES-pH 7.5, 1.0 mM $MgCl_2$), was added to 25 µl antibiotic and serum free culture medium and mixed gently (tube 1) and the appropriate DharmaFECT reagent (1.5 µl) with serum free medium (SFM) 48.5 µl added to tube 2. The cells were transfected with Dharmafect 1 reagent because in preliminary experiments it showed the best transfection efficiency of the four DharmaFECT transfection reagents 1, 2, 3 and 4 (Perbio Science, UK) as determined using siGlO (red), transfect indicator fluorescent oligonucleotide (Perbio Science, UK). The content of each tube was mixed gently by carefully pipetting up and down and incubated for 5 minutes at room temperature. Contents of tube 1 (siRNA) was added to tube 2 (transfection reagent 1) and mixed by gently pipetting up and down forming the transfection medium and was incubated for 20 minutes at room temperature. 400 µl of medium free of antibiotics and serum was then added to the transfection medium. The transfection medium was then added to cells after the removal of culture medium from the wells. They were incubated at 37°C in a CO_2 incubator for 24 or 96 hours for mRNA and protein determination by qRT-PCR and western blotting respectively.

2.12 Subjects for study

2.12.1 Human Brain Tissue

Snap-frozen CNS tissues were obtained from the UK Multiple Sclerosis Tissue Bank, Division of Neuroscience and Mental Health, Imperial College London (REC reference number 08/MRE09/31) (see appendix IV). Tissue blocks were from people with MS, including blocks containing normal appearing white matter (NAWM). The normal controls blocks were from donors with no history of MS and were from similar brain regions as blocks from MS donors. The UK Multiple Sclerosis Tissue Bank prepared the tissue blocks using a coronal slicing method. Briefly, a single coronal cut through the mamillary bodies was used to separate the brain into anterior (A) and posterior (P) halves (Figure 2.5 A). It was then cut into 2 cm thick slices, the first anterior to the mamillary bodies is A1, the next is A2 etc. The first slice posterior to mamillary bodies is P1, the next is P2 etc (Figure 2.5 B). Each slice was then divided into 2X2 cm blocks, with letters for the rows, and numbers for the columns given after the anterior or posterior slice number e.g. MS58 P1D3: MS58 indicates the case number, P1 (P) is the posterior portion of the brain and (1) is the number of slice, 1 is nearest the mamillary bodies. D3 indicates the portion of the brain in row D of that slice and column (3) as described above (see Figure 2.5). Each block was frozen by immersing in isopentane, pre-cooled on a bed of dry ice. Then all tissue blocks were stored at -80°C.

2.13 Scoring of MS Lesions

2.13.1 Haematoxylin and Eosin (H&E) / Oil-Red O Staining (ORO)

In this study H&E/ORO staining was performed on MS, NAWM and control human brain tissue to re-characterise it for histology and grade lesion inflammation. Grading was carried out by two independent observers.

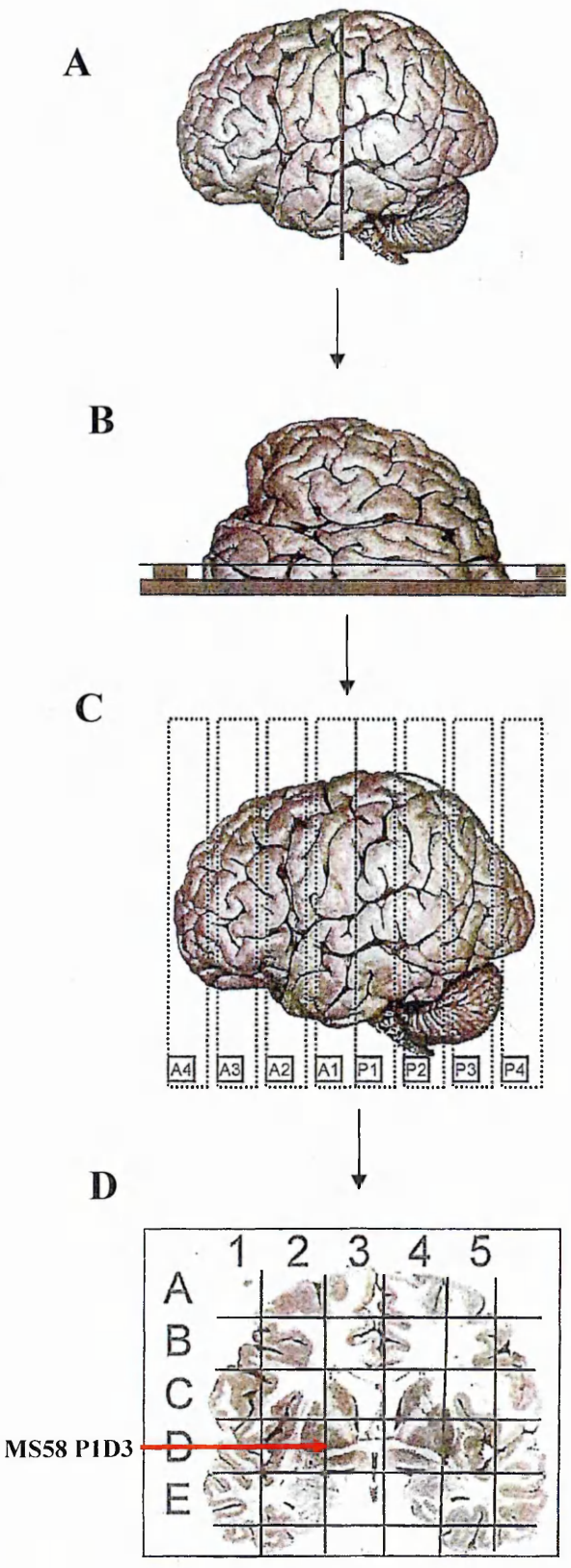


Figure 2.5: Human brain tissues cut into 2X2 cm blocks (Adapted from the UK Multiple Sclerosis Tissue Bank).

Ten-micrometre thick cryosections were cut using a cryostat, mounted on polysine–coated glass slides and stored at -80°C until required. These sections were stained with H&E/ORO and graded by using a four-point scale to determine the extent of inflammation and demyelination (Plumb *et al.*, 2005, Plumb *et al.*, 2006, Haddock *et al.*, 2006). Negative grade in H&E/ORO corresponded to no staining. H&E sections were scored + a small amount of cellular infiltrate in the perivascular region, ++ moderate cellular infiltrate and grade +++ an abundant number of cellular infiltrates in perivascular region. H&E is a stain that uses the basic dye haematoxylin to detect basophilic structures within a tissue section. Basophilic structures; such as the nucleus, ribosomes, and RNA-rich cytoplasmic regions produce a blue or purple colour. The acidic dye, eosin stains eosinophilic structures, generally proteins which can be extracellular or intracellular e.g. cytoplasm, connective tissues, and collagen which are stained pink.

ORO was scored + in the presence of few ORO positive cells, ++ moderate number of ORO positive cells in a MS lesion and grade +++ an abundant number of ORO positive cells. ORO is a neutral lipid dye which indicates areas of myelin breakdown and demyelination by staining myelin debris engulfed by macrophages.

H&E staining was performed in Coplin jars. Tissue sections (MS, NAWM and controls) were removed from -80°C and fixed in 4% PFA for 5 minutes. Sections were immersed in filtered Harris' haematoxylin (Sigma-Aldrich, UK) for 1 minute followed by a rinse with tap water. Then sections were stained with eosin (Sigma-Aldrich, UK) for 2 minutes and rinsed with water again. Slides were then dehydrated with 50, 70, 80, 95 and 100% (v/v) ethanol for 2 minutes each. Sections were placed in xylene (Sigma-Aldrich, UK) for 4 minutes and then mounted with DPX (mountant for the histology, Sigma-Aldrich, UK).

The ORO stain was prepared by adding 1g ORO powder to 100 ml 60% triethyl phosphate (TEP) and heated to 100°C for 5 minutes whilst stirring continuously. Then the ORO solution was filtered (warm), left for 1 hour and then filtered again when cooled. ORO staining was also performed in Coplin jars and sections were fixed with 4% PFA for 15 minutes at room temperature. Sections were washed in tap water and

immersed in 60% TEP for 2 minutes. Then the slides were incubated in filtered ORO stain for 30 minutes at room temperature. Slides were rinsed again with tap water. The nuclei of the tissue was counterstained with 20% Harris haematoxylin for 2 minutes and rinsed in tap water prior to mounting with aqueous mountant glycerol gelatin (stored at 4°C Sigma-Aldrich, UK) which was heated in water bath at 60°C to melt prior its use. H&E/ORO stained sections were visualised under an Olympus BX60 light microscope, scored with the 4 point scale (-, +, ++, +++) method and images were captured with a Cool Snap Pro (Cybernetics) colour digital camera and analysed by LabWorks Image Acquisition and Analysis Software.

2.14 Immunohistochemistry for Versican (V0/V2) Neoepitopes in MS and Normal CNS Tissue

2.14.1 Immunofluorescence for Versican (V0/V2) Neoepitopes

The rabbit anti-human versican (V0/V2) neoepitopes polyclonal antibody (Perbio Science, UK) was used in this study, which was previously verified for its specificity by Westling *et al* (2004). Determination of the versican neopitopes was performed to identify ADAMTS-mediated versican cleavage via their glutamyl peptidase activity. According to Westling *et al* (2004), the cleavage of versican (V0/V2) by ADAMTSs in human brain *in vivo* is similar to the cleavage of aggrecan in cartilage and spinal cord by ADAMTSs in that it occurs at a specific site after a glutamate residue (Rodriguez-Manzaneque *et al.*, 2002, Lemons *et al.*, 2001).

Sections were allowed to reach room temperature. Around the tissue a border circle was drawn with a wax pen (ImmEdge hydrophobic barrier pen) (Vector laboratories, UK) to define the tissue area. A deglycosylation step was required for versican (V0/V2) ADAMTS-generated neoepitope detection to unmask the antigen. Sections were treated with chondroitinase ABC (0.25 IU/ml) for 90 minutes at 37°C. They were washed twice with PBS for 5 minutes, blocked with 0.5% normal goat serum in PBS buffer for 30 minutes at room temperature then the primary antibody (versican V0/V2 neoepitopes antibody) 200 µL (1:100), was added and incubated on sections overnight at 4°C in a

moist chamber. Following 3 x 5 minute PBS washes, secondary antibody (1:800) was applied in PBS as shown in Table 2.5 and was incubated for 2 hours at room temperature in a moist humid chamber in the dark. Slides were washed 3 times with PBS and a Sudan Black B (SBB) staining step was carried out.

All slides were stained with SBB. 1% SSB is used to reduce or eliminate lipofuscin auto-fluorescence in the brain. Tissue lipofuscin is a fluorescent pigment that accumulates with age in the cytoplasm of cells in the CNS (Goddard *et al.*, 2001). SBB stain was prepared by dissolving 1g of SBB powder in 100 ml 70% ethanol, with stirring for 2 hours in the dark in the fume hood followed by filtration (Schnell *et al.*, 1999). Slides were stained with SBB for 5 minutes in the dark and slides then rinsed 6-8 times with PBS. Sections were then mounted with Vectashield mounting media (Vector Laboratories, Inc, Burlingame, USA), containing 4'-6-Diamidino-2-phenylindole (DAPI), which forms fluorescent complexes with dsDNA, enabling nuclei visualisation.

Sections were graded on a 4-point scale. Devoid of versican (V0/V2) neoepitopes immunoreactivity were graded -, sections showing versican (V0/V2) neoepitopes only associated with the blood vessels were graded +, sections with blood vessel and ECM staining versican (V0/V2) neoepitopes expression were graded ++ and sections with an abundance of extracellular staining versican (V0/V2) neoepitopes expression were graded +++. Grading was carried out blind by two observers.

2.14.2 Immunofluorescence for Markers von Willebrand factor (vWF), Human Leucocyte Antigen DR (HLA-DR) and Myelin Oligodendrocyte Glycoprotein (MOG) in Human Brain Tissue

Tissues were characterised using markers for the detection of endothelial cells of the blood vessels (vWF) and activated macrophage/microglial cells (HLA-DR), indicative of active lesions. MOG was also immunostained to determine regions of demyelination, indicative of MS lesions. These markers were immunostained with different antibody dilutions or incubation durations as shown in Table 2.5.

Briefly, sections were fixed with ice-cold acetone for 10 minutes at room temperature. Slides were air dried and a border line drawn around the tissue as previously described in section 2.14.1. Slides were incubated with 10% normal rabbit serum in PBS as blocking buffer for 30 minutes at room temperature. Then the primary antibody a mouse anti-human (vWF, HLA-DR and MOG) was applied. Details of the antibodies and their optimal dilutions are shown in Table 2.5. The primary antibodies were washed 3 times for 5 minutes each with PBS, followed by applying the secondary antibody with optimal dilutions and incubation times (Table 2.5). This was followed by washes (3 x 5 minutes in PBS) and then staining with SBB and mounting as previously described in section 2.14.1.

2.14.3 Dual Immunofluorescence

Dual-label immunofluorescence was performed to determine if there is any co-localisation of versican (V0/V2) neoepitopes produced by ADAMTS-mediated versican cleavage with other markers. Dual staining was based a sequential detection method with monoclonal antibody to either the endothelial marker (vWF) or indication of deymelination (MOG) with the polyclonal antibody to versican (V0/V2) neoepitopes. Sections were treated with chondroitinase ABC (deglycosylation) and the monoclonal mouse anti-vWF or monoclonal mouse anti-MOG were added to the slides according to the optimal dilution and incubation time required for each marker as shown in Table 2.5. This was followed by secondary detection antibody donkey anti-mouse alexa fluor 488 (Invitrogen, Molecular probes, 1:500 in PBS) for 2 hour in the dark at room temperature. This was followed by 3 x washes 5 minute in PBS and the versican (V0/V2) neoepitopes antibody with staining as previously described for the single-labelled immunoflourescence staining in section 2.14.1. Secondary antibodies were washed off in the same manner as primary antibodies, treated with SBB and mounted with DAPI as described in section 2.14.1.

2.15 Expression of ADAMTS-1 Protein in Human Brain Tissue by Western Blotting

Briefly, 15 µg/ lane of the protein samples were loaded into pre-cast gels and after transferring the protein samples to the membrane, the optimal blocking buffer 2% NFDM in TBST was added to the blots on an orbital shaker for 1 hour. Blots were washed 3 X 5 minutes each with TBST, and then the primary ADAMTS-1 antibody (Santa Cruz Biotechnology) was applied (1:100) and incubated at 4°C overnight. Blots were washed 3 X for 5 minutes each in TBST, then the secondary antibody was added (1:2000) in TBST and incubated for 2 hours at room temperature. The blots were washed with TBST 3 X for 5 minutes each with a final wash 1 X 5 minutes with TBS to remove excess Tween. Bands were detected as previously described in section 2.10.2.3 using the ECL detection method.

2.16 Expression of Versican (V0/V2) Neoepitopes in Human Brain Tissue by Western Blotting

An additional step was required for the versican (V0/V2) neoepitopes detection in human brain tissue by western blotting. This was an enzymatic deglycosylation step to enable the proteoglycan to run as a discrete band(s) on SDS-PAGE. Because the glycosaminoglycan chains are susceptible to chondroitinase ABC, the samples were treated with chondroitinase ABC to digest the GAGs, allowing samples run as discrete bands and the antibody to more easily recognise epitopes. The chondroitinase ABC (Sigma-Aldrich, UK) treatment used in study was as previously described by (Gerard *et al.*, 1993). Briefly, 12 IU/ml stock of the chondroitinase ABC in 0.1 M Tris/HCL containing 0.5 M sodium acetate was prepared. This was further diluted to get a concentration of 1 mIU/ml of chondroitinase ABC containing 10% protease inhibitor cocktail (P2714, Sigma-Aldrich, UK). This chondroitinase preparation was diluted 1:1 with protein sample (in 1% SDS). Incubation was at 37°C for 4 hours for

deglycosylation. The deglycosylated protein samples were denatured by the boiling at 95°C for 5 minutes then run on SDS-PAGE. The western blot was performed as previously described in section 2.10.2.2.

2.17 Statistical Analysis

To ascertain whether the difference in relative ADAMTS-1, -4 and -5 mRNA expression levels ($2^{-\Delta C_T}$) between RetA differentiated and undifferentiated SHSY-5Y cells, the Mann-Whitney U test was performed. The Mann-Whitney test is a non-parametric unpaired T-test used to assess the differences between two means. The Mann-Whitney test was also used to determine the difference between mRNA levels of ADAMTS-1, -4 and -5 in the RetA differentiated SK-N-DZ neuroblastoma cell line.

The Kruskal-Wallis test was used to determine any statistically significant differences in the stability of housekeeping genes and also of relative fold increases/decreases ($2^{-\Delta\Delta C_T}$) in ADAMTS-1, -4, -5, ADAM-17 and TIMP-3 mRNA expressions between untreated or cytokine treated cells. The Kruskal-Wallis test is a non parametric test equivalent to parametricto one way analysis of variance, used for comparing three or more means of independent experiments. Kruskal-Wallis gives an overall P value. This was followed by Dunnett's post hoc test in this study to calculate a P value for each particular cytokine treatment relative to the control sample (untreated sample) to ascertain whether differences were statistically significant. Both Mann-Whitney U and Kruskal-Wallis tests were performed using GraphPad Prism Inc.

Results were expressed as +/- standard error of the mean (SEM), for three independent experiments. Statistically significant differences were represented in Figures by * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$.

Table 2.5: Details of the primary and secondary antibodies used in immunohistochemistry of human brain

Antibodies	Species raised in	Isotype	Dilution (PBS)	Incubation	Source /Cat. No.
Versican V0/V2 Neo Polyclonal Antibody (P)	Rabbit	IgG	1:100 (10 µg/ ml)	O/N at 4 °C	Perbio science , UK/ PA3-119
vWF/F8/86 (m)	Mouse	IgG1, kappa	1:25 (9.6 µg/ml)	O/N at 4 °C	Dako,UK / M 0616
HLA-DR (m)	Mouse	IgG2b	1:50 (0.5 µg/ml)	1 hour at room temperature	Novocasta / NCL-LN3
MOG (m)	Mouse	Unknown	1:100 (unknown)	O/N at 4 °C	Kind gift from Dr Stephen McQuaid Queens University Belfast
*** Anti-rabbit Alexa–fluor 568**	Goat	IgG	1:800	2 hour at room temperature	Molecular Probes, Invitrogen, UK/A11011
***Anti-mouse Alexa-fluor 568**	Goat	IgG	1:800	2 hour at room temperature	Molecular Probes, Invitrogen, UK/ A11004
***Anti-mouse Alexa-fluor 488**	Donkey	IgG	1:500	2 hour at room temperature	Molecular Probes, Invitrogen, UK
Isotype control	Rabbit	IgG	same concentration as V0/V2 neoepitope antibody	same incubation for V0/V2 neoepitope antibody	Abcam, UK/ ab27478
Isotype control	Mouse	IgG1 kappa	same concentration as vWF antibody	same incubation for vWF antibody	Abcam, UK/ ab18448
Isotype control	Mouse	IgG2b	same concentration as HLA antibody	same incubation for HLA antibody	Abcam, UK/ ab18428

* Secondary Antibodies, (P) polyclonal, (m) monoclonal, O/N overnight.

Chapter 3

ADAMTS-1, -4 and -5 Expression and Modulation in Neuroblastoma Cell Lines

3.1 Introduction

It has been suggested that ADAMTS degradation of CSPGs may increase access of inflammatory cells promoting axonal damage. Alternatively, breakdown of CSPGs, by ADAMTSs may enable axonal regeneration and neurite outgrowth which is inhibited by CSPGs. Previously it has been demonstrated in our laboratory that ADAMTS-1, -4 and -5 are expressed by astrocytic cell lines (U373-MG and U87-MG) primary astrocytes and CHME3 microglial cells (Cross *et al.*, 2006a). Also Sasaki *et al*, (2001) showed that ADAMTS-1 was expressed by N1E-115; a neuroblastoma cell line (Sasaki *et al.*, 2001). ADAMTSs, especially ADAMTS-1, -4, -5, and -9, are expressed in brain (Hurskainen *et al.*, 1999, Jungers *et al.*, 2005) and it is interesting that each of these proteases is active in cleaving PGs. Several of the ADAMTSs have been shown to be elevated in human neurodegenerative disease and animal models of brain injury. ADAMTS-1, but not ADAMTS-5, appears to be up-regulated in Down's syndrome, Pick's disease and Alzheimer's disease (Miguel *et al.*, 2005).

IL-1β is elevated in the CSF of MS patients and in lesions in the animal model of MS, EAE (Wang and Shuaib, 2002). TNF also has been shown to be elevated in CSF and serum in MS patients with a correlation to disease severity (Munoz-Fernandez and Fresno, 1998). The expression of ADAMTS-4 and ADAMTS-1 mRNA was markedly elevated in the hippocampus of rats in response to kainate-induced excitotoxic lesion (Yuan *et al.*, 2002). An increase in ADAMTS-1 mRNA has also been demonstrated by Sasaki *et al.* (2001) who found that ADAMTS-1 was expressed at very low levels by rat hypoglossal motor neurons but following nerve injury, mRNA levels were rapidly increased, indicating that these proteases may be increased in response to injury or during an inflammatory response. However, research into whether neurons express ADAMTSs with aggrecanase activity has received little attention. ADAMTS-1, -4 and -5 have not been studied in neuronal cells in relation to the pathogenesis of MS. Therefore, an analysis of ADAMTS-1, -4 and -5 mRNA expression levels in SHSY-5Y and SK-N-DZ human neuroblastoma cells was performed using qRT-PCR.

3.1.1 Objectives

- To characterise the two neuroblastoma cell lines (SHSY-5Y and SK-N-DZ) used in this study, by detecting a neuronal marker neurofilament L (NF L).
- To determine the morphological effect of RetA on both neuroblastoma cells utilised in this study.
- To validate and optimise the qRT-PCR method for the detection of ADAMTS-1, -4 and -5 and TIMP-3 gene expression
- To determine the modulation of ADAMTS-1, -4, -5, ADAM-17 and TIMP-3 mRNA expression in undifferentiated or differentiated SHSY-5Y and SK-N-DZ by proinflamatory cytokines.

3.2 Results

3.2.1 Characterisation of the Neuroblastoma Cell Lines

The SHSY-5Y and SK-ND-Z neuroblastoma cell lines used in this study were spontaneously transformed cells from a female patient with neuroblastoma as shown in Table 2.2. Both cell lines propagate via mitosis and can be differentiated to extend neurites to the surrounding area. The cells grow as neuroblastic cells with epithelial like morphology with multiple, short, fine cell processes (neurites). To determine whether the human neuroblastoma cells that were used in this study had neuronal features, ICC was performed with a NF L antibody as described in section 2.3. NF L, is one of the neurofilament subunits present in neurons, neuronal processes, and peripheral nerves and is a specific neuronal cell marker.

Neuroblastoma cell lines (SHSY-5Y and SK-N-DZ) were plated into 8-well chamber slides at a density of 5 x 10^4/500 µl media/well and allowed to adhere for 24 hours. They were incubated with primary antibody against NF L followed by rabbit anti-mouse IgG-FITC secondary antibody (green). Nuclei were counterstained using propidium iodide (orange). Both SHSY-5Y and SK-N-DZ cells stained for NF L indicating a neuronal phenotype (Figures 3.1 A and C). No staining was observed when NF L antibody was omitted (replaced by PBS) from the protocol as a negative control for non-specific binding of the secondary antibody (Figures 3.1 B and D).

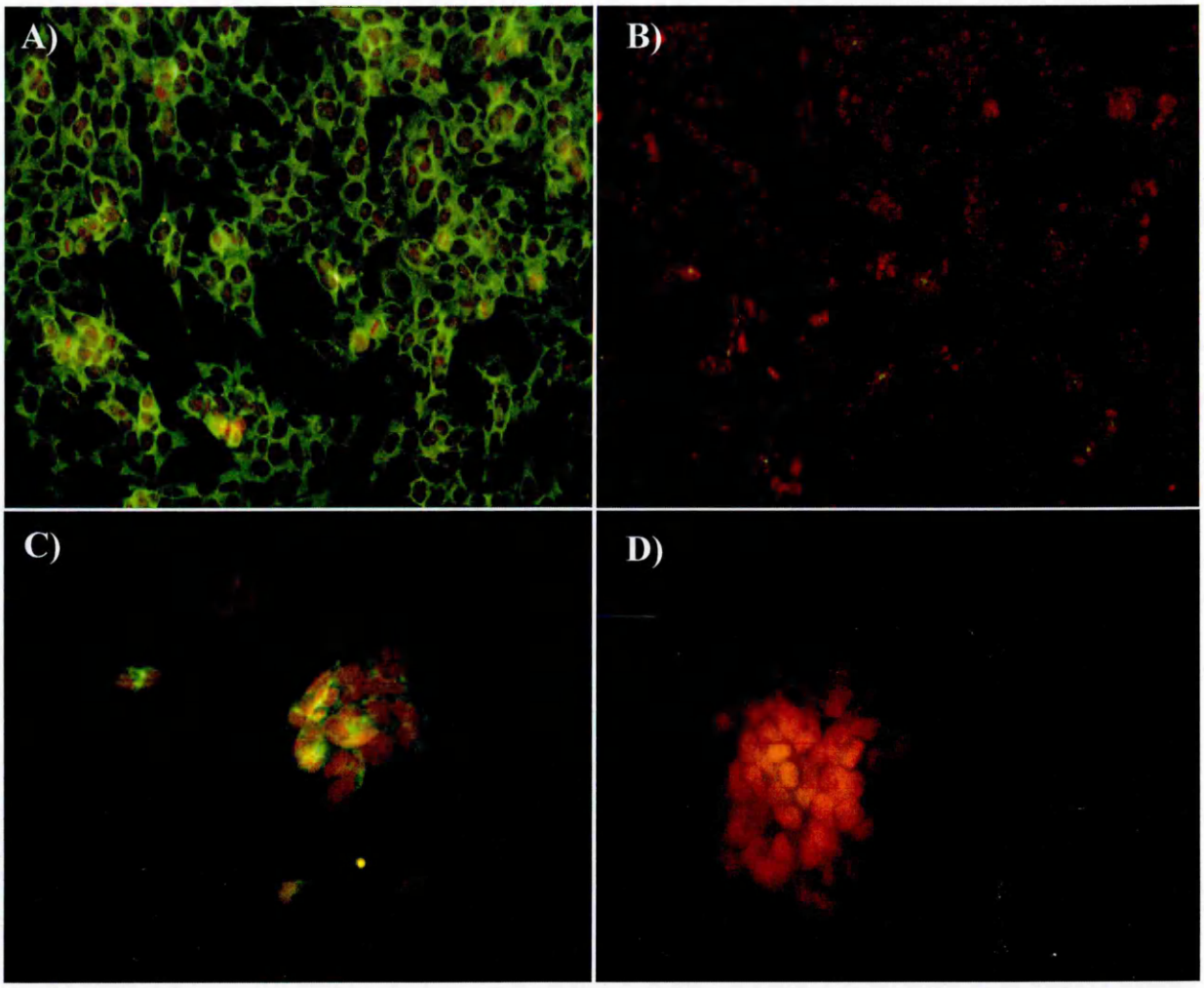


Figure 3.1: Characterisation of the neuroblastoma cell lines (SHSY-5Y and SK-N-DZ) without RetA used in this study. A) SHSY-5Y cells staining (green) with anti-NF L antibody. B) No primary antibody (SHSY-5Y cells), as a negative control. C) SK-N-DZ cells staining (green) with anti-NF-L antibody and D) omission of the primary antibody (SK-N-DZ) as negative control. Nuclei of the cells were counterstained using propidium iodide (orange). Images were captured with an Olympus BX60 fluorescence microscope with Cool Snap Pro an integrated solution colour digital camera (Olympus Media Cybernetics, Silver Spring, USA) (x200).

3.2.2. Neuroblastoma Cell Lines Differentiation with Retinoic Acid (RetA)

Cells were cultured as previously described in section 2.2.1 and treated with RetA as described in section 2.5. Figure 3.2 shows the differences in cell morphology between A) untreated and B) RetA (10^{-5} M) treatment of the SHSY-5Y cells and C) untreated and D) RetA treatment of the SK-N-DZ cells. Cell images were taken on day seven of culturing with RetA. Cells treated with RetA appeared to have decreased proliferation compared to untreated cells in that they were observed to grow more slowly than untreated cells. The morphology of RetA treated cells has also changed. Arrows indicate that RetA can enhance or elongate the neurite out growth processes, suggestive of differentiation to a more neuronal phenotype.

3.3 Expression of ADAMTS-1, - 4 and -5 mRNA in SHSY-5Y Cells

In this study, the expression and modulation of ADAMTS-1, -4, -5 and TIMP-3 mRNA after proinflammatory cytokine (24 hour) treatment (described in section 2.4) was analysed in SHSY-5Y human neuroblastoma cell line by qRT-PCR (described in section 2.9.5). These cells were also treated with RetA as previously described, to induce neuronal differentiation, to assess whether ADAMTS-1, -4 and -5 mRNA expression levels are affected by RetA treatment.

3.3.1 Sample Preparation for qRT-PCR

After proinflammatory cytokine treatment with IL-1β or TNF (0-100 ng/ml) as described in section 2.4, total RNA was extracted from SHSY-5Y cells. Then the samples were separated by agarose gel electrophoresis (described in section 2.7). Figure 3.3 shows a representative SHSY-5Y extracted RNA gel with intact and good quality RNA as determined by the presence of discrete bands for 28S and 18S rRNA subunits.

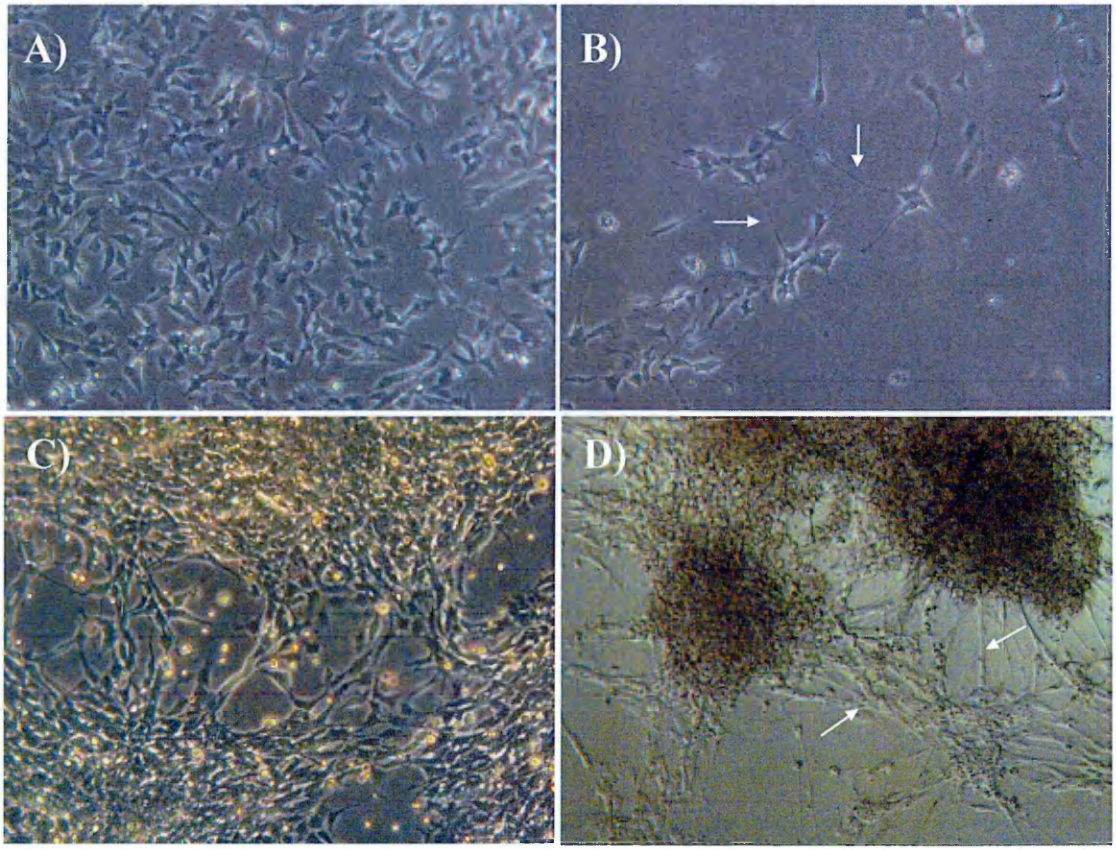


Figure 3.2: Morphological affect of RetA on neuroblastoma cell lines. SHSY-5Y cell morphology A) untreated B) treated with RetA. SK-N-DZ morphology C) untreated D) treated with RetA. RetA treatment was for 7 days. Images were captured on a light microscope (Leica) (x200).

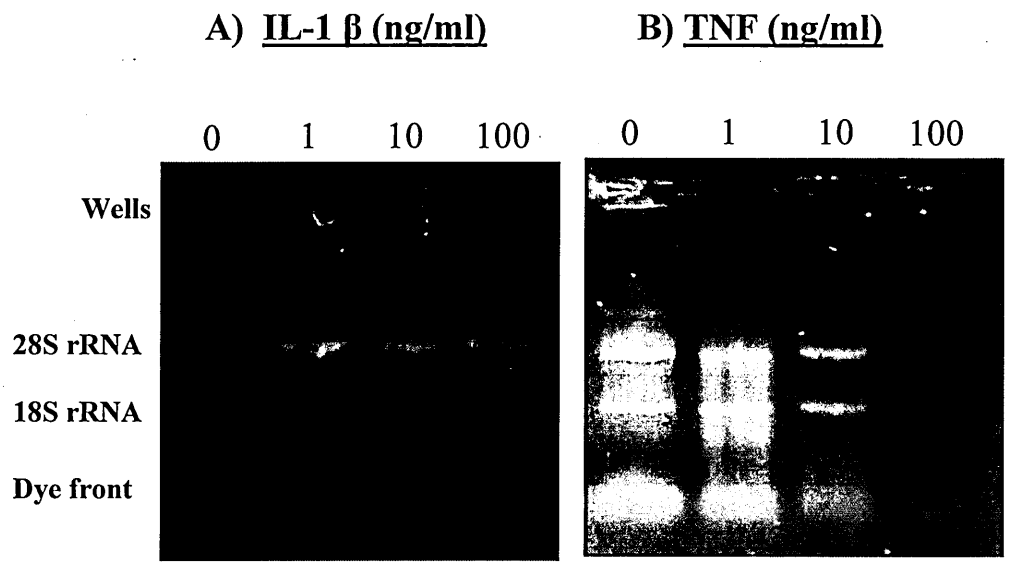


Figure 3.3: RNA extraction from SHSY-5Y cells. Cells treated with IL-1 β (A) or TNF (B) for 24 hour treatment. Upper band shows 28S rRNA and lower bands show 18S rRNA.

3.3.2 qRT-PCR Validation, Normalisation and Housekeeping Genes

It is essential to rule out variations between samples when detecting mRNA expression. These variations can be introduced at a number of stages throughout the experimental protocol (RNA extraction as described in section 2.6.2 and reverse transcription into cDNA as described in section 2.9.5). A widely used approach is to normalize mRNA levels of the target to an internal reference or housekeeping gene.

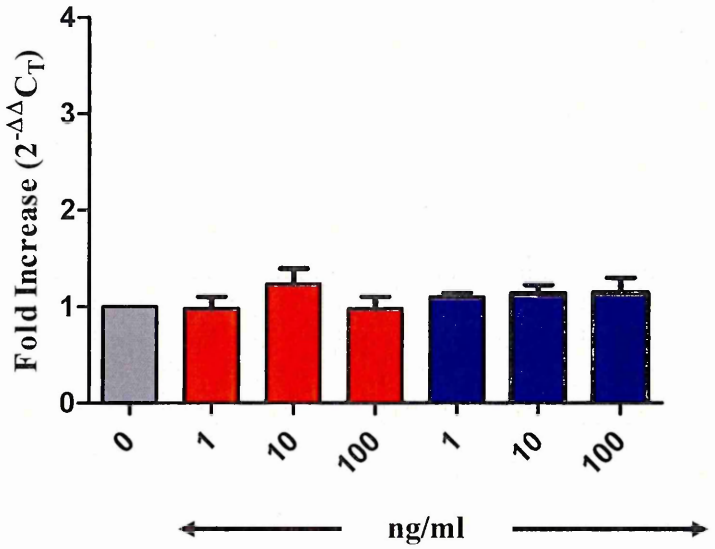
The strategy here in this study to validate the qRT-PCR was by comparing the mRNA expression of three housekeeping genes GAPDH, cyclophilin-A and RNAP-II in the SHSY-5Y neuroblastoma cell line under the same conditions i.e. proinflammatory cytokine treatment of cells with IL-1β at 1, 10 and 100 ng/ml concentrations as described in 2.4. Results showed that RNAP-II was the most stable housekeeping gene as its relative mRNA expression was constant either used as reference or as a target compared to the untreated sample (control) (Figure 3.4 A and C). When using GAPDH and cyclophilin-A (Figure 3.4 B) changes were observed on the cytokine treatment (IL-1β at 1 ng/ml). However, no statistical significance was seen with these housekeeping genes. Statistics were performed by Kruskal-Wallis non parametric test, followed by Dunnett's test using the Graph Pad Prism software.

3.3.3 Comparison of ADAMTS-1, -4 and -5 mRNA Expression in SHSY-5Y Cells with and without RetA Differentiation

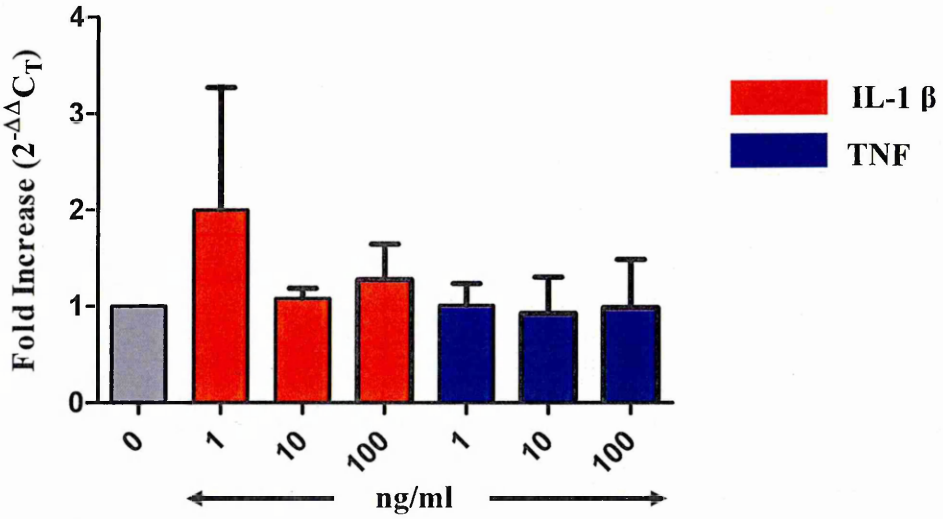
qRT-PCR analysis of relative expression levels ($2^{-\Delta C_T}$) demonstrated that the SHSY-5Y cell line expressed ADAMTS-1, -4 and -5 mRNA levels with and without RetA differentiation in this study (Figure 3.5). Results were expressed as +/- standard error of the mean (SEM), for three independent experiments. Representative amplification plots are shown in appendix I.

Figure 3.4: Validation of housekeeping genes (qRT-PCR) for normalising mRNA expression in SHSY-5Y cells. A) Cyclophilin A vs RNAP-II, B) GAPDH vs cyclophilin A and C) RNAP-II vs GAPDH. Results showed that RNAP-II was the most stable housekeeping gene as its relative mRNA expression was constant either used as reference or as a target. Results expressed are relative to untreated control sample (grey bar) (A, B and C). When using GAPDH and cyclophilin-A (B) changes were observed on the cytokine treatment (IL-1β at 1 ng/ml). However, no statistical significance was seen with these housekeeping genes. Results are expressed as means ± SEM (n=3 independent experiments).

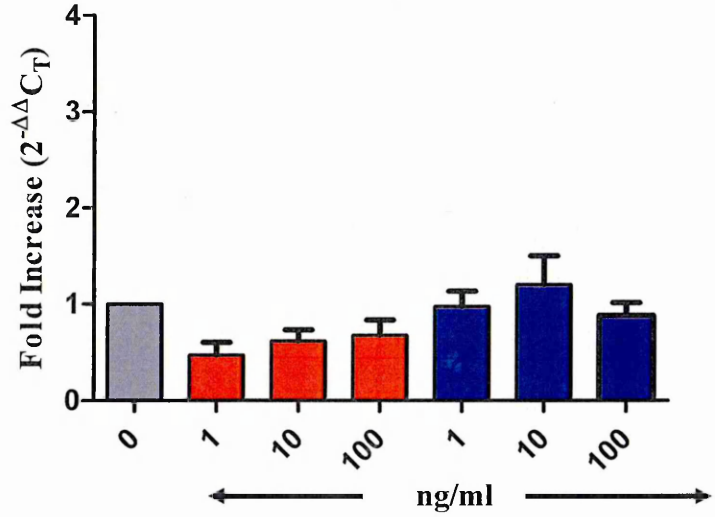
A)


Fold Increase ($2^{-\Delta\Delta C_T}$)
0
1
2
3
4
0
1
10
100
1
10
100
ng/ml

B)


Fold Increase ($2^{-\Delta\Delta C_T}$)
0
1
2
3
4
0
1
10
100
1
10
100
ng/ml
IL-1 β
TNF

C)


Fold Increase ($2^{-\Delta\Delta C_T}$)
0
1
2
3
4
0
1
10
100
1
10
100
ng/ml

A statistically significant increase was observed in differentiated cells compared with undifferentiated cells in terms of ADAMTS-1 mRNA expression ($p<0.05$, Mann-Whitney). There was an increase in ADAMTS-4 expression in differentiated cells compared to undifferentiated cells, but this was not statistically significant. There was low expression of ADAMTS-5 mRNA in both treated and untreated cells (Figure 3.5).

3.3.4 Effect of Pro-inflammmatory Cytokines on ADAMTS-1, -4, and -5 , ADAM-17 and TIMP-3 mRNA Expression Levels in SHSY-5Y Cells

To detect the modulation of ADAMTS-1, -4 and -5 and ADAM-17 expression in the SHSY-5Y cells, they were treated with pro-inflammatory cytokines (IL-1β or TNF), which are implicated in the pathogenesis of MS. Relative quantification of mRNA levels of ADAMTS-1, -4 and -5 from cytokine stimulated cells compared to untreated cells was calculated by the comparative cycle threshold C_T method ($2^{-\Delta\Delta C_T}$) (see section 2.9.3).

Figures 3.6 (A, B and C) show no statistically significant effects ($p>0.05$) on ADAMTS-1, -4 and -5 mRNA expression in SHSY-5Y cells differentiated with RetA (+RetA) or undifferentiated (-RetA), following 0-100 ng/mL treatment with IL-1 or TNF. Furthermore, there was no effect of the cytokines on ADAM-17 or TIMP-3 mRNA expression (Figure 3.6 D and E respectively) in the SHSY-5Y cells with and without RetA. Results shown are the combined results of three independent experiments.

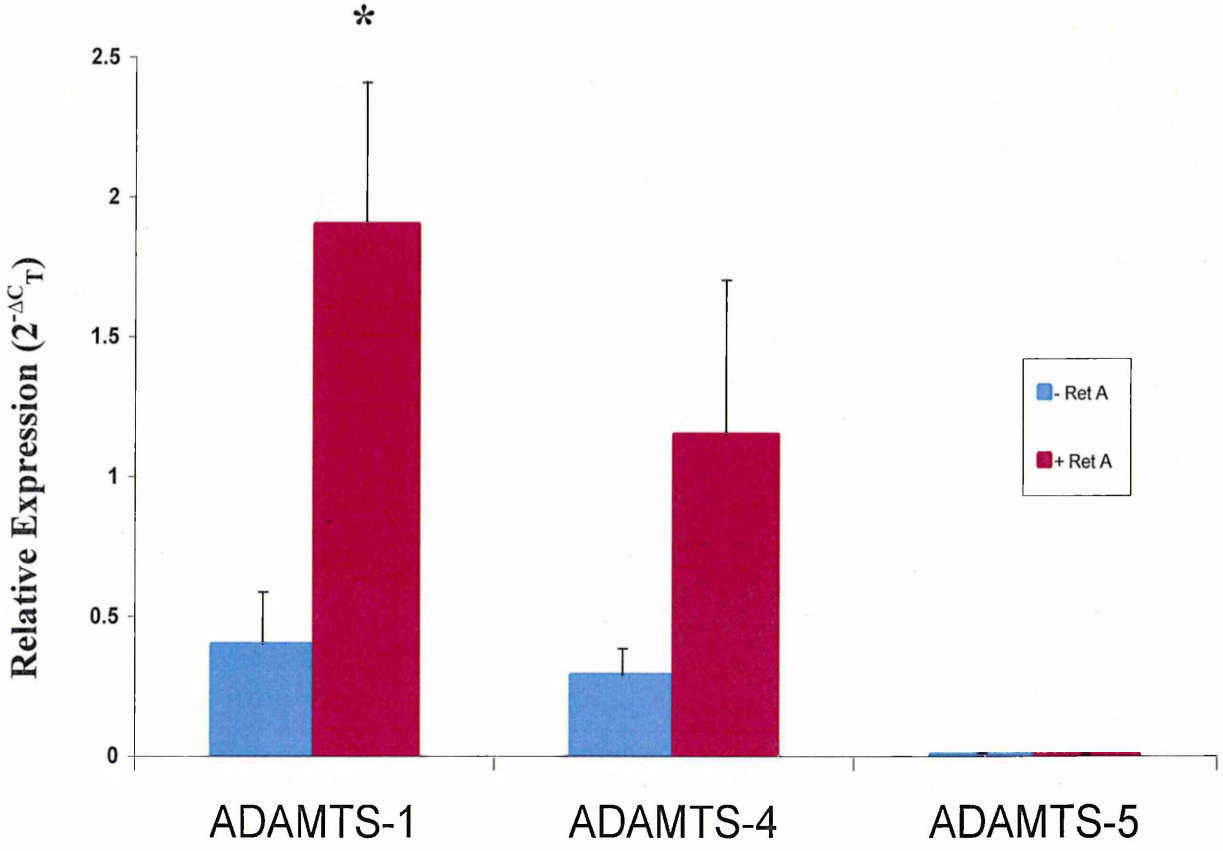
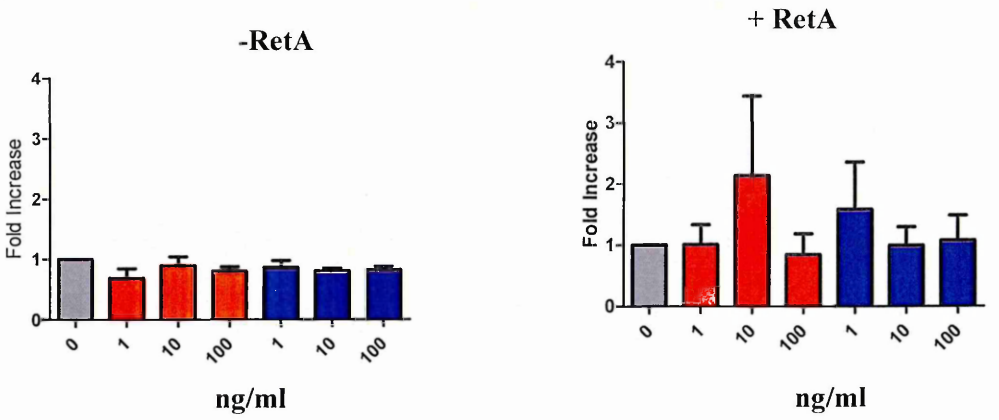


Figure 3.5: ADAMTS-1, -4 and -5 mRNA expressions in SHSY-5Y cells. Comparison of ADAMTS-1, -4 and -5 mRNA expression in SHSY-5Y cells with RetA differentiation (10^{-5}M) compared to control (undifferentiated cells), the signal is normalised to the reference gene (RNAP-II). Results are expressed as mean ± SEM of three independent experiments (*P<0.05, Mann-Whitney test n=3).

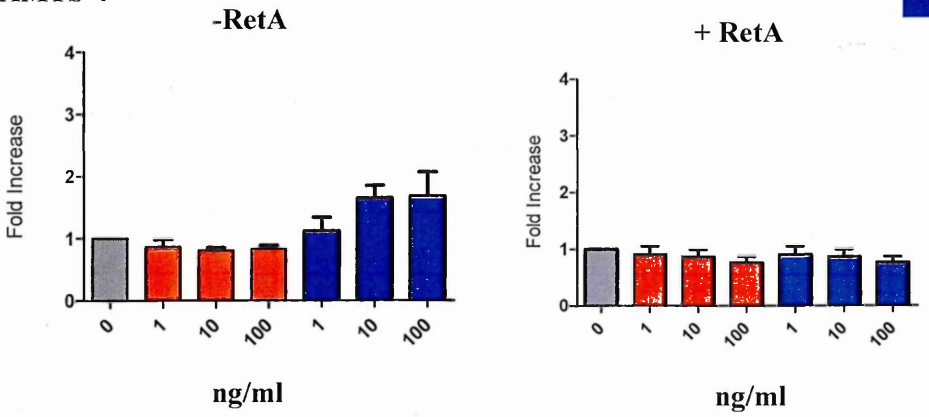
Figure 3.6: ADAMTS-1, -4 and -5 mRNA expression in SHSY-5Y cells following cytokine treatment. ADAMTS-1, -4 and-5 mRNA expression in SHSY-5Y cells with and without RetA differentiation following 24 hours of treatment with IL-1 or TNF (1-100 ng/ml). The signal is normalised to the reference gene (RNAP-II) and relative to the untreated control sample. Results are expressed as mean ± SEM, n= 3 independent experiments.

A) ADAMTS-1

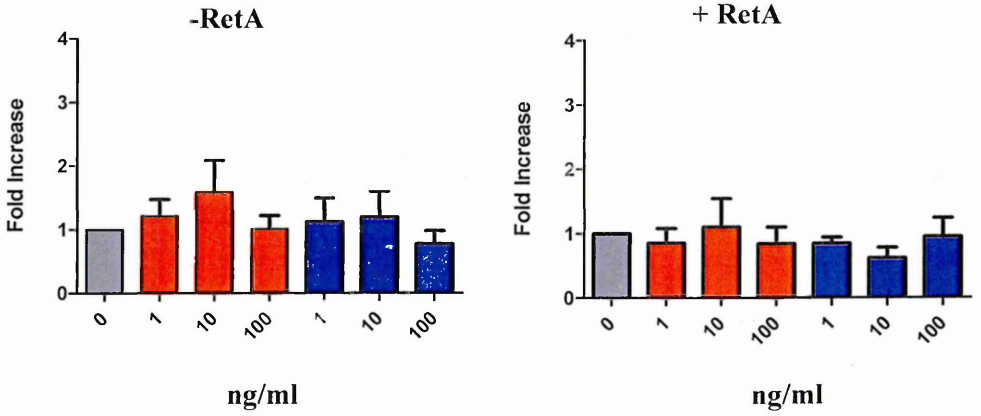


IL-1 β
TNF

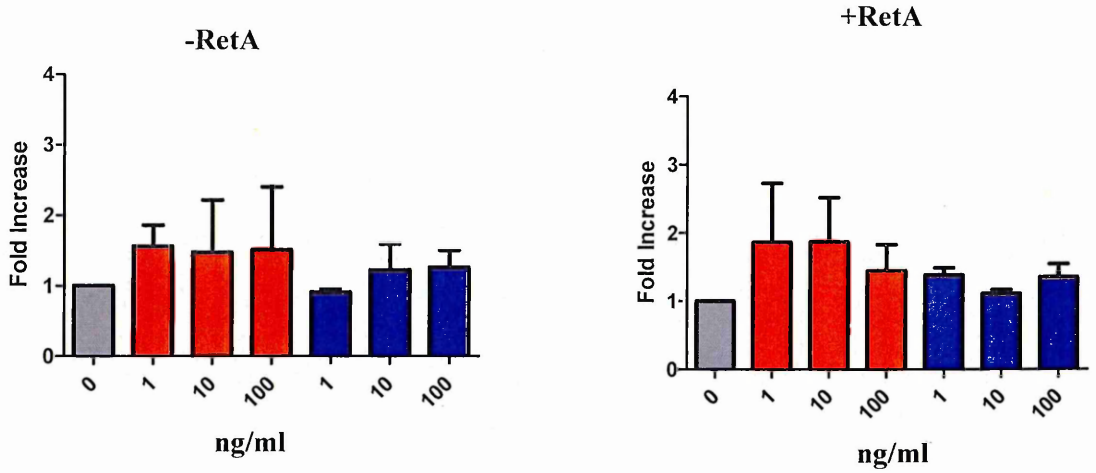
B) ADAMTS-4



C) ADAMTS-5



D) ADAM-17



E) TIMP-3

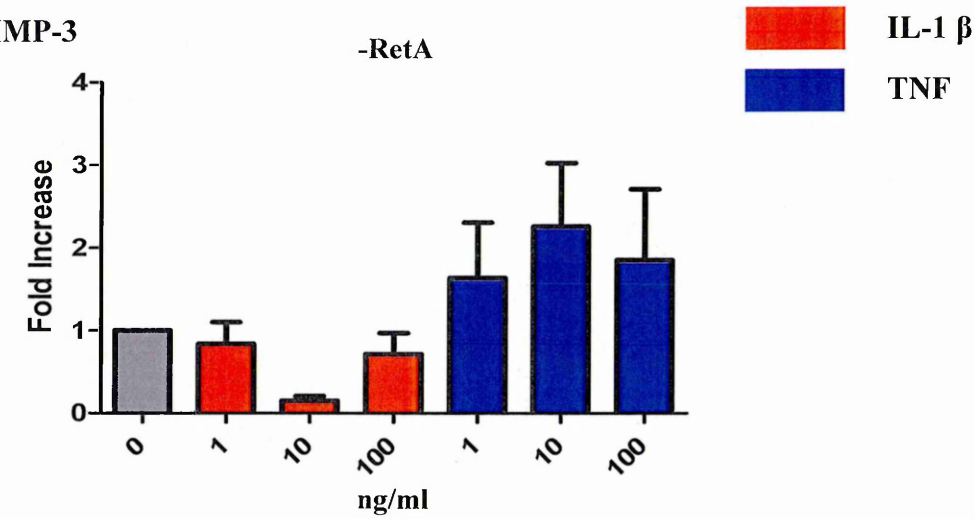


Figure 3.6 (continued): ADAM-17 and TIMP-3 mRNA expression in SHSY-5Y cells following cytokine treatment. D) ADAM-17 (+RetA and –RetA) and E) TIMP-3 (-RetA only) mRNA expression in SHSY-5Y cells following 24 hours of treatment with IL-1 or TNF (1-100 ng/ml). The signal is normalised to the reference gene (RNAP-II) and relative to the untreated control sample. (n=3 independent experiments).

3.3.5 Comparison of ADAMTS-1, -4, -5 and TIMP-3 mRNA Expression in Cytokine Treated SK-N-DZ Cells with RetA Differentiation

SK-N-DZ cells were used as another example of a neuroblastoma cell line, used as a neuronal model, to compare with the SHSY-5Y neuroblastoma cell line. The SK-N-DZ cells were characterised with neuronal marker NF L, differentiated with retinoic acid and treated with proinflamatory cytokines. RNA was extracted, cDNA was synthesised and used as a template for the qRT-PCR, as described in Chapter 2.

qRT-PCR analysis of relative expression levels ($2^{-\Delta C}{}_T$) demonstrated that the SK-N-DZ cell line constitutively expressed ADAMTS-1, -4, -5 mRNA levels with RetA differentiation with no detectable TIMP-3 expression (Figure 3.7). Representative amplification plots are shown in appendix II.

3.3.6 Effect of Pro-Inflammmatory Cytokines on ADAMTS-1, -4, -5 and TIMP-3 mRNA expression levels (qRT-PCR) in Differentiated (+RetA) SK-N-DZ Cells

To detect the expression and modulation of ADAMTS-1, -4 and -5 in differentiated SK-N-DZ cells, they were treated with pro-inflammatory cytokines (IL-1β or TNF) as described in section 2.4. The analysis of relative gene expression data using qRT-PCR was performed using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001).

Figure 3.8 A shows a statistically significant increase ($p<0.05$) in ADAMTS-1 mRNA expression in differentiated SK-N-DZ cells with IL-1 β (10 ng/ml). Furthermore, ADAMTS-4 mRNA expression level were significantly increased with 10 ng/ml and 100 ng/ml TNF treatment (Figure 3.8 B). However, ADAMTS-5 mRNA expression levels showed no statistically significant changes following either IL-1 or TNF treatment (Figure 3.8 C).

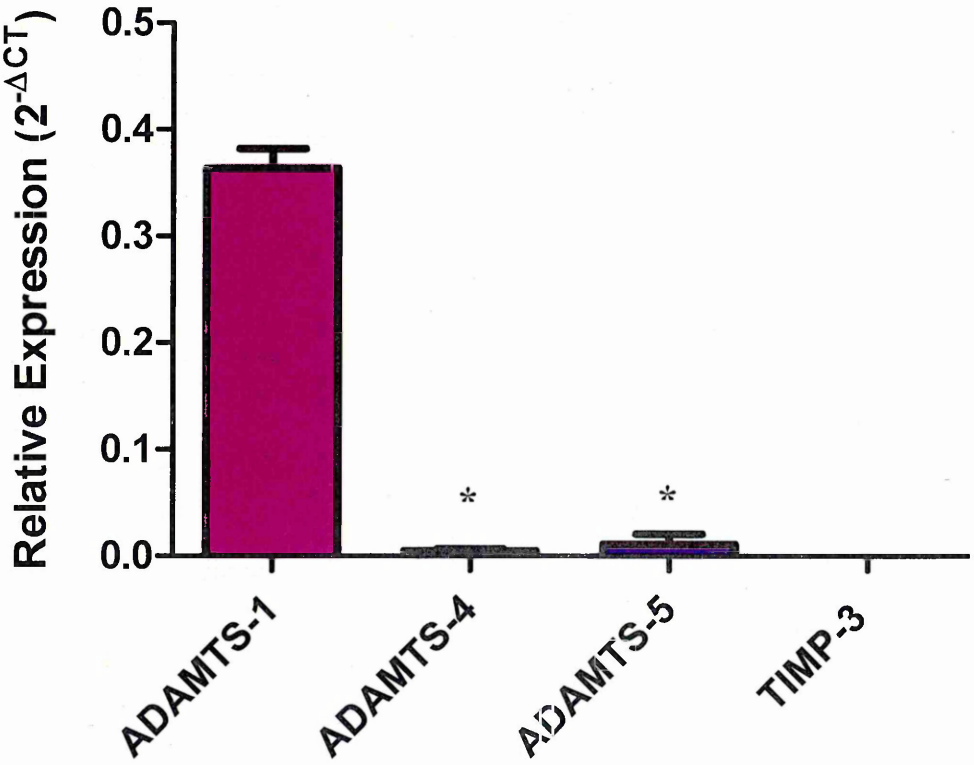
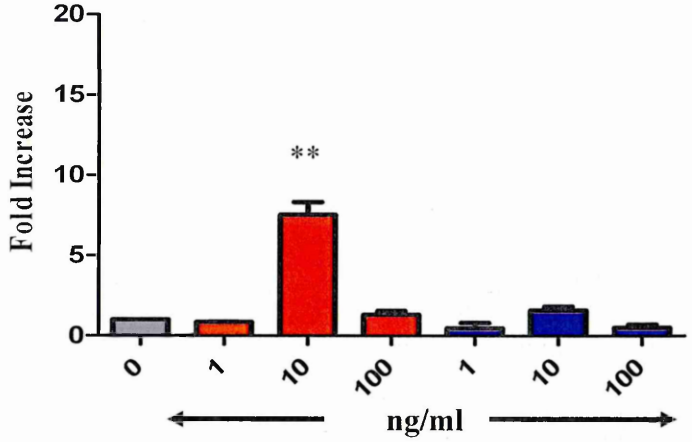


Figure 3.7: ADAMTS-1, -4, -5 and TIMP-3 mRNA expression in SK-N-DZ cells differentiated with RetA. The signal is normalised to the reference gene (RNAP-II). There was no TIMP-3 expression and results are expressed as +/- standard error of the mean (SEM), for three independent experiments. A statistically significant difference was observed in the ADAMTS-1 mRNA expression compared to ADAMTS-4 and -5 (*$P<0.05$, Mann-Whitney test).

Figure 3.8: ADAMTS-1, -4 and -5 mRNA expression in RetA differentiated SK-N-DZ cells following 24 hours of IL-1 or TNF treatment (0-100 ng/ml). The signal is normalised to the reference gene (RNAP-II) and relative to the untreated control sample. Results are expressed as +/- standard error of the mean (SEM), for three independent experiments. A) ADAMTS-1, B) ADAMTS-4 and C) ADAMTS-5 * $P<0.05$, ** $P<0.01$, *** <0.001(Kruksal-Wallis followed by Dunnet's post test).

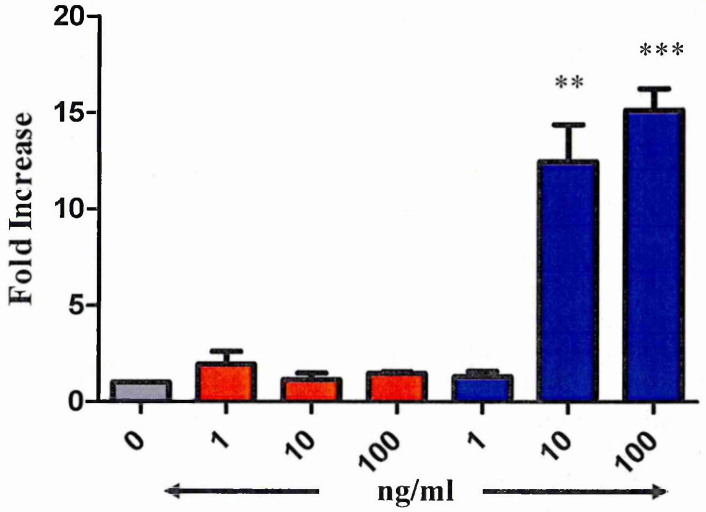
A) ADAMTS-1



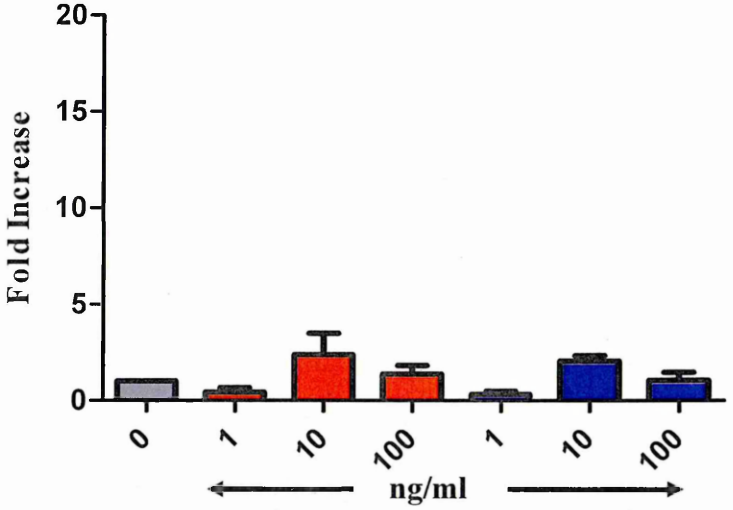
IL-1 β

TNF

B) ADAMTS-4



C) ADAMTS-5



3.4 Discussion

3.4.1 ADAMTS-1,-4, -5 and TIMP-3 Expression in Neuroblastoma Cell lines

The objectives of these studies were to determine whether ADAMTS-1,-4, -5 and their endogenous inhibitor TIMP-3 are expressed in SHSY-5Y and SK-N-DZ neuroblastoma cell lines. Production of ADAMTSs and TIMP-3 by neuronal cells may be important in the pathogenesis of MS. Increased ADAMTS production by neurons may enable neurite outgrowth into regions of axonal damage or loss, via breakdown of CSPG rich ECM, to enable repair. The enzyme-inhibitor balance may be important in this process. These cell lines were used as a model for neuronal cells in this study. Previous studies have demonstrated that neuroblastoma cells can constitute a useful neuronal model system for *in vitro* study. They show one of the highest rates of induction to differentiation into more neuronal CNS cells of all cancer cells (Thiele, 1998).

The SHSY-5Y cell line has been widely used as a model of neurons since the early 1980s as these cells possess many biochemical and functional properties of neurons. It shows neuronal marker enzyme activity (tyrosine and dopamine-β-hydroxylases), specific uptake of norepinephrine and expresses neurofilament proteins and nerve growth factor receptors. In addition, SHSY-5Y cells can proliferate in culture for long periods maintaining their phenotype, a requirement for the development of an *in vitro* cell model. SHSY-5Y cells have the potential to differentiate upon treatment with RetA leading to production of cells with neuronal morphology (Encinas *et al.*, 2000). The cell lines are the nearest alternative to primary cells which would be difficult if not impossible to obtain from humans and of reduced relevance if obtained from animals.

In vitro studies with cytokine treatment such as IL-1 β and TNF, which are involved in the pathogenesis of MS could induce ADAMTSs. Under basal conditions, the SHSY-5Y and SK-N-DZ cells express ADAMTS-1, -4 and -5 mRNA. Previous studies in our laboratory revealed that U373-MG, U87-MG, primary astrocytes and CHME3 microglial cells expressed ADAMTS-1, -4 and -5 (Cross *et al.*, 2006a). Sasaki *et al*,

(2001) reported that ADAMTS-1 is expressed in N1E-115 neuroblastoma cell line (Sasaki *et al.*, 2001). This suggests that CNS cells may express ADAMTS-1, -4 and -5.

ADAMTS-1 is the predominant GEP of the ADAMTSs expressed by both SHSY-5Y and SK-N-DZ cells, whereas ADAMTS-5 mRNA is expressed at low levels in both cell lines. RetA is an active metabolite of vitamin A, involved in development and differentiation of the nervous system. The RetA has an essential role during development and maturation of the CNS, mainly in the generation of neurons by the increase of neurite outgrowth (axons and dendrites) (Clagett-Dame *et al*, 2005). Culturing SHSY-5Y cells in the presence of RetA demonstrated an impact on the morphology of the cells, which agrees with Encinas *et al.*, (2000). Microscopic images of the cells in this study showed apparent differentiation with extension of cells to produce neurite-like outgrowths.

Differentiated SHSY-5Y cells (RetA) showed a statistically significant ($p<0.05$) increase in ADAMTS-1 expression when compared to cells cultured without RetA. The increase of ADAMTS-1 in differentiated cells could lead to the breakdown of CSPGs and allow the neurite outgrowth as CSPGs are normally inhibitory. There was no difference in mRNA expression for ADAMTS-4 and -5 in both treated and untreated cells with RetA. A study done by East *et al* (2007), showed an increase in ADAMTS-5 mRNA in cartilage explants with RetA treatment for only three days, whereas in our study differentiated SHSY-5Y cells were cultured for 7 days and there were no differences in ADAMTS-5 mRNA expression levels. It may indicate differential ADAMTS -5 expression between different cell types.

ADAMTS expression could be regulated at different stages including, pre-transcriptional (e.g. cytokine modulation of expression) post-transcriptional (e.g. alternative splicing) and post-translational (e.g. prodomain or C-terminal cleavage) regulation. Stability of the ADAMTS expression levels in normal physiology avoids the alterations that could result in neurodegenerative diseases.

There was no modulation of ADAMTS-1, -4 and -5 or ADAM-17 mRNA expression found in the SHSY-5Y neuroblastoma cell line with either IL-1β or TNF, at the concentrations used in this study, compared to controls. However, ADAMTS-1 mRNA expression in differentiated SK-N-DZ on treatment with IL-1β at the 10 ng/ml, showed a significant increase (~ 8 fold change). A higher concentration (100 ng/ml) seemed to be inhibitory. There is also a significant increase in ADAMTS-4 mRNA expression with TNF treatment (10, 100 ng/ml). However, ADAMTS-5 showed no statistically significant change after proinflammatory cytokine treatments. In a previous study in glial cells, a gradual increase in ADAMTS-4 mRNA and protein expression in response to TNF treatment was demonstrated. In the same study, IL-1β did not significantly alter expression of ADAMTS-4 mRNA and protein levels by astrocytes and only increased ADAMTS-4 mRNA expression at 1 ng/ml (Cross *et al.*, 2006a). TIMP-3 mRNA expression in SHSY-5Y cells indicated that there was no modulation on cytokine treatment and in the SK-N-DZ cell line there was no TIMP-3 expression. The differences in the expression and modulation of ADAMTSs in both neuronal cell lines used in this study could be due to differences in their selected phenotypes and cell populations. The SK-N-SH is the parent cell line for both SHSY-5Y and the SK-N-DZ contains cells with three different phenotypes: neuronal (N type), Schwannian (S type), and intermediary (I type). The SHSY-5Y cell line is a comparatively homogeneous neuroblast-like cell line (N type) (Joshi *et al.*, 2006).

In this study there was no modulation of ADAM-17 mRNA expression levels with proinflammatory cytokines in both differentiated and undifferentiated SHSY-5Y cells. This *in vitro* investigation indicates that neuronal ADAM-17 may not play a role in the pathogenesis of MS. It has been previously demonstrated that there is upregulation of ADAM-17 in MS lesions and that endothelial cells; parenchymal astrocytes and activated macrophages or microglia are responsible for the expression of ADAM-17 within MS brain tissue (Plumb *et al.*, 2006).

Chapter 4

Expression of ADAMTS-1 Protein

4.1 Introduction

The data in chapter 3 indicates that ADAMTS-1 mRNA was the most highly expressed, compared with ADAMTS-4 and -5 by both the SHSY-5Y and SK-N-DZ neuroblastoma cells. Furthermore, ADAMTS-1 has been previously observed to be expressed in the N1E-115 neuroblastoma cell line (Sasaki *et al.*, 2001). ADAMTS-1 gene was first identified as inflammation and cancer cachexia-related (Kuno *et al.*, 1997). It appears to be necessary for normal growth, fertility, and organ morphology and function (Shindo *et al.*, 2000). Kuno *et al,* (1997) indicated that ADAMTS-1 gene expression is induced in the kidney and heart of mice treated with lipopolysaccharides. Moreover, ADAMTS-1 mRNA could be induced by stimulating colon 26 cells, an adenocarcinoma cell line, with an inflammatory cytokine, interleukin-1 *in vitro*, suggesting a possible role of ADAMTS-1 in various inflammatory processes (Kuno *et al.*, 1997).

Cross *et al* (2006) demonstrated that ADAMTS-1, -4 and -5 are constitutively expressed in normal and EAE spinal cord and showed changes in expression of mRNA and protein levels during different stages of EAE. ADAMTS-1 had the lowest level of expression of the ADAMTSs, although it was the only ADAMTS with increased mRNA levels in animals with EAE (pre-disease) compared to normal controls (Cross *et al.*, 2006b). Sasaki *et al* (2001) found that ADAMTS-1 was expressed at very low levels by rat hypoglossal motor neurons but following nerve injury, mRNA levels were rapidly increased (Sasaki *et al.*, 2001).

Therefore, a study was performed to analyse ADAMTS-1 protein levels, by western blotting, in SHSY-5Y cells. In order to study the expression of ADAMTS-1 at the protein level, three commercially available antibodies were tested from Abcam (ab391940), Triple Point Biologics (RP1-ADAMTS1) and Santa Cruz Biotechnology (sc-31080).

The aim of the study was to optimise an ADAMTS-1 antibody (or antibodies) for the detection of ADAMTS-1 by western blotting and verify its specificity. This antibody could then be used in further experiments.

This aim was achieved by the following objectives.

4.1.1 Objectives

- To test the specificity of ADAMTS-1 antibody by the pre-incubation with immunising blocking peptide.

- To knockdown ADAMTS-1 in SHSY-5Y cells using an siRNA method and detect any reduction of ADAMTS-1 at mRNA (qRT-PCR) and protein (western blotting) levels.

4.2. Results

4.2.1 Optimisation of Abcam and Triple Point Biologics Antibodies to Detect ADAMTS-1 Protein by Western Blotting

Western blot optimisation is an important procedure to verify the immuno-detection of target protein and to eliminate non specific bands. Protein samples were extracted from SHSY-5Y cells (section 2.6.3) and their protein levels determined as previously described in section 2.8. The western blotting protocol used was as described in sections 2.10.2. and 2.10.2.1. Bands of approximately 51 kDa and 87 kDa were obtained with the Abcam and Triple Point Biologics ADAMTS-1 antibodies respectively (Figure 4.1 A and B). The ADAMTS-1 87 kDa band could represent the full length active form and the 51 kDa band a product of C-terminal proteolytic processing (Cross *et al.*, 2006a, Haddock *et al.*, 2006). However, with both of these antibodies the zymogen, with a molecular weight of 105 (according to the manufacturer's information) was not present. The immunogen for both of these antibodies is a synthetic peptide based on the carboxyl end of the human ADAMTS-1 that recognizes the last 28 amino acids of the protein. Anti-β actin was detected by β actin immunoblotting (42 kDa) as a reference protein (Figure 4.1 C). The Abcam and Triple Point Biologics ADAMTS-1 antibodies gave results which were not reproducible.

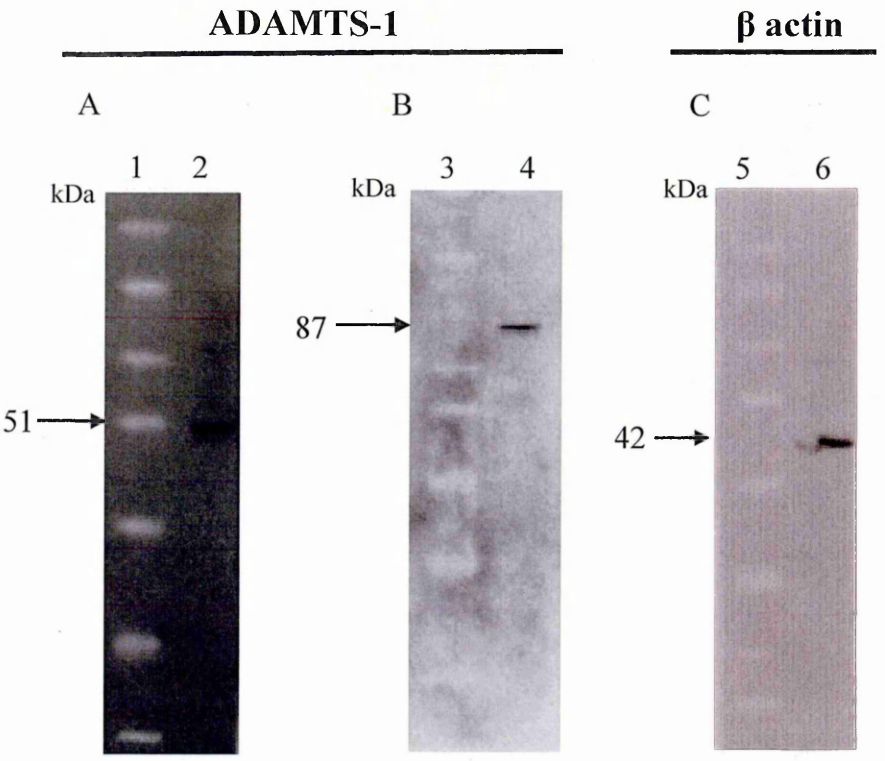


Figure 4.1: ADAMTS-1 protein optimisation by western blotting in SHSY-5Y cells. (A) Abcam (1:100) in TBST, (B) Triple Point Biologics (1:500) in TBST, (C) anti- β actin (1:1,000) in 5% TBST. Lane: 1, 3, 5 – molecular weight markers, Lane: 2, 4, 6 - protein samples.

4.2.2 Optimisation of Santa Cruz Biotechnology Antibody to Detect ADAMTS-1 Protein by Western Blotting in Human Neuroblastoma Cell Lines

Optimisation of Santa Cruz Biotechnology antibody to detect ADAMTS-1 protein by Western Blotting in human neuroblastoma cell lines was performed as described in section 2.10.2.2. The antibody showed a band of ~ 37 kDa for ADAMTS-1 which may represent a degraded or processed form of ADAMTS-1. ADAMTS-1 zymogen (110 kDa) was not present or the active mature form (85 kDa) which are detectable with this antibody, according to manufacturers information (Figure 4.2 A). Recombinant human ADAMTS-1 (rhADAMTS-1) was used as positive control. Figure 4.2 B shows an approximately 63 kDa molecular band which is around the predicted molecular mass of rhADAMTS-1 according to the manufacturer's instructions.

4.2.3 Determination of ADAMTS-1 Santa Cruz Biotechnology Antibody Specificity

The Santa Cruz Biotechnology antibody was tested for its specificity because it was the only antibody producing reproducible results in this study and a blocking peptide was available.

4.2.3.1 ADAMTS-1 Santa Cruz Biotechnology Antibody and Blocking Peptide

Peptide blocking of the Santa Cruz ADAMTS-1 antibody was performed according to the manufacturer's instructions using its commercially available peptide. This enabled determination of the ADAMTS-1 specific band. The western blotting method was performed as described in section 2.10.2.2. Samples were run from SHSY-5Y protein extracts. Again the antibody showed a band of ~ 37 kDa for ADAMTS-1 (Figure 4.3

A). Also demonstrated was almost a complete removal of this band following incubation of the primary antibody (0.4 μg/ml) with 2 μg/ml of ADAMTS-1 immunising peptide for 2 hours at room temperature (Figure 4.3 B).

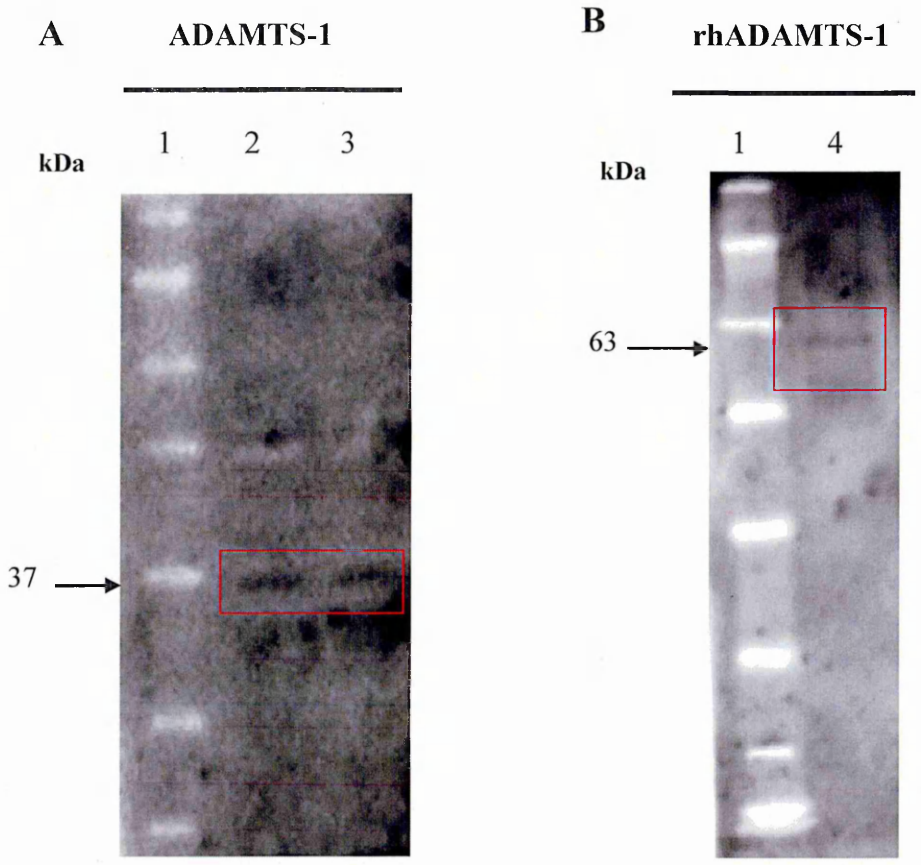


Figure 4.2: Detection of ADAMTS-1 by western blotting. (A) ADAMTS-1 detected in SHSY-5Y and SK-N-DZ cells using Santa Cruz Biotechnology antibody (1:500) in SHSY-5Y (lane 2) and SK-N-DZ (lane 3) cells showed ~ 37 kDa ADAMTS-1 bands. (B) Shows that ADAMTS-1 Santa Cruz Biotechnology antibody with rhADAMTS-1 protein detects a ~ 63 kDa band (lane 4), which is around the predicted molecular weight of the protein according the manufacturer's information, 66 kDa (R&D systems) under reducing conditions. Lane 1 indicates the molecular weight markers in both blots A and B.

SHSY-5Y Protein for ADAMTS-1 and Blocking Peptide

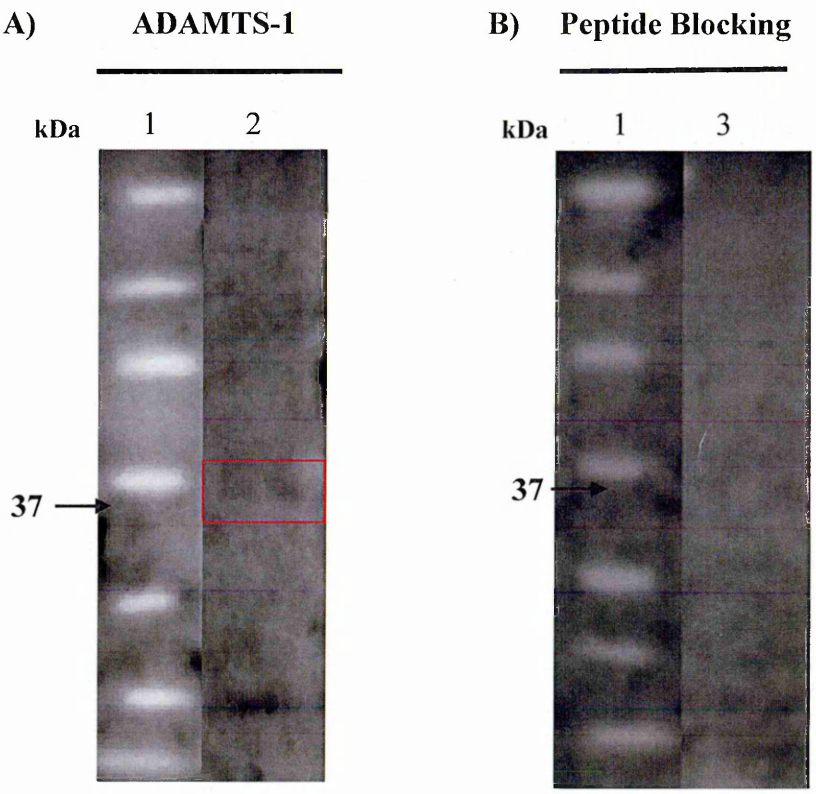


Figure 4.3: Western blot analysis of ADAMTS-1 antibody specificity using blocking peptides (Santa Cruz Biotechnology). A) The antibody demonstrated a reactive band ~ 37 kDa (lane 2). Blocking antibody by pre-incubation with the immunising peptide lowered the intensity of the 37 kDa immunoreactive band (lane 3). Lane 1: molecular weight markers (A and B) (n=1).

4.2.4 ADAMTS-1 siRNA Knockdown in the SHSY-5Y Neuroblastoma Cell Line

An ADAMTS-1 siRNA knockdown experiment was performed, as described in section 2.11.2, to confirm ADAMTS-1 mRNA expression in SHSY-5Y cells by qRT-PCR and to verify the specificity of the Santa Cruz Biotechnology ADAMTS-1 antibody.

4.2.4.1 Optimisation of Transfection

Optimisation of transfection was previously described in sections 2.11.3.1 and 2.11.3.2. Slides of transfected cells were examined under the microscope at 400 x magnification and the assessment of nuclear fluorescence was by counting the cells with a bright, red nuclear fluorescence, a signal of transfection success. The number of fluorescent cells out of 100 cells was counted. This allows easy optimisation of conditions that resulted in efficient siRNA delivery into cells because the siGLO transfection indicators are specially designed for nuclear localisation. Figure 4.4 shows the transfection reagents 1, 2, 3 and 4 efficiencies of optimisation. The most efficient transfection reagent for the SHSY-5Y cells was the transfect 1 reagent as it showed the highest rate of successful transfection (78.5%) red nuclear signal or siGLO uptake positive transfected cells, compared to the transfection reagents 2, 3 and 4 (Table 4.1).

4.2.4.2 ADAMTS-1 siRNA Knockdown in the SHSY-5Y Neuroblastoma Cell Line Determined at the mRNA (qRT-PCR) and Protein (Western Blotting) Levels

The ADAMTS-1 in SHSY-5Y cells was knocked down using the siRNA method which was performed as described in section 2.11.2. In section 3.3.3 ADAMTS-1 showed the most abundant mRNA expression levels and a significant up-regulation with RetA differentiation in SHSY-5Y cells. To confirm the ADAMTS-1 mRNA expression in SHSY-5Y cells by qRT-PCR, the SHSY-5Y cells were transfected for 24 hour with siRNA for ADAMTS-1 and GAPDH as a positive control. The mRNA levels for

ADAMTS-1 and GAPDH were determined by qRT-PCR as previously described in chapter 2. Results showed that ADAMTS-1 was knocked down ~ 66% and GAPDH was by ~98% at the mRNA level compared to the untreated sample (no treatment) (Figure 4.5).

The SHSY-5Y cells were transfected with ADAMTS-1 siRNA for 96 hour to determine the specificity of the Santa Cruz Biotechnology antibody. At the protein level, by densitometric analysis, ADAMTS-1 showed ~ 67% knockdown of the 37 kDa band (Figure 4.7 A). Western blotting was performed with detection of GAPDH using anti-GAPDH antibody as a positive control. All samples constitutively expressed GAPDH protein (40 kDa) with a clear reduction in the intensity of the GAPDH knockdown sample, compared to the untreated (cells without transfection medium) and non target samples (scrambled siRNA), which were used as negative controls. Western blotting was performed for β actin to verify equal loading levels (15 μg/ lane) (Figure 4.6 A). Non specific immune-reactivity was determined by omission of the primary antibody to detect any non specific reaction of the secondary antibody and as a negative control (Figure 4.6 B). Densitometric semi-quantitation of the bands as integrated optical density (IOD) was performed as previously described (Cross *et al.*, 2006) (Figure 4.7).

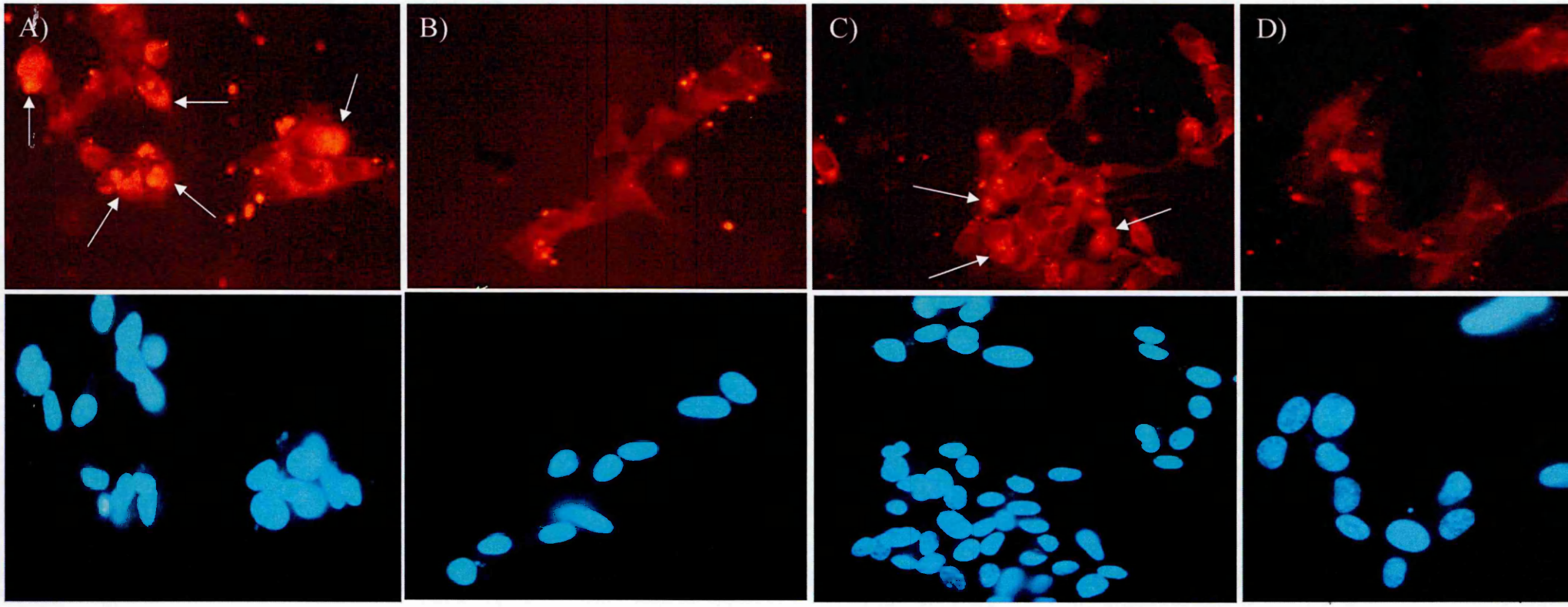


Figure 4.4: Transfection reagent efficiencies optimisation. Red nuclear staining (white arrows) is indicative of positive siGLO red uptake: TransFECT 1 (A TransFECT 2 (B), TransFECT 3 (C) and TransFECT 4 (D). The lower plates indicate the nuclei staining with Hoechst 33342.

Table 4.1: Transfection reagents efficiencies in the siRNA experiment in SHSY-5Y (mean of two determinations).

Transfection reagents with SiGLO	Efficiency %
DharmaFECT 1	78.5
DharmaFECT 2	33.0
DharmaFECT 3	45.0
DharmaFECT 4	25.0

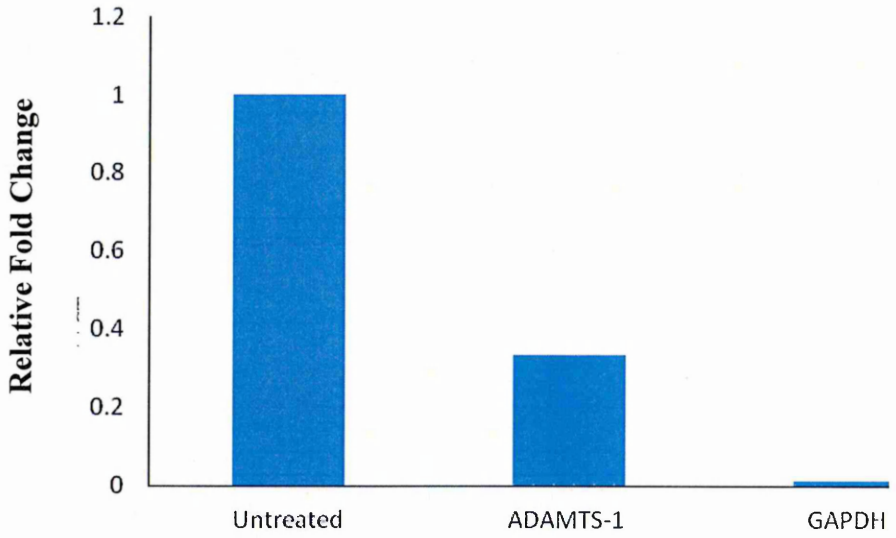


Figure 4.5: qRT-PCR of the ADAMTS-1 and GAPDH after siRNA knockdown in SHSY-5Y cells, GAPDH siRNA was used as a positive control. GAPDH showed a 98% reduction in the mRNA and ADAMTS-1 66% knockdown in mRNA level compared to untreated SHSY-5Y cells (no treatment) (n=1).

SHSY-5Y Protein for ADAMTS-1 siRNA Knockdown

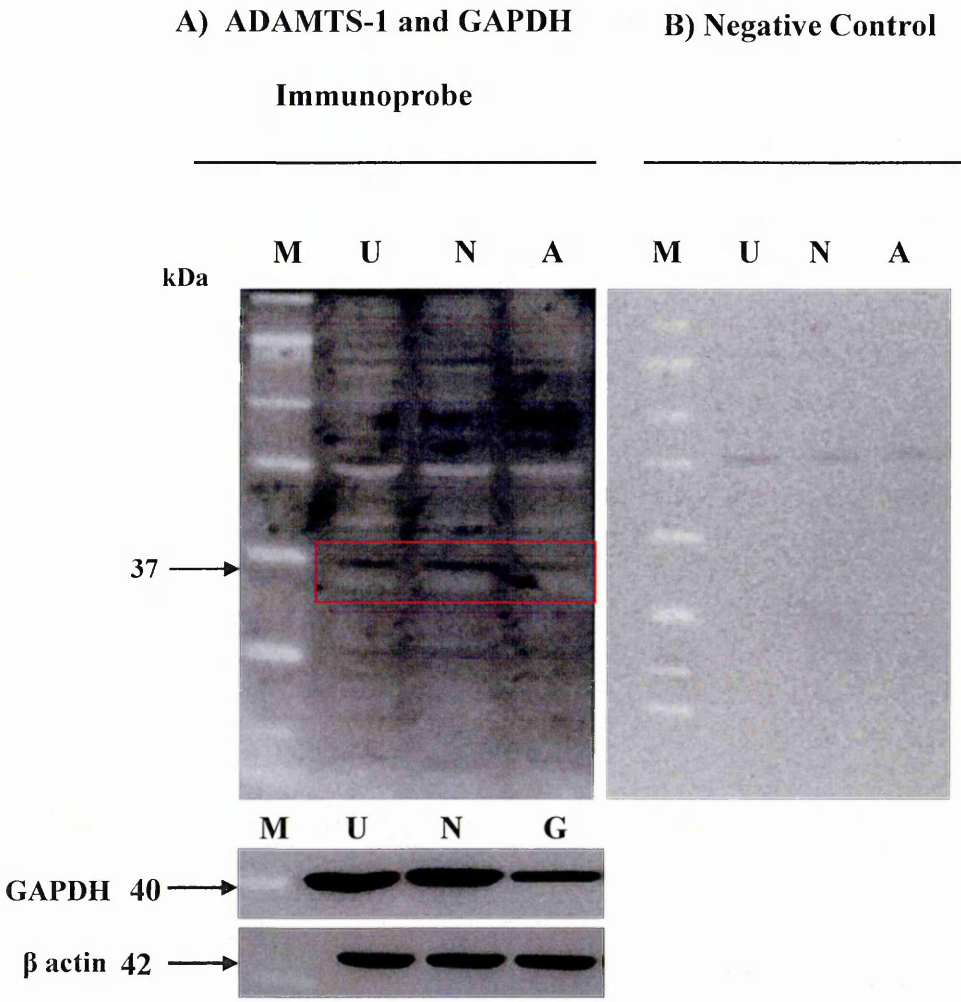
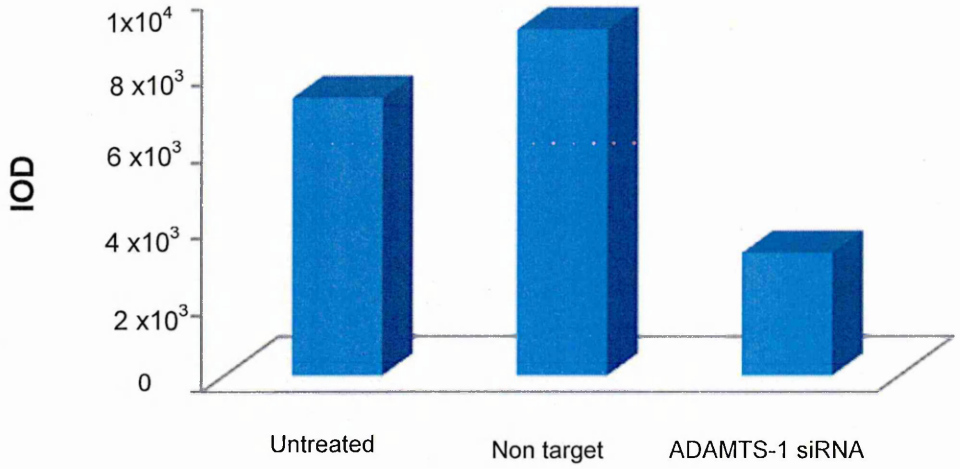


Figure 4.6: Western blotting of ADAMTS-1 and GAPDH after 96 hour siRNA transfection in SHSY-5Y cells (A) shows ADAMTS-1 detected by Santa Cruz Biotechnology antibody (1:500 in TBST), GAPDH as positive control and β-actin to verify the equal loading of protein samples (15µg/ lane). The ADAMTS-1 band at ~37 kDa was reduced in the ADAMTS-1 siRNA sample (A) compared to untreated (U) and non target (N) siRNA protein samples. The GAPDH antibody showed a band at the predicted molecular size of GAPDH (40 kDa) in all samples (GAPDH siRNA (G), U and N, which was reduced in the GAPDH siRNA (G) sample compared to the untreated and non target samples. β actin antibody showed comparable bands (42 kDa) β actin protein in all samples. (B) Omitting the primary antibody as a negative control (n=2).

(A)



(B)

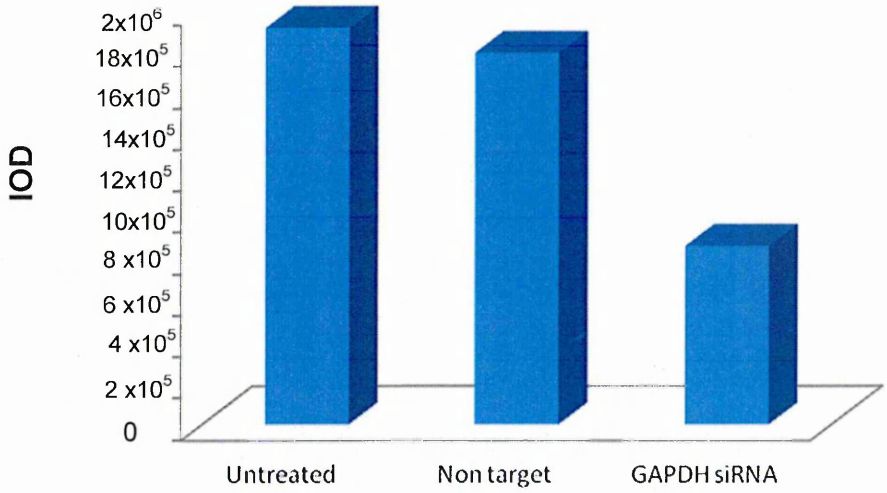


Figure 4.7: IOD of the ADAMTS-1 and GAPDH in SHSY-5Y cells. (A) Shows that ADAMTS-1 protein siRNA sample was approximately one third compared to the non target sample and approximately half to untreated SHSY-5Y cells after siRNA knockdown. (B) GAPDH (positive control) was reduced after siRNA knockdown compared to untreated and non target sample. Y-axis represents the densitometric results (IOD).

4.3 Discussion

4.3.1 The Optimisation of Antibodies and the Detection of ADAMTS-1

In chapter 3 using qRT-PCR, human neuroblastoma cell lines *in vitro* were demonstrated to constitutively express ADAMTS-1, -4 and -5 mRNA. ADAMTS-1 mRNA appeared to show the highest expression levels, ADAMTS-4 and ADAMTS-5 mRNA were lower. There is little known about neuronal ADAMTS expression. The SHSY-5Y cells, used as a neuronal cell model in this study, showed on siRNA knockdown ~ 66% reduction of ADAMTS-1 mRNA, which is consistent with previous results in chapter 3 that SHSY-5Y cells express ADAMTS-1 mRNA. Therefore, the main objective of this study was to detect ADAMTS-1 protein in the SHSY-5Y neuroblastoma cell line and determine the specificity of an ADAMTS-1 antibody to verify it for further uses. Initially, ADAMTS-1 immunoprobed blots with antibodies from three different sources showed immunoreactive bands with dissimilar sizes. The Abcam ADAMTS-1 antibody showed ~ 51 kDa band, which could represent a form produced by proteolytic cleavage similar to one previously described by Cross *et al.* (2006). However, the difference was that the previous study by Cross *et al.* (2006) used a polyclonal carboxyterminal Santa Cruz Biotechnology ADAMTS-1 antibody for the detection of the protein in primary astrocytes (Cross *et al.*, 2006a). Also, a 50 kDa ADAMTS-1 form has been demonstrated previously by Haddock *et al.*, 2006 in human brain tissue by using a Triple Point Biologics antibody.

The other antibody used in this study (Triple Point Biologics) has shown a ~87 kDa band which could represent the full length active form. Abcam and Triple Point Biologics antibodies, being directed against the C-terminus, can recognise processed forms of ADAMTSs (Haddock *et al.*, 2006). The Abcam and Triple Point Biologics antibodies were unsuitable to be used in further studies because they produced unreproducible results (repeated 5 times) and did not have available blocking peptides of the immunizing peptides to test specificity. Therefore, the antibody that was used further in this study was from the Santa Cruz Biotechnology. Using the Santa Cruz Biotechnology antibody, western blots showed a ~ 37 kDa ADAMTS-1 band. It is

unknown whether these three antibodies to ADAMTS-1 from Abcam, Triple Point Biologics and Santa Cruz Biotechnology recognised similar or overlapping epitopes though all were directed to the C- terminus of ADAMTS-1. No information was available from the suppliers regarding exact binding regions of the antibodies in terms of epitopes or domains. A previous study by Miguel *et al* (2005), using a Santa Cruz Biotechnology antibody to ADAMTS-1 showed 37 kDa and 50 kDa ADAMTS-1 bands. Interestingly, both bands were fivefold over-expressed in neurodegenerative diseases (Miguel *et al.*, 2005). Also another company (Sigma-Aldrich, UK) reports that ADAMTS-1 C-terminal antibody (A 4476) detects bands at 34 kDa and 50 kDa, which represent ADAMTS-1 fragments, helping to confirm that ADAMTS-1 could be present as a fragment at ~37 kDa.

The 37 kDa ADAMTS-1 protein band was reduced on ADAMTS-1 siRNA knockdown and blocked on pre-absorption of the antibody with the immunizing peptide. The 37 kDa band could represent a form produced by C-terminal proteolytic processing or may represent protein polymorphisms or splicing variants (Miguel *et al.*, 2005). The peptide blocking and the siRNA knockdown experiments were able to confirm the specificity of the antibody to ADAMTS-1. According to the data in this chapter the most suitable antibody for ADAMTS-1 protein detection is from Santa Cruz Biotechnology and could be used in further investigations because it the only antibody that produced reproducible results and had an available blocking peptide.

These data suggest that neuronal cells may express ADAMTS-1 at both mRNA and protein levels. Data obtained from a previous study (Miguel *et al.*, 2005) and from this one showing that the neuronal model produces a ~ 37 kDa ADAMTS-1 band.

Chapter 5

Versican (V0/V2) Neoepitopes in Normal and MS Human Brain

5.1 Introduction

Key components of CNS ECM are proteoglycans of the lectican family, also known as or hyalectans as they bind to hyaluronan (Figure 1.3). They include CSPGs aggrecan, brevican, neurocan, phosphacan, appican and versican. Their role is to provide structural support as well as influencing cell behaviour through a wide variety of physiological brain processes e.g. development, repair, proliferation, cell signalling and interaction with other ECM components (Bellail *et al.*, 2004).

There are four versican isoforms (V0, V1, V2 and V3) which result from alternative splicing. Versican V0 contains the two GAG-attachment domains, GAGα and β, whereas versican V1 and V2 contain only GAG-β and GAG-α respectively. Versican V3, the smallest lectican, lacks both of these alternatively spliced elements and consequently carries no GAGs as shown in Figure 1.4. The V2 isoform is largely present in the CNS. Its cellular origin in the CNS is most likely oligodendrocytes (Schmalfeldt *et al.*, 2000).

In healthy adult tissue, protein synthesis, modification, and degradation is a continuous feature of the physiological system (controlled and balanced) in the body. In many pathological conditions, this stable equilibrium is unbalanced leading to an increased rate of protein breakdown. Modulations in synthesis and degradation of the CSPGs may contribute to pathological processes. In damaged CNS, accumulated CSPGs may inhibit neurite outgrowth and prevent axonal regeneration (Asher *et al.*, 2000). CSPG could form barriers to the regenerating nerve fibres in the central and peripheral nervous system (Bronner-Fraser, 1994, Landolt *et al.*, 1995, Fitch and Silver, 1997). CSPGs neurocan (Friedlander *et al.*, 1994), brevican (Yamada *et al.*, 1997) and phosphacan (Milev *et al.*, 1994) demonstrated *in vitro* neurite outgrowth inhibiting activities. Conversely, the removal of chondroitin sulphate promotes neuronal regeneration (Bradbury *et al.*, 2002, Moon *et al.*, 2001).

It has also been shown that *in vitro*, the V2 splice variant inhibits axonal outgrowth and migration (Fidler *et al.*, 1999, Niederost *et al.*, 1999, Schmalfeldt *et al.*, 2000). This

inhibiting activity of versican can be reduced, but not eliminated, by removing chondroitin sulfate chains, indicating that multiple domains of versican are involved in controlling axon regeneration.

ADAMTSs are proteins synthesised on the ER. They undergo N-terminal processing, resulting in the removal of the signal peptide in the ER and a subsequent removal of the prodomain, often in the trans Golgi. The prodomain is generally considered to preserve enzyme latency and is also important for correct protein folding and secretion (Cao *et al.*, 2000). With the exception of ADAMTS-10 and ADAMTS-12, all currently known ADAMTSs contain a proprotein convertase cleavage site containing furin recognition sequences. Pro-protein processing has been demonstrated for ADAMTS-1 (Rodriguez-Manzaneque *et al.*, 2000), ADAMTS-7 (Somerville *et al.*, 2004), ADAMTS-9 and ADAMTS-12 (Cal *et al.*, 2001). It has also been observed that intracellularly, pro-ADAMTS-4 co-localises with furin (Wang *et al.*, 2004). Additional proteolytic processing or cleavage occurs within the ancillary C-terminal domains of the ADAMTSs. This C-terminal processing affects both substrate specificity and localisation of these enzymes (Kashiwagi *et al.*, 2004).

ADAMTS-1, -4 and -5 are known for their aggrecanase activity. Also it is known that ADAMTS-1 and -4 can cleave versican, and ADAMTS-4 can cleave brevican. Therefore, these enzymes could play a significant role in brain ECM turnover. ADAMTSs are endopeptidases, cleaving substrate proteins into smaller polypeptide fragments. The result of protein cleavage is the generation of new N- and C-termini. These termini form neoepitopes as they are antigenically different from the sequence of the intact protein. The cleavage of the core protein of CSPGs generates neoepitopes. Antibodies that recognise the cleavage specific sites, neoepitopes, generated by proteolysis of the aggrecan and versican by glutamyl endopeptidases such as ADAMTS-1, -4 and -5, neoepitope antibodies, are now commercially available. They are important products in determining the enzymes responsible for tissue turnover and breakdown (Mort and Buttle, 1999).

Sandy *et al*, 2001 demonstrated that mature human aorta contains a 70-kDa versican fragment, which reacts with a neoepitope antibody to the C-terminal peptide sequence DPEAAE. This protein could correspond to the G1 domain of versican V1 (G1-DPEAAE441), which has been generated *in vivo* by proteolytic cleavage by one or more members of the ADAMTSs at the Glu441-Ala442 bond, within the sequence DPEAAE441-A442RRGQ (Sandy *et al.*, 2001). The V2 isoform, which is a predominant brain proteoglycan, is composed of the G1 domain, GAG-α domain, and the G3 domain. Westling *et al.*, 2004 demonstrated that versican V2 protein core is cleaved by ADAMTS-4 at the Glu405–Gln406 bond in the GAG-α domain which generates a 64 kDa glial hyaluronate binding protein (GHAP) fragment (Westling *et al.*, 2004). The same study suggests that it is possible that a small amount of this neoepitope is generated in the human brain by cleavage of V0 versican at the same cleavage site (Glu405–Gln406). V0 versican also contains the GAG-α domain, so this would also generate the 64 kDa fragment. An antibody produced using the same neoepitope peptide by Westling *et al* (2004) (JSCNIV) was used in this study to detect neoepitopes produced by ADAMTS cleavage of versican.

5.1.1 Versican (V0/V2) Neoepitopes in Human Brain Tissues

Versican is a proteoglycan known to be expressed during embryonic development (Landolt *et al.*, 1995, Stigson *et al.*, 1997, Perissinotto *et al.*, 2000) and found during adult life in most of the connective and nervous tissues (Yamagata *et al.*, 2003, Schmalfeldt *et al.*, 1998, Asher *et al.*, 2002). It was isolated and characterized from bovine aorta and chick embryo fibroblasts (Oegema *et al.*, 1979, Kimata *et al.*, 1986, Shinomura *et al.*, 1990) and cloned and sequenced in cultured chick and human cells (Shinomura *et al.*, 1993). Several studies recognized that there are three isoforms, V1, V2, and V3 which could be generated by alternative splicing from V0 transcript (Dours-Zimmermann and Zimmermann, 1994, Zako *et al.*, 1995).

In normal adults, versican appears to be largely in vascular constitutions. Versican is abundantly expressed by vascular smooth muscle cells (Lemire *et al.*, 1999, Lee *et al.*,

2001). Furthermore, previous studies on normal and diseased blood vessels *in vivo* have suggested that versican could represent a major constitutive ECM component of the adult endothelium (Riessen *et al.*, 1994, Bode-Lesniewska *et al.*, 1996). Cattaruzza *et al* (2002) demonstrated that versican isoforms can be expressed by the human brain as shown in Table 5.1. They also showed expression patterns of V0-V2 splice variants in different human organs (Cattaruzza *et al.*, 2002).

Table 5.1: Transcriptional versican isoforms in normal human tissues

Tissue/organ	Versican isoform			
	V0	V1	V2	V3
1. Spleen	+	+	--	+
2. Stomach	+	+	-	+
3. Mammary gland	+	+	+	+
4. Liver	--	+	--	--
5. Brain	+	+	+	+
6. Heart	+	+	+	+
7. Testis	--	--	--	--
8. Thymus	+	--	+	--
9. Placenta	+	+	--	+
10. Salivary gland	+	+	+	--
11. Kidney	+	+	+	+

"This was originally published in Journal of Biological Chemistry. Cattaruzza S, Schiappacassi M, Ljungberg-Rose A Spessotto P, Perissinotto P, Morgelin M, Mucignat M T, Colombatti A, Perris R. Distribution of PG-M/Versican Variants in Human Tissues and *de Novo* Expression of Isoform V3 upon Endothelial Cell Activation, Migration, and Neoangiogenesis *in Vitro*. J. Biol. Chem. 2002; 277: *47626-47635*. © the American Society for Biochemistry and Molecular Biology."

The aim of this work was to determine the versican cleavage by ADAMTS GEP activity in normal and MS CNS tissue as an index of enzyme activity and their role in MS pathogenesis. This was done by detecting neoepitopes of versican V2 using an antibody specific for the new C-terminal at the sequence NIVSFE405 which is a product of cleavage of versican V0 or V2 at the Glu405-Gln406 bond generated on ADAMTS cleavage as shown in Figure 5.1 (Westling *et al.*, 2004). This aim was achieved by the following objectives.

5.1.1 Objectives

- To study CSPG breakdown by ADAMTSs in post-mortem tissue via the production of versican (V0/V2) neoepitopes of ECM components in brain tissue. These neoepitopes were detected using a commercial antibody (Perbio Science, UK Ltd) by both immunohistochemistry and western blotting.

- To determine whether the expression levels of the versican (V0/V2) neoepitopes showed any differences in MS post mortem tissues compared to normal control brain tissue.

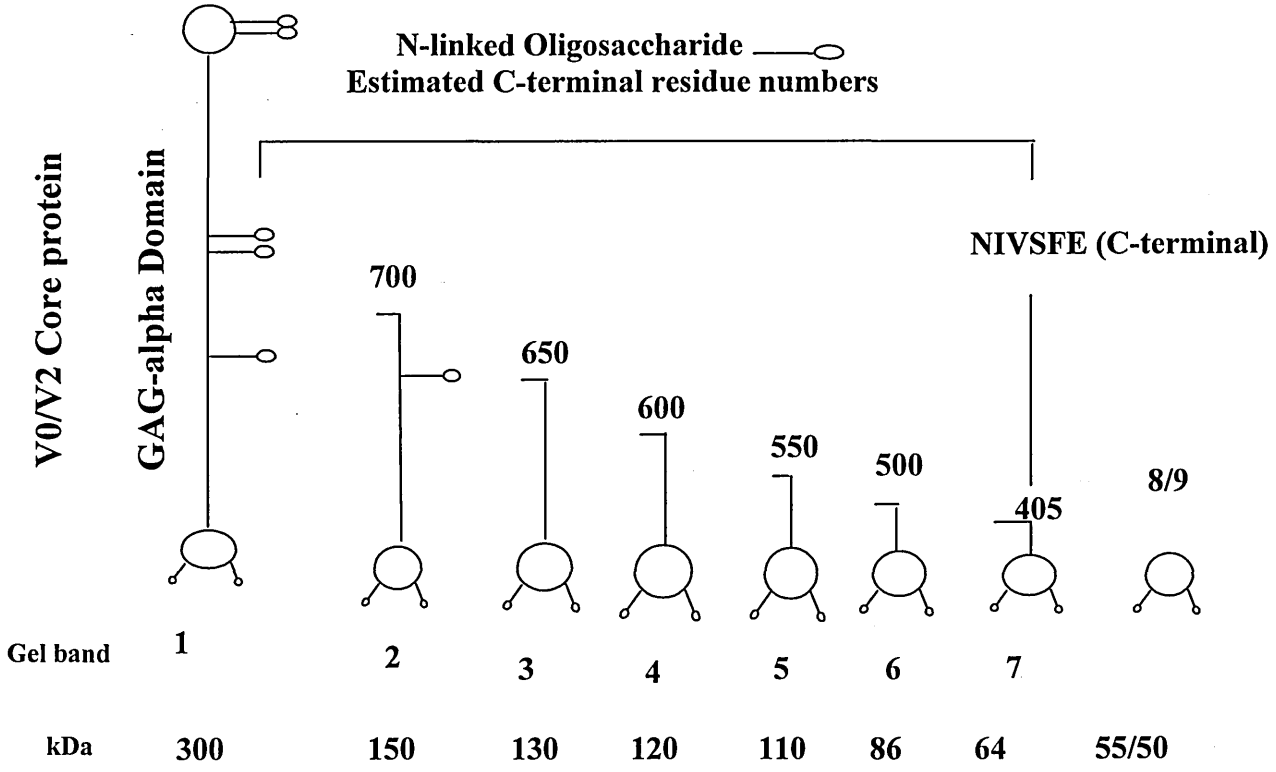


Figure 5.1: A schematic diagram of the versican (V0/V2) core glycoprotein species. There are a number of possible enzyme cleavage sites of versican (V0/V2) producing species detected in the human brain. The versican (V0/V2) neoepitopes antibody used in this study detects a C-terminus neoepitope at 64 kDa, produced by ADAMTS cleavage, by western blotting (species 7) (Adapted from Westling *et al.*, 2004).

5.2 Results

As previously described (section 2.12.1), tissues were obtained from the UK Multiple Sclerosis Tissue Bank, Division of Neuroscience and Mental Health, Imperial College London. Tissue blocks were from people with MS, including blocks containing NAWM and normal controls, people with no history of MS. White matter blocks containing MS lesions were from 3 females and 2 males with a mean age 55.2 years (range 37-71 yr). NAWM blocks were from 6 females and 1 male with a mean age of 62.6 years (range 46-86 yr). The tissue had a mean time from death to freezing of 17.9 h (range 5-33 h). 11 of the people with MS were diagnosed with secondary progressive MS and 1 with primary progressive MS. Five normal control brain tissue blocks were obtained from people with no history of MS, 3 female and 2 males with a mean age 61.2 (range 35-78 yr) see Table 5.2. Patient's details used in this study are summarised in Table 5.3. Tissue blocks were re-characterised in house using H&E and ORO as previously described in section 2.13.1.

5.2.1 Re-characterisation of the Human Brain Tissues (Blocks)

Ten-micrometre thick cryosections were cut, mounted on polysine–coated glass slides and stored at -80°C until required. The H&E or the ORO screening was performed on the tissue sections for lesion grading using a four point scale (negative –, +, ++, +++) as previously described in section 2.13.1 (Sanders *et al.,* 1993, Plumb *et al.,* 2002, Kirk *et al.*, 2003). This was performed to re-characterise the tissue used in this study. Tissue blocks with no inflammatory or ORO positive cells were scored negative. H&E, grade + contained a small number of cells in the perivascular region, grade ++ moderate cellular infiltrate and grade +++ had an abundant number of cells in the perivascular region. Figure 5.2 A shows an MS plaque x40, B shows +++ H&E staining for an MS lesion with perivascular cellular infiltrate. ORO, was scored + for a few scattered ORO positive cells, ++ frequent ORO positive cells in a MS lesion and grade +++ had an abundant number of ORO positive cells. Figure 5.2 E shows an ORO positive (grade +++) (demyelination) lesion in an MS patient x40. At higher magnification x200 the

presence of myelin breakdown in the activated lipid-laden macrophages with ORO positive (red) is evident (Figure 5.2 F). Figure 5.2 for normal control brain showed a negative score (C and G) as did NAWM (D and H) for H&E and ORO. ORO positive and perivascular cellular infiltrates were classified as lesional MS; those with ORO negative were classified as chronic plaques. A few of the NAWM blocks that were obtained from the UK Multiple Sclerosis Tissue Bank, when they were re-characterised, showed inflammation. The microscope settings were kept consistent when comparing images.

Table 5.2: Case Material

Type of Block (number of patients)	Mean age ± SD (Range)	Sex (F:M)	Mean DAI ± SD (hour)	Number of sample blocks
MS (5)	55.2 yr ±12.7 (37-71 yr)	3: 2	19.6 ±5.0	10
NAWM (7)	62.6 yr ±14.2 (46-86 yr)	6: 1	12.6 ±8.3	12
NC (5)	61.2 yr ±16.1 (35-78 yr)	3: 2	23.8 ±8.9	9

DAI, death autopsy interval; NC, normal control; NAWM, normal appearing white matter.

Table 5.3: Summary of case details in this study

Case No.	Blocks	Age (Years)	Sex (F/M)	Diagnosis	DAI (h)	No. of blocks studied			Cause of Death
						MS	NAWM	NC	
MS1	(MS058)	51	F	SPMS	15	2			MS
MS2	(MS060)	55	M	SPMS	16	2			Aspiration of gastric contents, MS
MS3	(MS080)	71	F	SPMS	24	1			Heart failure
MS4	(MS090)	62	M	SPMS	17	3			MS
MS5	(MS092)	37	F	SPMS	26	2			MS
MS6	(MS103)	77	F	SPMS	7		2		Pneumonia
MS7	(MS067)	86	F	SPMS	11		1		Bronchopneumonia
MS8	(MS057)	77	F	PPMS	9		3		General deterioration, lung infection
MS9	(MS093)	57	F	SPMS	25		2		Pneumonia, multiple sclerosis
MS10	(MS071)	78	F	SPMS	5		2		Metastatic carcinoma of bronchus
MS11	(MS100)	46	M	SPMS	7		1		Pneumonia
MS12	(MS159)	55	F	SPMS	24		1		Amitriptyline overdose
NC1	(CO14)	64	M	Normal	18			2	Cardiac failure
NC2	(CO22)	69	F	Normal	33			2	Lung cancer
NC3	(CO25)	35	M	Normal	22			2	Carcinoma of the tongue
NC4	(CO26)	78	F	Normal	33			2	Myeloid leukaemia
NC5	(CO28)	60	F	Normal	13			1	Ovarian cancer

DAI, death autopsy interval; NC, normal control; NAWM, normal appearing white matter; SPMS, secondary progressive MS; PPMS, primary progressive MS. Block number indicates the patient from which the block derives.

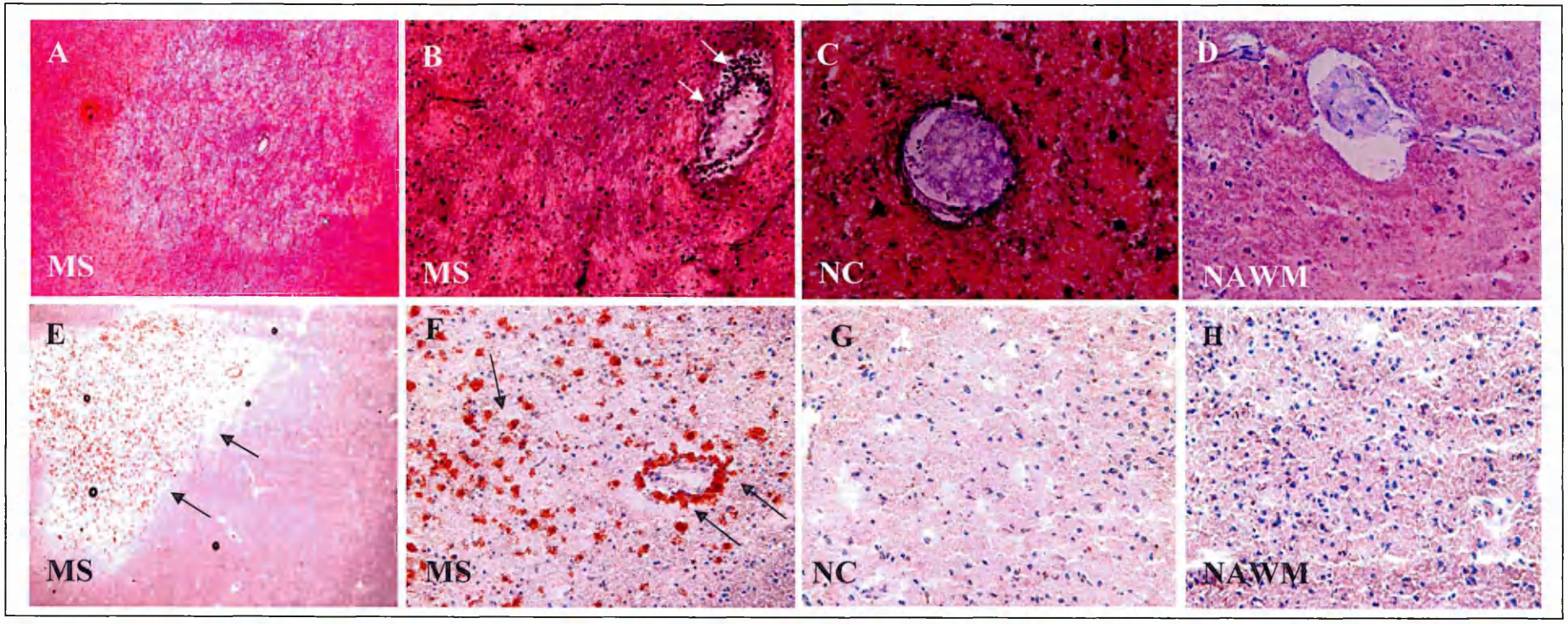
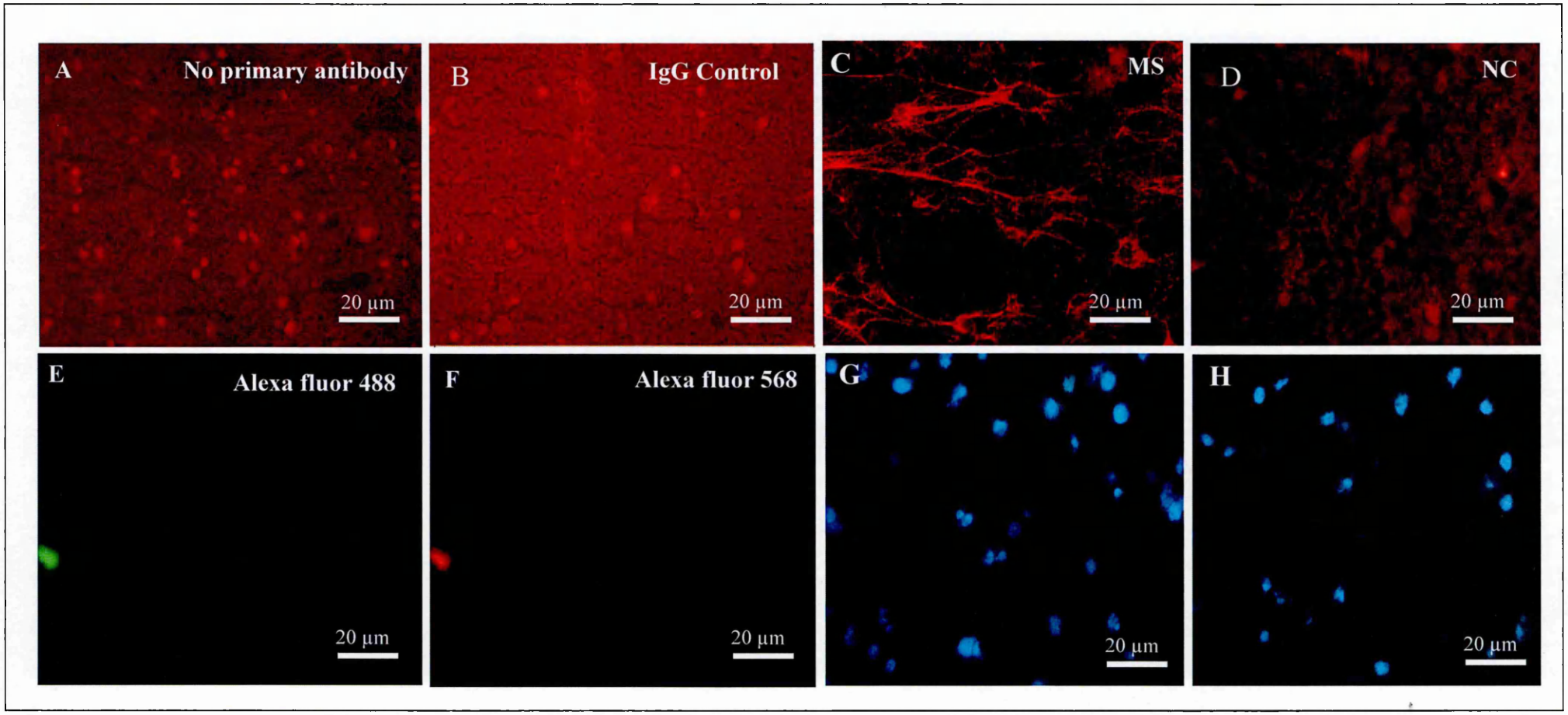


Figure 5.2: Olympus BX60 light microscope images of H&E and ORO staining for MS tissue and control brain. A) H&E staining of an MS tissue blo with a lesion at low magnification x40 or B) at x200 with cellular perivascular infiltration indicating inflammation (white arrows). E) Low-power image x of an MS lesion (demyelination) with ORO positive staining (red) (black arrows) or F) Higher magnification x200, where the presence of myelin breakdo in the activated lipid-laden macrophages is seen with ORO positive red staining, nuclei are counterstained with haematoxylin (dark blue). Both norm control (H&E (C) and ORO (G)) and NAWM tissues (H&E (D) and ORO (H)) show no cellular infiltration and no ORO positive cells respectively.

Immunofluorescence for versican neoepitopes was performed as previously described in section 2.14.1. Sections were graded on a 4-point scale as previously described (Haddock *et al.*, 2006, Plumb *et al.*, 2006). Figure 5.3 shows negative controls A, omission of primary antibody and B, IgG class to detect the non specific background. Both showed red fluorescence background nuclear-like staining in the tissue but no staining resembled staining of the ECM with versican (V0/V2) neoepitopes in the tissues. Sections that showed no versican (V0/V2) neoepitopes staining (immunoreactivity) were graded -, those which showed versican (V0/V2) neoepitopes only associated with the blood vessels were graded +, sections with blood vessel and ECM staining of versican (V0/V2) neoepitopes expression were graded ++ and those with an abundance of ECM staining were graded +++. Figure 5.4 summarises results from the 31 human brain tissues (blocks) studied of which 10 were MS lesion blocks, 12 were NAWM and 9 were normal control blocks. 9 of the MS blocks showed ++/+++ immunoreactivity out of 10. All normal control blocks were within - or + grading. NAWM showed variable immunoreactivity results (Figure 5.4). Within a lesional MS tissue, (MS58 P1D3), versican (V0/V2) neoepitope expression was increased (+++) with an abundance of ECM staining (Figure 5.3 C). In comparison, a normal control brain shows the absence of the versican (V0/V2) neoepitope staining in the ECM (Figure 5.3 D). As shown in Figure 5.5, in control tissues (CO25 P1B2 (B) and CO25 P1C2 (C)), shows versican (V0/V2) neoepitopes localised to blood vessels (determined using vWF as a marker for the blood vessels (Figure 5.5 A)), but were absent in the ECM (Figure 5.5 B and C).

Versican (V0/V2) immunostaining was heterogeneous in its distribution between sections. Four blocks out of nine of the normal control brain tissues demonstrated low (Grade +) versican (V0/V2) neoepitope expression whilst the remaining showed undetectable levels of expression and none of the normal control samples showed a ++/+++ grading. Higher expression levels of the versican (V0/V2) neoepitopes immunoreactivity were observed in lesional MS tissue (grading ++/+++) showing nine MS blocks out of ten, which is higher compared to normal control brain tissues and could indicate more activity of ADAMTSs in MS compared to normal control brain tissues (Figure 5.4).

Figure 5.3: Versican (V0/V2) neoepitope and negative controls in human brain tissue. Omitting the primary polyclonal rabbit versican (V0/V2) neoepitope antibody to detect non specific binding of the secondary antibody (A), IgG class control used instead of the primary antibody (B), negative controls for dual staining to detect the background due to the non specific binding of the secondary antibodies labelled with alexa fluor 488 (green) (E) or the secondary labelled with alexa fluor 568 (red) fluorescence (F). Versican (V0/V2) neoepitopes positive staining in a lesional MS section **(MS58 P1D3)** (C) and in a normal control tissue **(CO22 P1C3)** no versican (V0/V2) neoepitopes staining in the ECM (D), plates show the nuclei counterstained with DAPI (G, H). Images were captured with an Olympus BX60 fluorescence microscope, x400.


A
No primary antibody
20 µm
B
IgG Control
20 µm
C
MS
20 µm
D
NC
20 µm
E
Alexa fluor 488
20 µm
F
Alexa fluor 568
20 µm
G
20 µm
H
20 µm

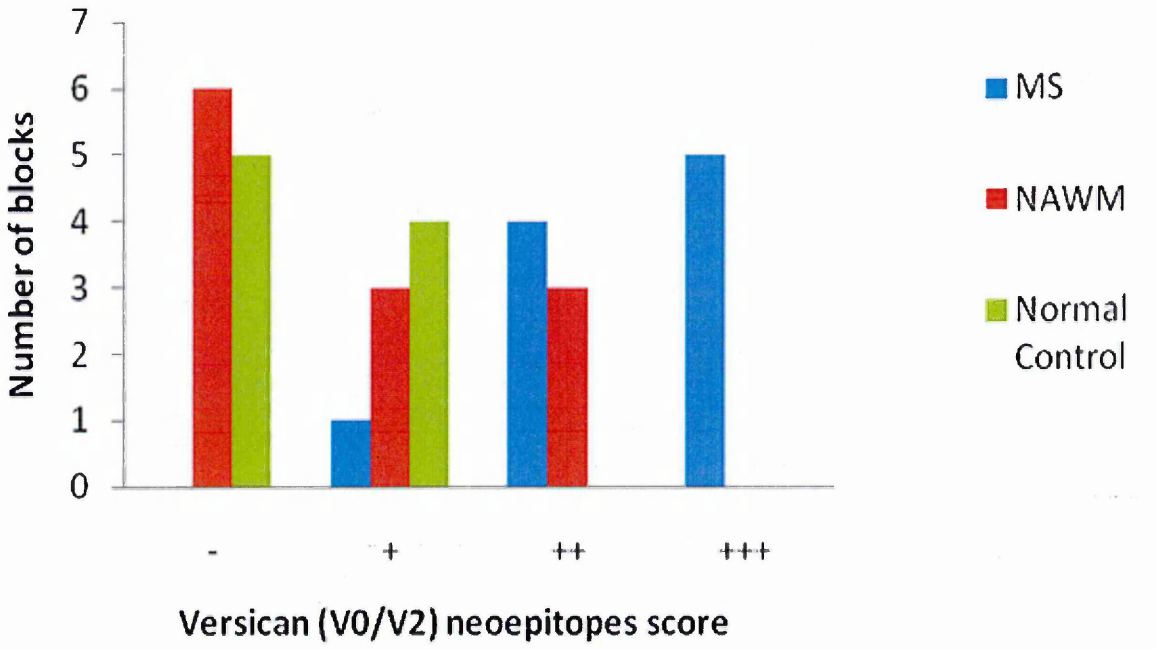


Figure 5.4: Versican (V0/V2) neoepitopes scoring in human brain tissue. The majority of MS cases were scored ++/+++, normal control brain tissue blocks showed only – or + scoring and NAWM showed variable scoring results – to ++.

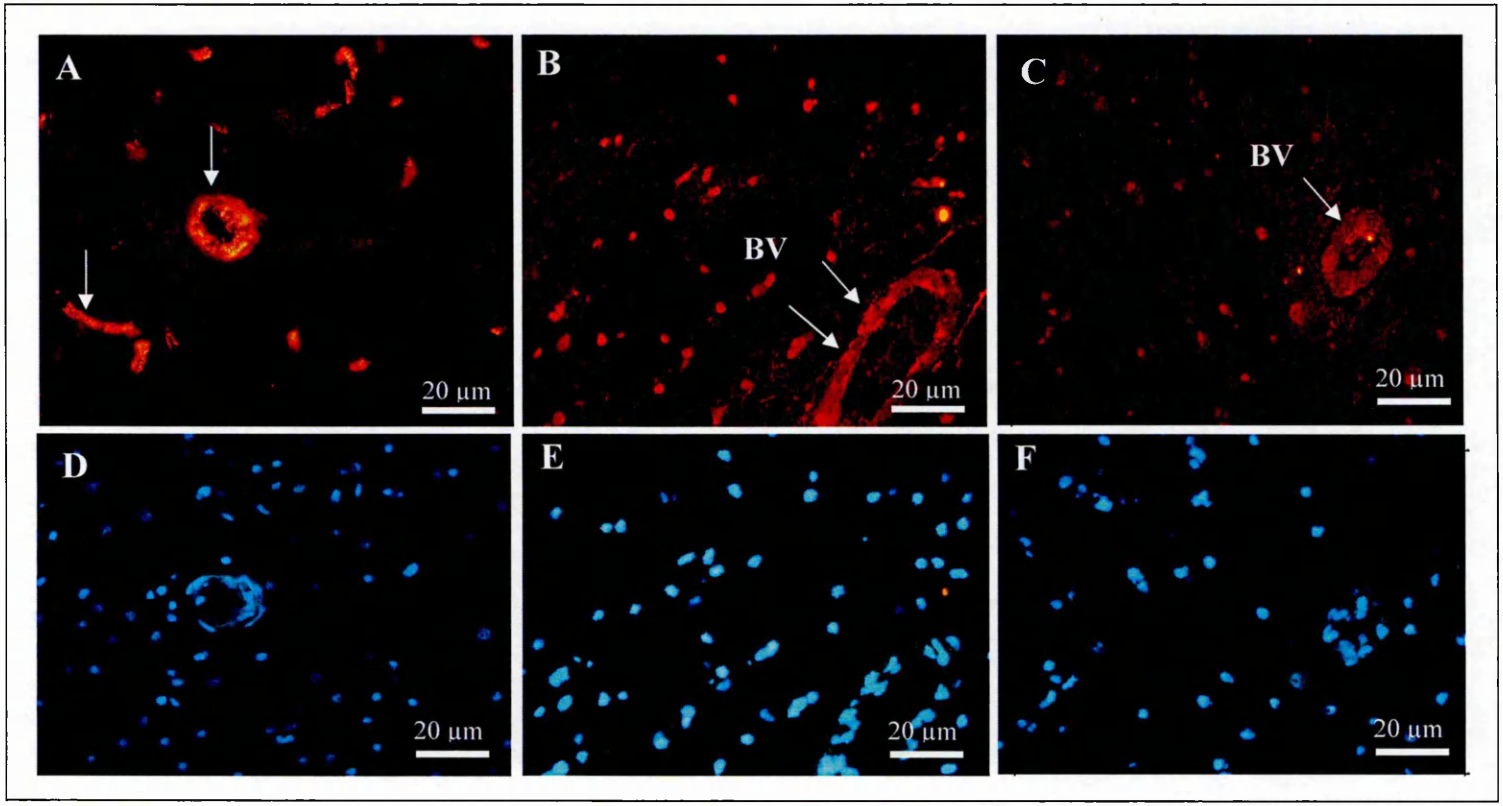


Figure 5.5: Versican (V0/V2) neoepitopes in normal control brain tissue. Images were captured with an Olympus BX60 fluorescence microscope x400. vWF expression in human brain tissue (A), versican (V0/V2) neoepitopes in normal controls (CO25 P1B2) (B) and CO25 P1C2 (C), which shows negative staining in the ECM and white arrows indicate the expression in the blood vessels (BV). Lower plates show the nuclei of the cells counterstained with DAPI (D, E and F).

5.2.3 Human Brain Tissues Classification According to HLA-DR Immunostaining

HLA-DR is a transmembrane human MHC class II family member and consists of a 34 kDa (alpha) and 28 kDa (beta) subunits. It is expressed primarily on cells on which it presents antigenic peptides for recognition by the T cell receptor on CD4+ T cells. Undetectable or low expression levels of MHC class II antigen expression in normal brain has been previously observed; in response to inflammatory stimuli in MS, microglia become activated and up regulate MHC class II antigen expression (Hayes *et al.*, 1987). A number of lesional MS cases that had shown positive versican (V0/V2) staining were further characterised to determine the presence of active lesions by detection of HLA-DR using an HLA-DR mouse monoclonal antibody (Table 5.4). Immunohistochemistry was performed as described in section 2.14.2. The HLA-DR antibody optimal concentration used in this study is shown in Table 2.5 and was according to the manufacturer's recommendations. An isotype control (IgG2b) was used as a negative control, replacing the primary antibody to detect the non specific background staining. Non-specific staining could result from the binding of the primary antibody to the Fc-receptors on cells.

Cellular activation (HLA-DR) was graded using a four-point scale (negative, + , ++ , +++) as previously described (Sanders *et al*, 1993; van der Valk *et al*, 2000; Plumb *et al*, 2002; Kirk J *et al*, 2003), according to the abundance of HLA-DR positive cells and the intensity of the staining. Figure 5.6 A shows a negative control when replacing HLA-DR antibody with its isotype control (IgG2b), showing no reactivity with the tissue. Figure 5.7 A and B show images of an active MS lesion (MS58 P1C3 and MS80 P1A4 respectively) with HLA-DR staining which is compared to a normal control brain tissue (CO25 P1B2) where expression levels of HLA-DR were undetectable (Figure 5.7 C).

5.2.3.1 Expression of HLA-DR and Versican (V0/V2) Neoepitopes in Human Brain Tissues

Immunohistochemical examination of HLA-DR and versican (V0/V2) neoepitopes was carried out on serial sections from a tissue block as previously described in section 2.14. Figure 5.8 A) shows HLA-DR staining in a lesional MS section with corresponding DAPI staining of cell nuclei (Figure 5.8 D).

An increase in versican (V0/V2) neoepitopes expression was found associated with an increase in HLA-DR in MS (Figure 5.8 B and E). Normal control brain tissue showed low expression for HLA-DR (Figure 5.8 C and F).

Table 5.4: Human brain tissues (Blocks) re-characterisation

Case code	MS Tissue Bank Characterisation	MS classification	H&E	ORO	MOG/HLA	Tissue Re-characterisation	Versican (V0/V2) neoepitopes
MS58 P1C3	Lesion	SPMS	++	+++	Disrupted/+++	Active	+++
*MS58 P1D3			++	+++	Disrupted/+++		+++
*MS92 A2E3	Lesion	SPMS	+	-	ND/+	Chronic inactive	+++
MS92 P1B1			+	-ve	ND/+++	Chronic active	+
*MS90 P2B4	Lesion	SPMS	+/-	+/-	ND/++	Active	+++
*MS60 P2C7	Lesion	SPMS	+	+++	ND/++	Active	++
*MS80 P1A4	Lesion	SPMS	++	++	ND/+++	Active	++
*MS103 P1D3	NAWM	SPMS	+	-ve	ND/ND	NAWM**	++
*MS103 P1B3	NAWM		-ve	-ve	Evenly distributed /ND	NAWM	- ve
*CO28 P1C3	Control	Normal	-ve	-ve	Evenly distributed/-ve	Normal	+
*CO14 A2E5	Control	Normal	-ve	-ve	Evenly distributed/ND	Normal	- ve
*CO14 P2A2			-ve	-ve			- ve
*CO25 P1B2	Control	Normal	-ve	-ve	Evenly distributed/-ve	Normal	+
*CO22 P1C3	Control	Normal	-ve	-ve	Evenly distributed/ +	Normal	+

SPMS, secondary progressive MS; ND, not done; *V0/V2 neoepitopes were detected by western blotting in these samples; NAWM**, showing inflammation on re-characterisation. Coding of blocks to indicate location in the brain was previously described in section 2.12.1.

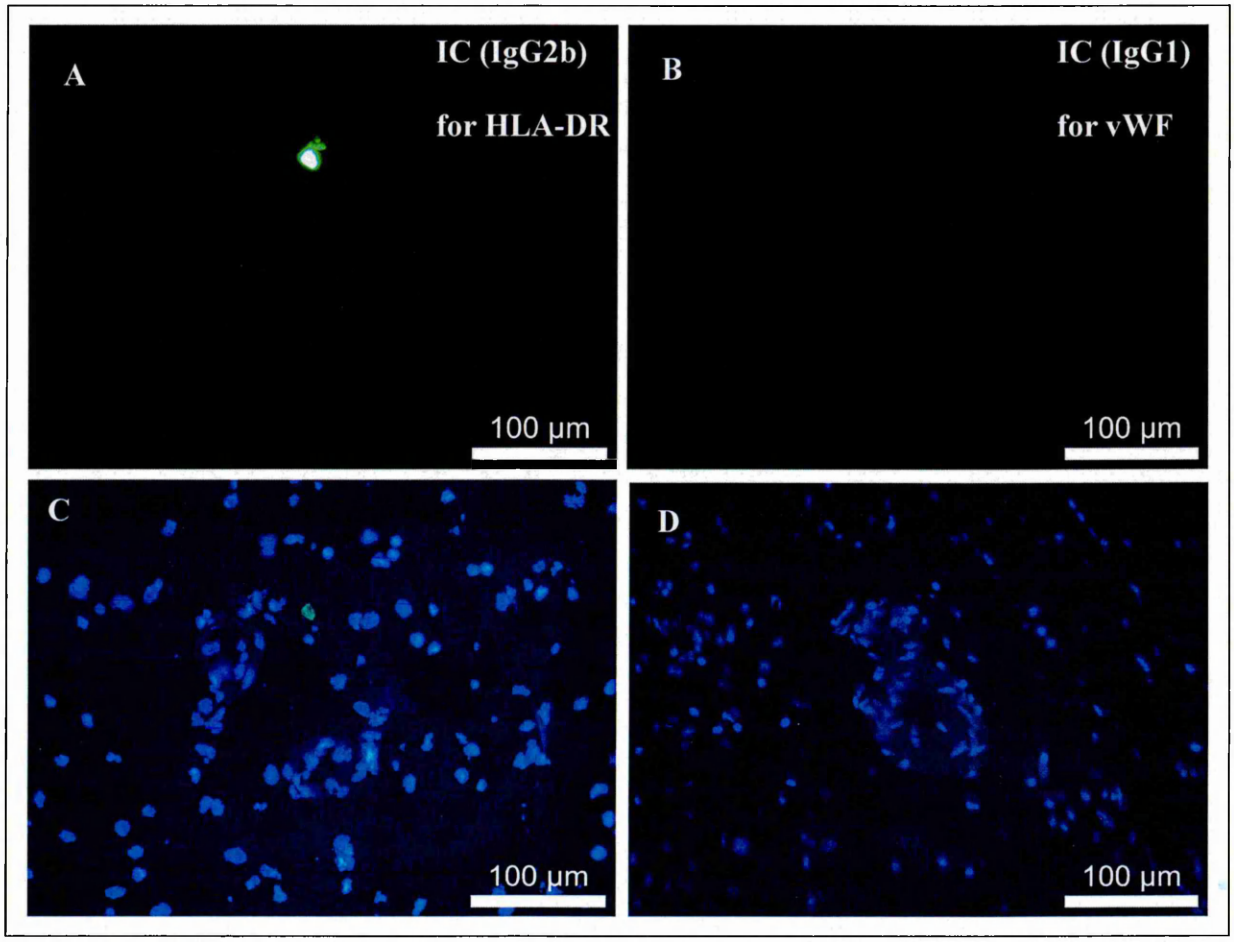


Figure 5.6: Negative controls. Isotype control (IC) IgG2b used instead of primary antibody anti HLA-DR at the same concentration (A), isotype control (IgG1) to detect the non specific binding of the anti-vWF primary antibody (B). Lower plates show nuclei counterstained with DAPI in blue.

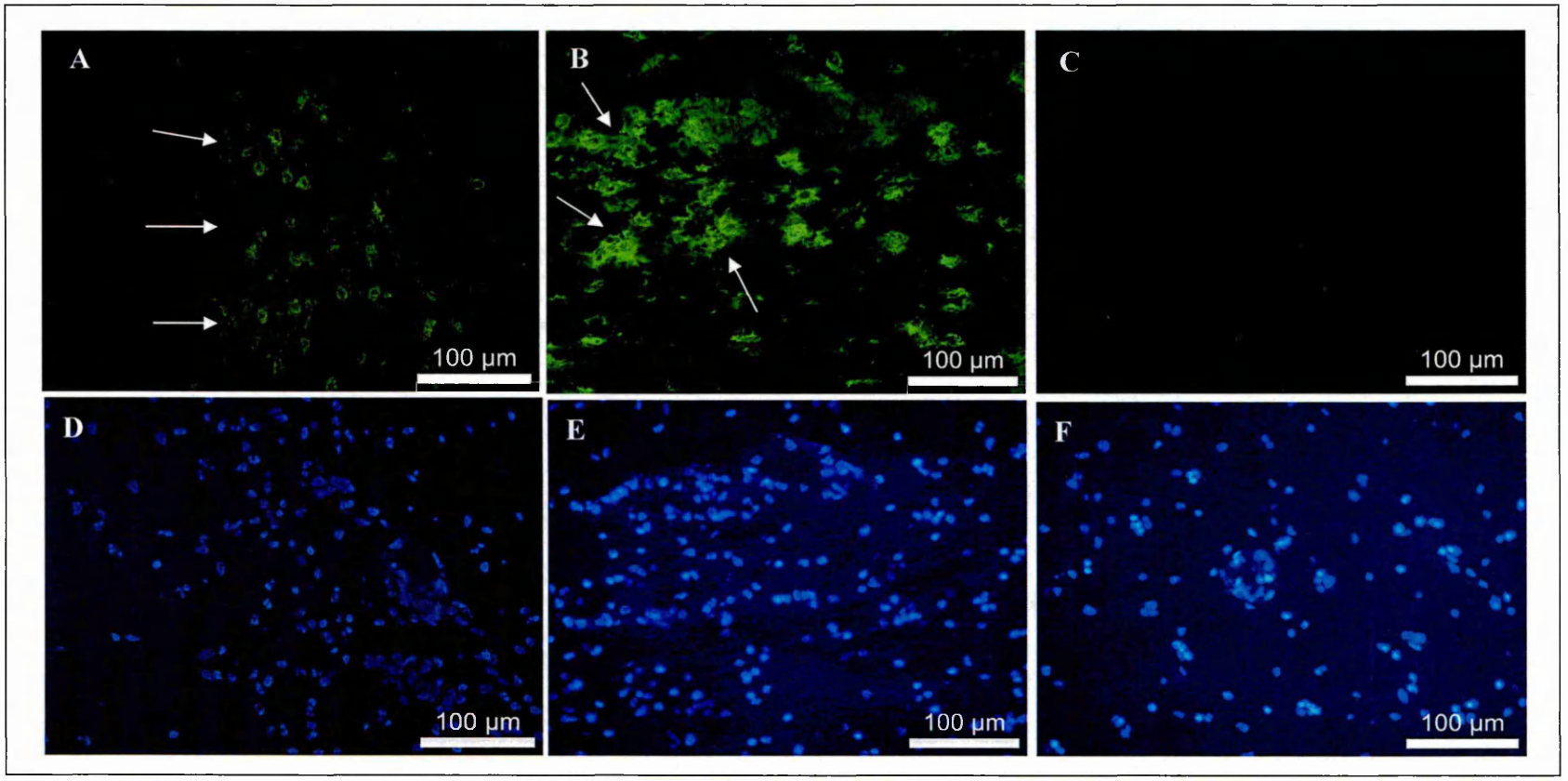
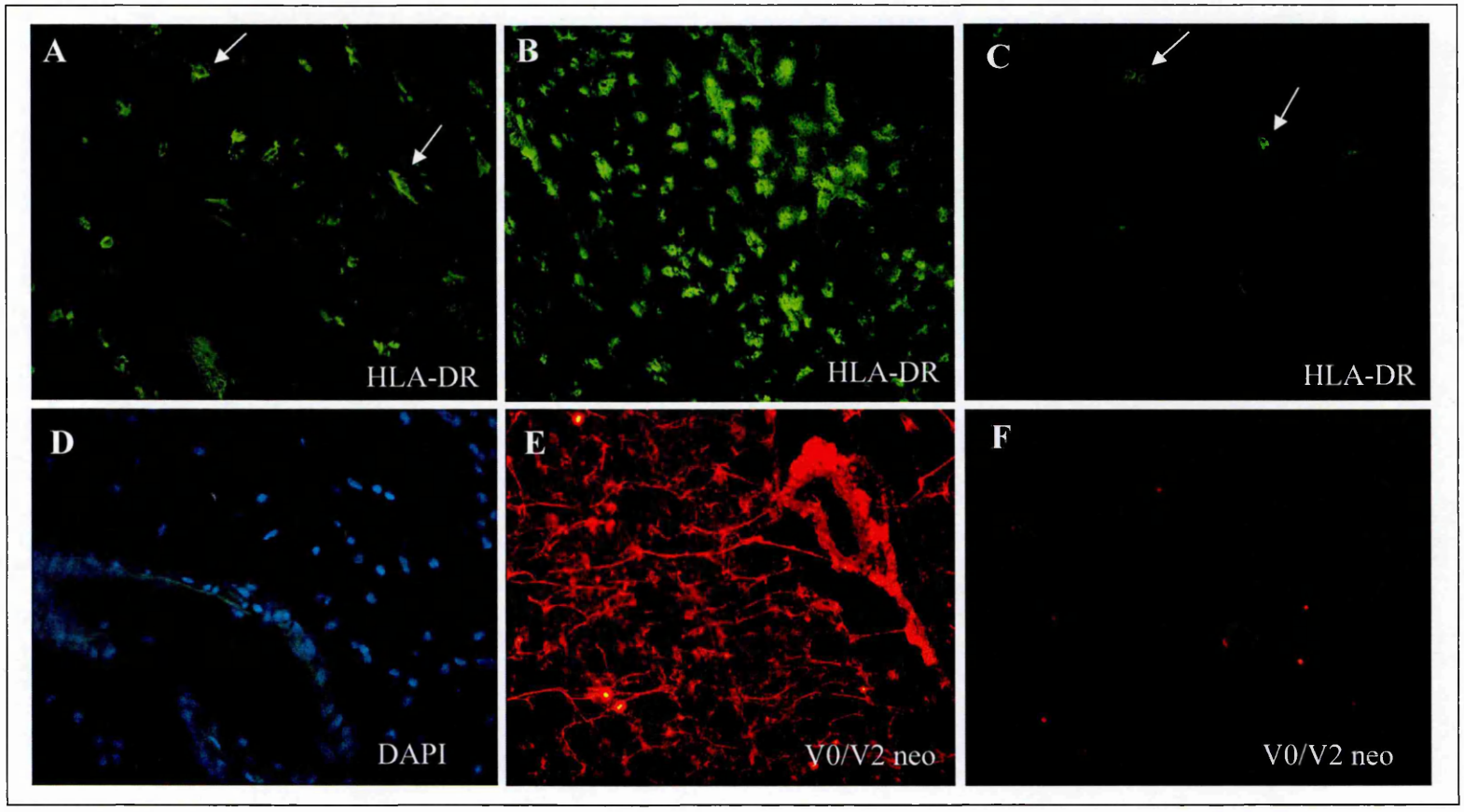


Figure 5.7: HLA-DR expression in human brain tissues indicating cellular activity. Immunostaining of HLA-DR positive cells in active MS lesions **(MS58 P1C3)** and **(MS80 P1A4),** A and B respectively (white arrows). Normal control tissue **(CO25 P1B2)** showing an undetectable HLA-DR immunostaining (C), Nuclei counterstained with DAPI (D, E, and F) x400.

Figure 5.8: Expression of versican (V0/V2) neoepitopes and HLA-DR immunoreactivity in an active MS lesion and control tissue. High levels of HLA-DR immunoreactivity were observed in an active MS lesion **(MS58 P1D3)** ((A) x400, (B) x200). Versican (V0/V2) neoepitopes expression in the same active MS lesion (E), compared to normal brain tissue showing a low HLA-DR expression (white arrows) (C) and showing no staining for versican (V0/V2) neoepitopes (F). Nuclei counterstained with DAPI (D). Immunohistochemical examination of each protein was carried out on serial sections from a tissue block.


A
HLA-DR
B
HLA-DR
C
HLA-DR
D
DAPI
E
V0/V2 neo
F
V0/V2 neo

5.2.4 vWF and Versican (V0/V2) Neoepitopes in Human Brain Tissues

The antibody used in this study detects both V0 and V2 splice variant neoepitopes. These splice variants could be expressed by endothelial blood vessels as reported by (Cattaruzza *et al.*, 2002). Both V0 and V2 have an α-GAG region in their core protein, which is cleaved by ADAMTSs. The new C-terminal produced on cleavage is recognised by this commercial antibody. Neoepitope expression could therefore also be associated with blood vessels. vWF is a marker for endothelial cells in blood vessels.

Single immunostaining was performed as previously described in section 2.14.3 using the mouse monclonal anti-vWF antibody at a 1:25 dilution, according to the manufacturer's recommendation. Immunostaining of the vWF marker was initially optimised in normal control brain tissue as shown in Figure 5.5 (A) and compared to control brain tissues stained with versican (V0/V2) neoepitopes (Figure 5.5 B and C). In addition, dual staining for vWF marker and versican (V0/V2) neoepitopes was performed (section 2.14.3) to investigate the localisation of the versican (V0/V2) neoepitopes with blood vessels and to investigate whether their expression is within the perivascular areas in MS tissue. Figure 5.9 shows co-localisation of versican (V0/V2) neoepitope with a blood vessel. It also shows positive staining of the versican (V0/V2) neoepitopes in the ECM.

Figure 5.9 continued (E and H) also shows representative images of blood vessels with vWF in MS sections, F and I also demonstrate perivascular and blood vessel versican (V0/V2) neoepitopes expression in the MS lesional blocks. Their co-localisation is shown in Figure 5.9 continued in G and J. Normal control brain tissue showed no versican (V0/V2) neoepitopes in a perivascular region but with some staining in the blood vessel (Figure 5.10 A) vWF (green), B) versican (V0/V2 neoepitopes (red) and DAPI C)). Negative controls for the dual staining were carried out by omitting the primary antibodies (Figure 5.3 (E and F).

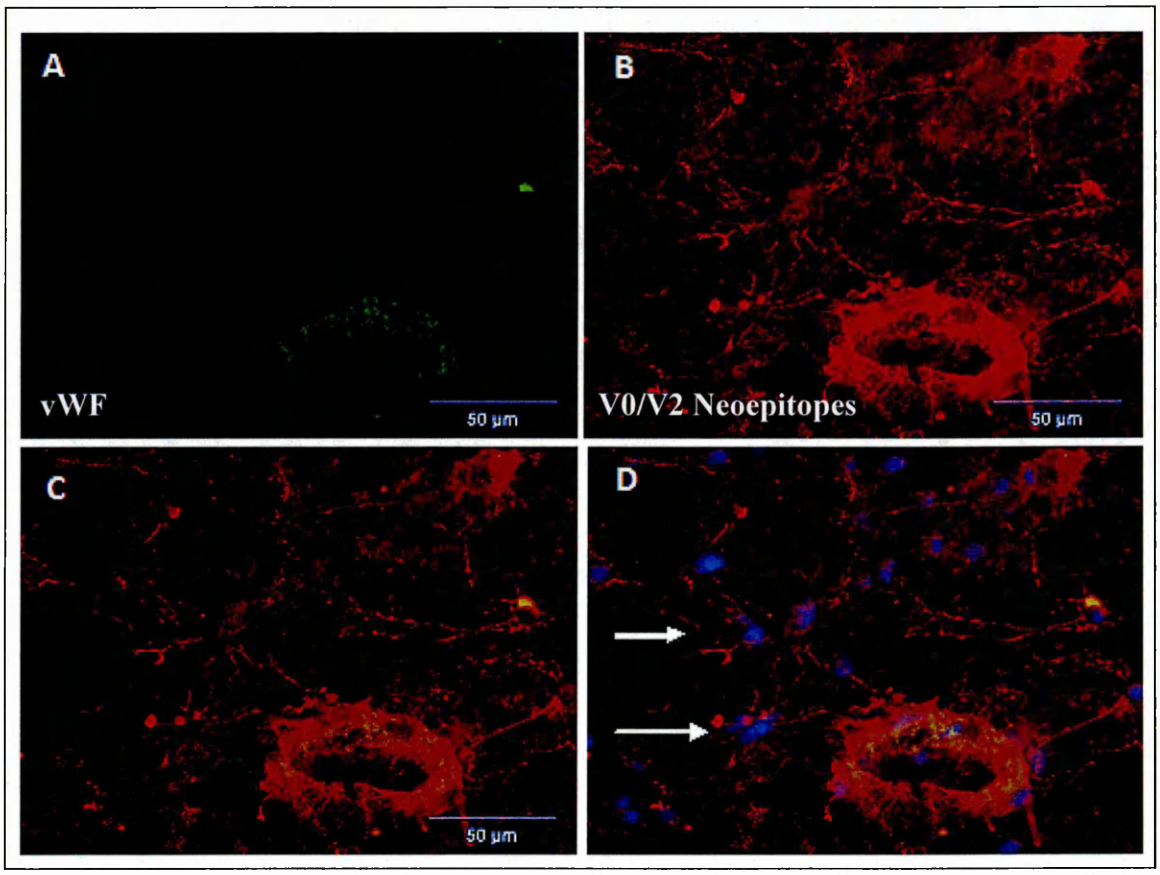


Figure 5.9: Expression of versican (V0/V2) neoepitopes in a perivascular region in lesional MS tissue (**MS58 P1D3)**. vWF expression (blood vessel) (green) (A), versican (V0/V2) neoepitopes (+++) in the ECM (perivascular region) (red) (B), their co-localisation (yellow) (C) and vWF, versican (V0/V2) neoepitopes (white arrows) and nuclei of cells counterstained with DAPI in blue (D) x400.

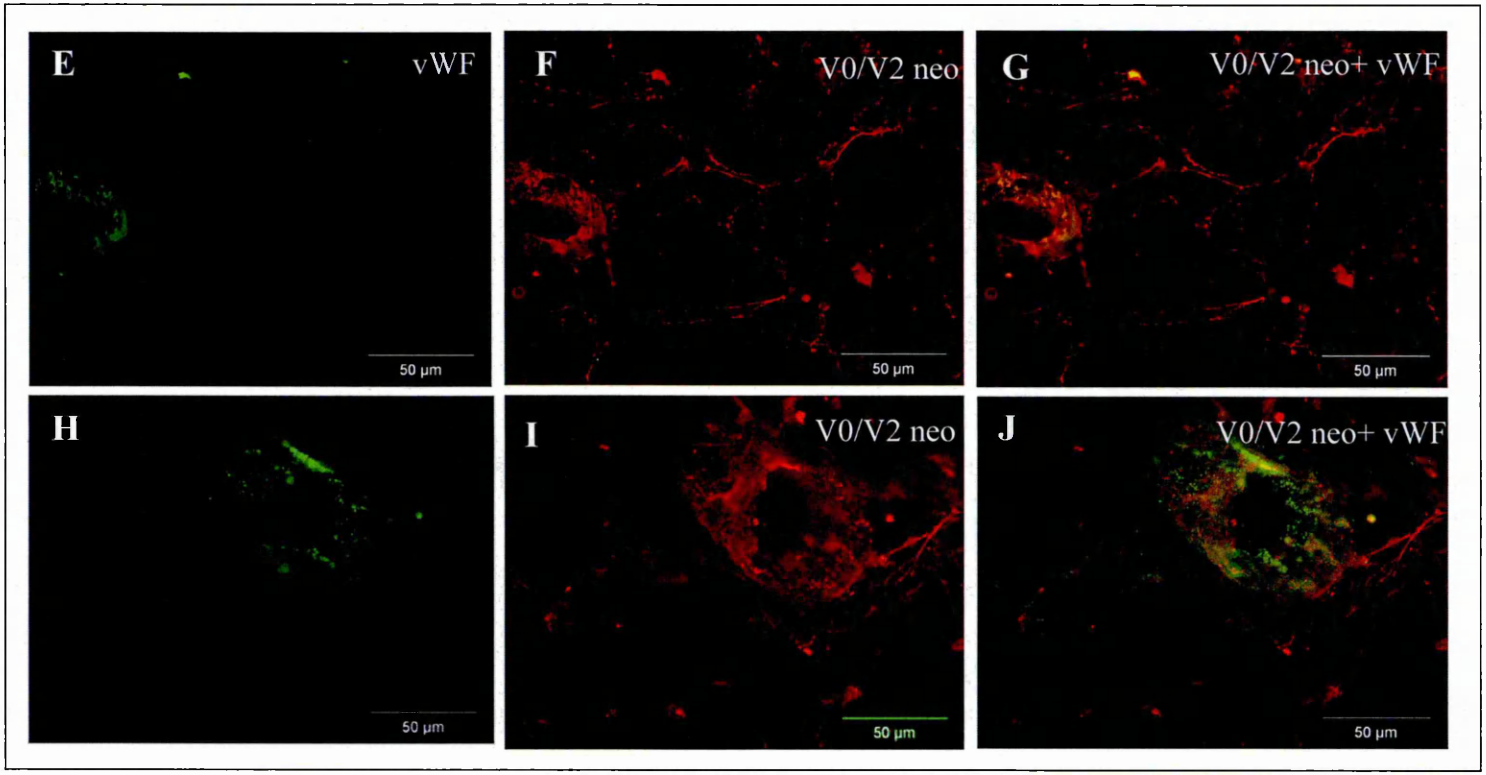


Figure 5.9 (continued): Expression of versican (V0/V2) neoepitopes in a perivascular region in lesional MS tissue **(MS80 P1A4)** and **(MS60 P2C7)**. vWF expression in a blood vessel (green) (E, H), versican (V0/V2) neoepitopes in the ECM (perivascular region) (red) (F,I), and their co-localisation (yellow) (G,J) x400.

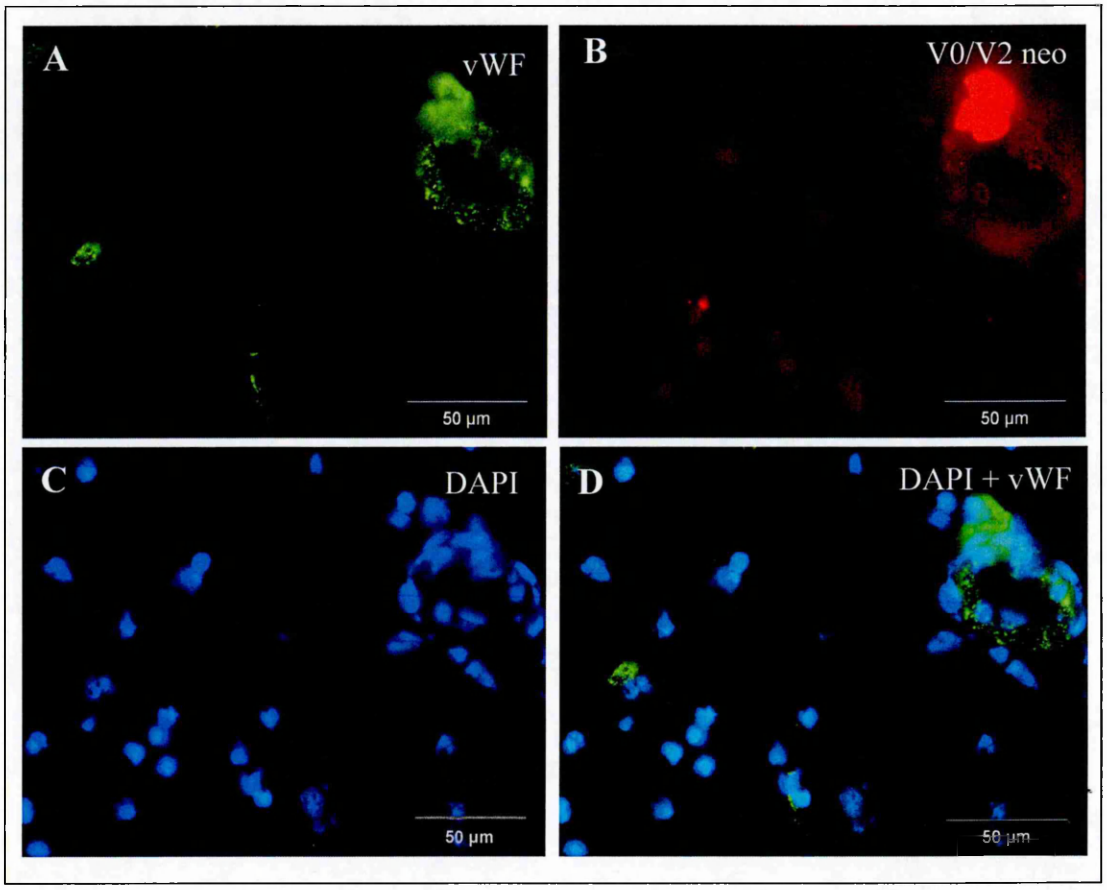


Figure 5.10: Expression of versican (V0/V2) neoepitopes in normal control brain tissue **(CO28 P1C3)** in the perivascular region. vWF expression in a blood vessel (green) (A), negative staining for versican (V0/V2) neoepitopes in the ECM and some staining is only in the blood vessels (perivascular region) (B), nuclei counterstained with DAPI (C) and their co-localisation with vWF x400(D).

5.2.5 Expression of MOG and Versican (V0/V2) Neoepitopes in Human Brain Tissues

MOG is a membrane myelin glycoprotein expressed on the cell surface of oligodendrocytes and the outermost surface of myelin sheaths. Due to its localisation, it is a primary target antigen involved in immune-mediated demyelination (Reder and Oger, 2004).

In this study the expression of MOG in human brain tissue was to determine the extent of demyelination. An anti-MOG antibody, provided as a gift by Dr. S. McQuaid, was used at his recommended dilution (Table 2.5). Figure 5.11 (A) shows a normal control tissue (CO22 P1C3) with a uniform pattern of expression of MOG and Figure 5.11 (B), lesional MS tissue (MS58 P1C3), shows loss and disruption of MOG. Following dual staining, as previously described in section 2.14.3., loss of myelin was found in areas where there was increased expression of versican (V0/V2) neoepitopes, indicative of increased ADAMTS activity in lesional MS tissue (Figure 5.12 A and B). Control and NAWM tissues showed an evenly distributed expression of MOG respectively (Figure 5.12 D and G).

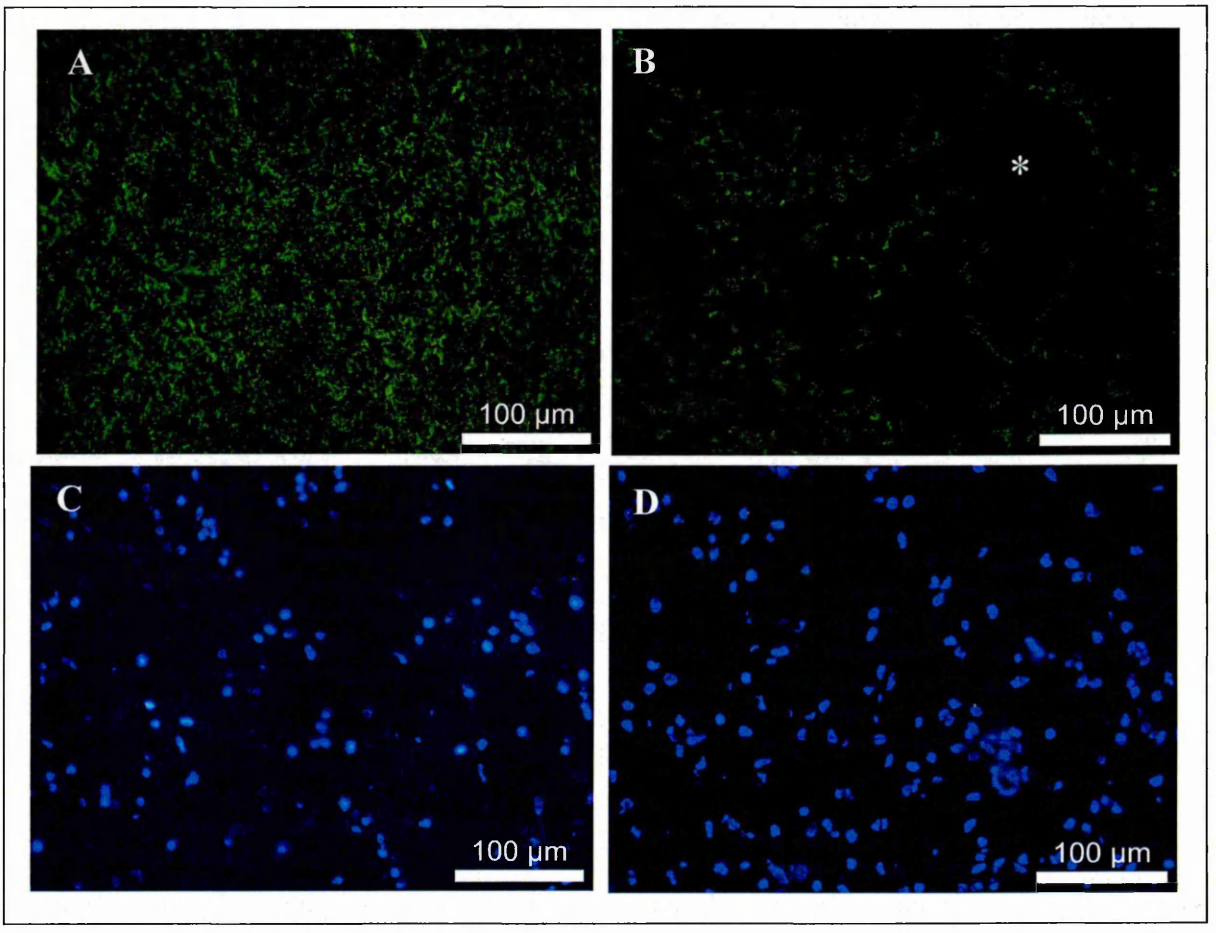
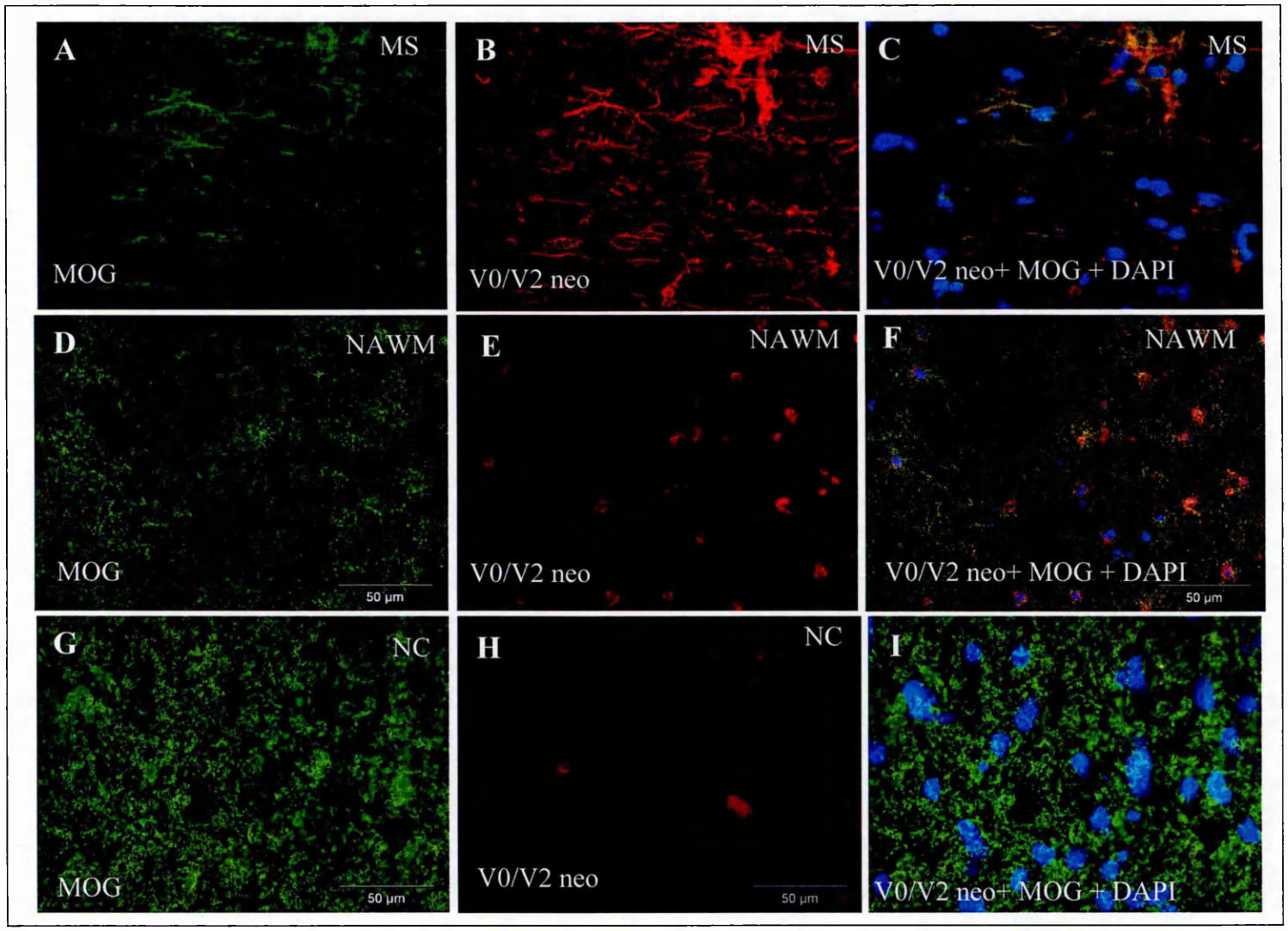


Figure 5.11: Expression of MOG in normal control and lesional MS tissues to determine the extent of demyelination. Normal control tissue **(CO22 P1C3)** showed an evenly distributed expression of MOG (A). Lesional MS **(MS58 P1C3)** (B) showed disrupted MOG, with areas of complete loss (asterisk). Nuclei counterstained with DAPI in blue (C and D x400).

Figure 5.12: Expression of versican (V0/V2) neoepitopes and MOG in lesional MS, NAWM and NC tissues. MOG in lesional MS **(MS58 P1C3)** (A), NAWM **(MS103 P1B3)** (D) and normal control (NC) **(CO28 P1C3)** (G) (green). Versican (V0/V2) neoepitopes expression in MS (B), NAWM (E) and NC (H) (red). Co-localisation of the versican (V0/V2 neoepitopes) with MOG and DAPI in MS (C), NAWM (F) and NC (I).


A
MS
MOG
B
MS
V0/V2 neo
C
MS
V0/V2 neo+ MOG + DAPI
D
NAWM
MOG
50 µm
E
NAWM
V0/V2 neo
F
NAWM
V0/V2 neo+ MOG + DAPI
50 µm
G
NC
MOG
50 µm
H
NC
V0/V2 neo
50 µm
I
V0/V2 neo+ MOG + DAPI

5.3 Expression of ADAMTS-1 and Versican (V0/V2) Neoepitopes in Human Brain Tissues by Western blotting

5.3.1 Protein Extracts from Human Brain Tissues

Serial cryostat sections (5 X 30 μm) of 2X2 cm human brain tissue blocks were collected in microcentrifuge tubes from 5 MS, 2 NAWM and 5 normal control tissue blocks as previously described in Plumb *et al.* (2006) and Haddock *et al.* (2006). The protein was extracted using Tri-reagent following the manufacturer's protocol and as described in sections 2.6.3. Protein concentrations were determined by the bicinchoninic acid assay as described in section 2.8. The western blotting method was performed as previously described in section 2.15 for ADAMTS-1 and section 2.16 for versican (V0/V2) neoepitope detection. ADAMTS-1 western blotting was performed on tissue from 3 MS cases and 4 normal controls using an ADAMTS-1 antibody from Santa Cruz Biotechnology. This antibody had been verified for its specificity as is previously described in chapter 4. In addition, pre-absorption of the antibody with its immunising peptide was performed as previously described in section 4.2.3.1 to confirm specificity of ADAMTS-1 antibody in western blots of protein extracts from CNS tissue (Figure 5.13 A, B).

The results demonstrated that ADAMTS-1 protein was present in both normal controls and in MS human brain tissues at full length (~107 kDa) and in two proteolytically cleaved forms ~50 and 49 kDa (Figure 5.13 A), which was verified by peptide blocking (Figure 5.13 B).

A band ~ 18 kDa was detected, which was not removed after pre-absorbing the antibody with its immunising peptide (Figure 5.13 B) indicating that it was non-specific. The β actin (42 kDa) was used to verify the equal loading of the samples (Figure 5.13 C). From Figure 5.14 it is difficult to make any conclusions. ADAMTS-1 expression is variable in normal controls and MS brains. More samples are needed to confirm any differences.

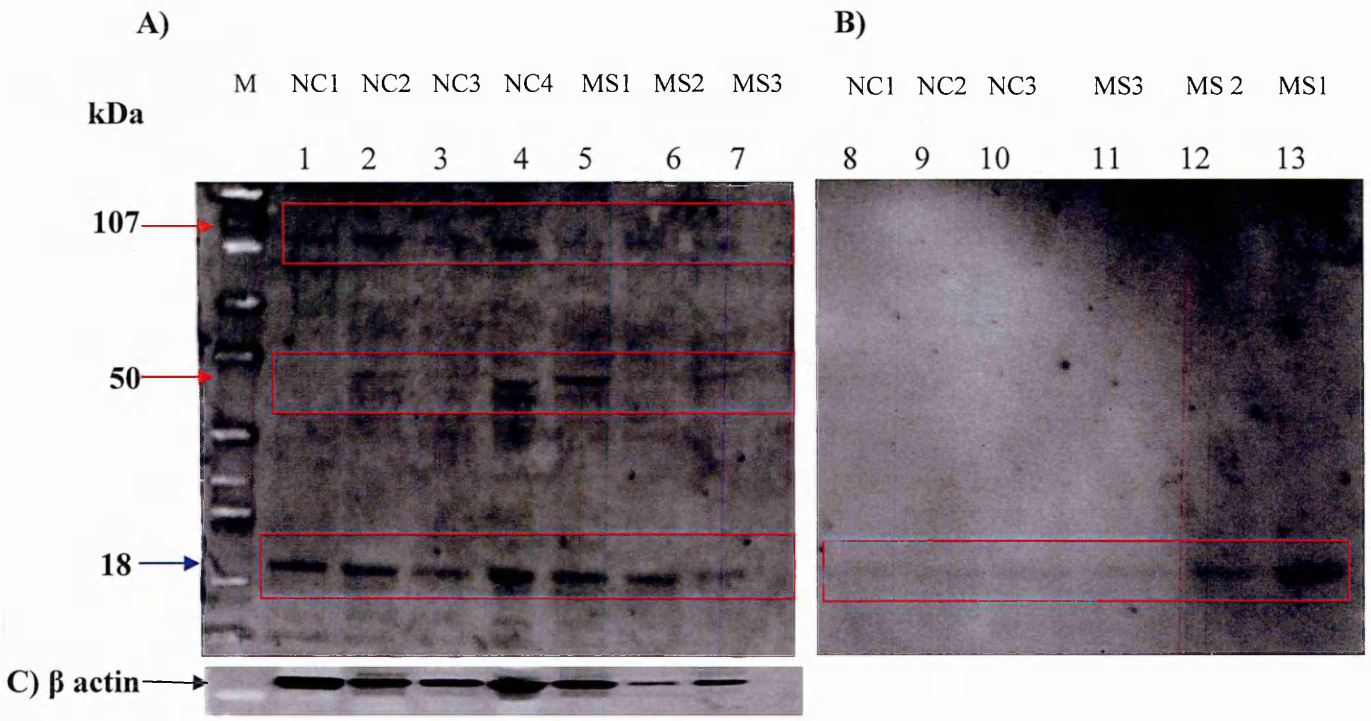


Figure 5.13: ADAMTS-1 protein expression in human brain tissue. Western blotting compares ADAMTS-1 protein level in normal (lanes 1- 4), MS (lanes 5-7) human CNS samples. Positions of the molecular weight markers are indicated on the left. Red arrows indicate the ADAMTS-1 bands ~ 107 kDa and doublets at ~50 kDa and 49 kDa. The pre-absorption of the ADAMTS-1 antibody with the immunizing peptide showed a low molecular weight band (18 kDa) which is non specific (blue arrow) (B). The β actin (42 kDa) control for the samples is also shown (C)

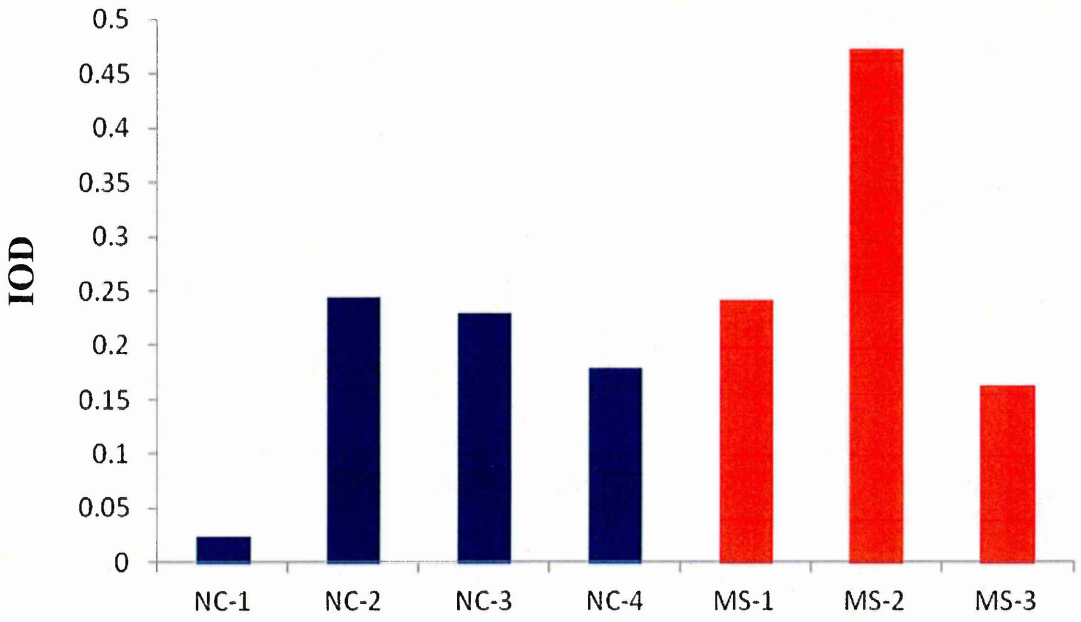


Figure 5.14: Densitometry of the ADAMTS-1 (all bands except the 18 kDa band) in protein extracts from human brain tissue samples. Integrated optical density (IOD) ratio is the IOD of protein of interest / IOD of internal control protein.

5.3.2 Expression of Versican (V0/V2) Neoepitopes in Human Brain Tissue by Western Blotting

Figure 5.15 shows the optimised western blot with A) negative control (no primary antibody) B) and C), the versican (V0/V2) neoepitopes detected by both the ECL and DAB immunodetection methods in human brain (NC and MS). Western blotting for the detection of versican (V0/V2) neoepitopes was carried out on 5 MS, 5 NC and 2 NAWM tissue samples as shown in Table 5.4. All the MS samples contained lesions and one NAWM sample showed inflammation according to the re-characterisation carried out as part of this work.

This study was to determine whether lesional MS differs from NAWM and NC tissues in their ADAMTS activity as determined by the presence of versican (V0/V2) neoepitopes by western blotting. Figure 5.15 shows that the expected 64 kDa versican (V0/V2) neoepitope fragment (Westling *et al.*, 2004) is present. All human brain tissue samples studied, whether from control or MS tissue showed the 64 kDa versican (V0/V2) neoepitope (Figure 5.16).

The intensities of the 64 kDa band in all samples, when analysed by densitometry, showed variable results when comparing MS and NC brain tissues. One of the 5 MS samples analysed here showed a greater intensity of the 64 kDa band compared to NC control samples. Surprisingly NAWM showed an increase in both blocks compared to controls (Figure 5.17). Because NAWM could be close to white matter lesions, axonal pathology and microglial activation may explain changes in so-called NAWM or it may be only artifactual. More samples need to be studied to determine if there are any consistent trends. Other bands were detected in these studied cases, which could represent other cleaved sites in the core protein for the versican V0 and V2 by other enzymes in addition to ADAMTSs. Bands of less than 64 kDa may represent additional cleavage of the 64 kDa species by ADAMTSs or other enzymes. Bands higher than 64 kDa e.g. 86 kDa band, may result from incomplete deglycosylation of the proteins.

Optimisation of the Versican (V0/V2) Neoepitopes in Human Brain Tissues (MS/NC) by Western Blotting

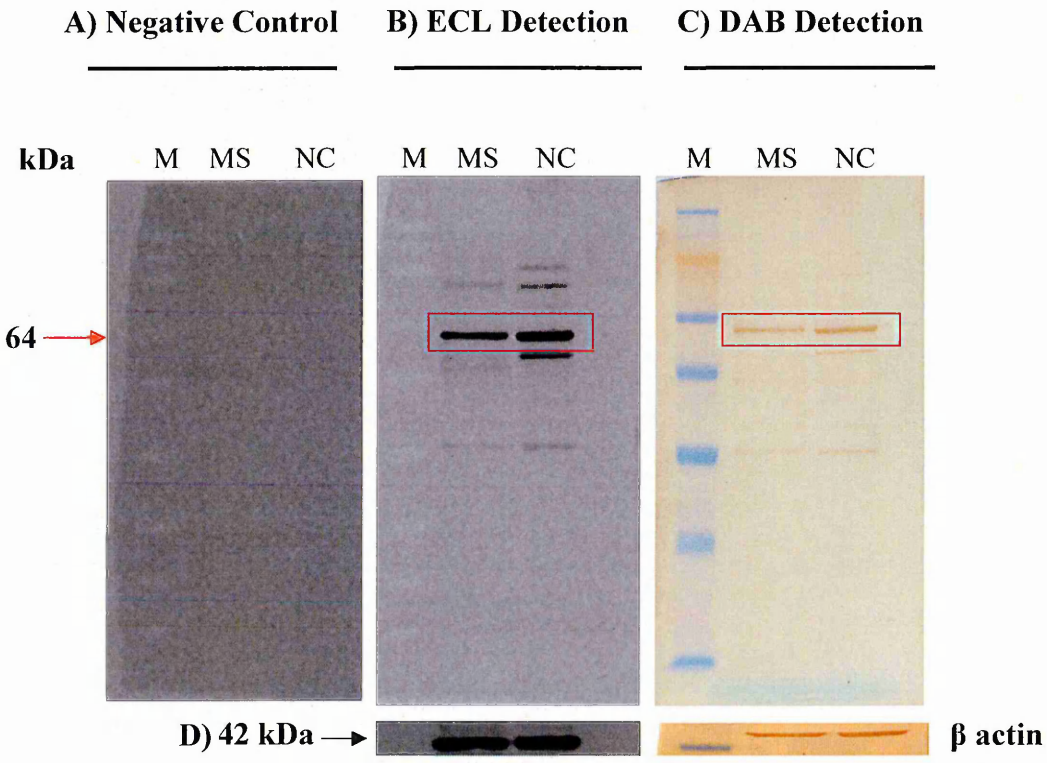


Figure 5.15: Western blot optimisation of detection of versican (V0/V2) neoepitopes in human brain, generated by ADAMTS cleavage. Negative control (omitting the primary antibody) (A). Red arrow indicates the position of the versicanase-generated 64 kDa fragment visualized in blot (B) ECL and DAB (C) detections. Positions of the molecular weight markers (M) are indicated at the left of each blot. The β actin control was to verify equal loading of protein samples (D)

Versican (V0/V2) Neoepitopes in Human Brain Tissue by Western Blotting

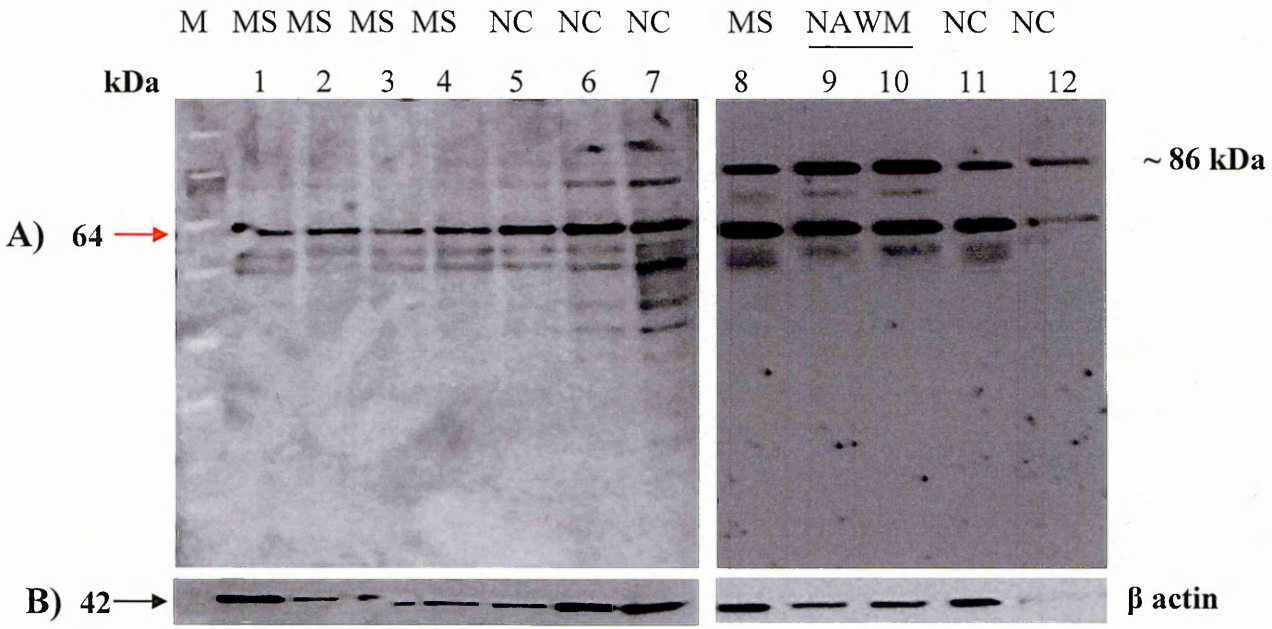


Figure 5.16: Western blots illustrating ADAMTS enzyme cleavage in post mortem tissue by the detection of ADAMTS-generated 64 kDa fragment using versican (V0/V2) neoepitope antibody. Western blotting compares versican neoepitope protein expression in normal (lanes 5, 6, 7, 11 and 12), NAWM (lanes 9 and 10) MS (lanes 1, 2, 3, 4 and 8) human CNS samples. The molecular weight markers (M) are indicated on the left. The red arrow indicates the position of the ADAMTS-generated 64 kDa fragment visualized. The β actin loading control for the samples is also shown (B).

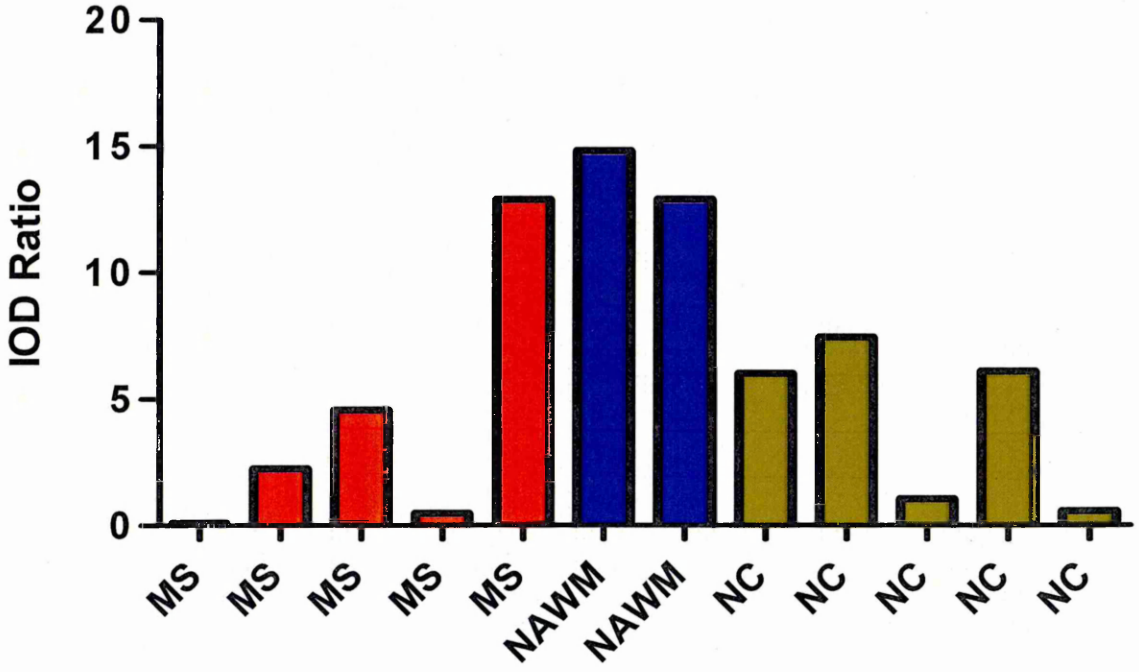


Figure 5.17: Densitometry of the 64 kDa versican neoepitope band in protein extracts from human brain tissue samples. IOD ratio is the IOD of protein of interest / IOD of internal control protein.

5.4 Discussion

The objective in this chapter was to study ADAMTS-1 protein expression and CSPG breakdown by ADAMTSs in post mortem tissue via the production of versican (V0/V2) neoepitopes in brain tissue. This was done by immunohistochemistry and western blotting. Also to show any differences in MS, MS tissues were compared to normal control brain tissue in terms of versican (V0/V2) neoepitopes expression.

The results demonstrated that ADAMTS-1 protein was present in both normal controls and in MS human brain tissues at full length (~107 kDa) and in two proteolytically cleaved forms ~50 and 49 kDa (Figure 5.13 A), which was verified by peptide blocking (Figure 5.13 B). A band ~ 18 kDa was detected, which was not removed after pre-absorbing the antibody with its immunising peptide (Figure 5.13 B) indicating that it was non-specific. Unfortunately, using this ADAMTS-1 antibody (Santa Cruz Biotechnology) for immunohistochemistry on human brain tissue gave no consistent results. Previous studies by Haddock *et al*, (2006), using western blotting, showed ADAMTS-1 bands at 62 kDa and a doublet at 53 and 50 kDa, which correspond to the mature active form and two forms produced by proteolytic cleavage at the carboxyl end, respectively. These were detected for all the normal and MS tissue samples run, no differences were observed (Haddock *et al*, 2006). These results showed some similarity with those of this study. The only difference is that the previous study used a C-terminal antibody that detected the mature form at 62 kDa whereas this study showed a band, possibly the zymogen form, at ~107 kDa. More samples should be analysed to determine if there are any differences between MS and normal control brain tissues with respect to ADAMTS-1 protein expression.

The versican V2 isoform has been proposed to be abundant in adult human, bovine and rat brains (Perides *et al.*, 1992; Schmalfeldt *et al.*, 1998; Sandy *et al.*, 2001). In this study, it was demonstrated that versican (V0/V2) neoepitopes were expressed in normal and MS brain, which has not previously been reported in MS or normal brain tissue immunohistologically. From a previous study using the same versican neoepitope

antibody a 64 kDa band indicated the presence of this neoepitope in human brain tissue (Westling *et al.*, 2004). Versican (V0/V2) neoepitope expression was increased, as observed by immunohistochemistry, in lesional tissue relative to normal control tissue, suggesting that changes in versican matrix degradation may result from changes in ADAMTS activity levels. It has been previously demonstrated that ADAMTS-4 is expressed in the CNS and shows an increased expression in MS compared to normal control tissues (Haddock *et al*, 2006). Also a previous study showed that TIMP-3, which is an endogenous inhibitor for these ADAMTSs, is decreased in MS (Haddock *et al*, 2006) and EAE (Plumb *et al*, 2005). This increase in the ADAMTS activity could indicate a role for the ADAMTS in lesion development and ECM turnover in MS.

Sobel and Ahmed demonstrated that in active MS lesions, versican was decreased in the ECM (Sobel and Ahmed, 2001). The decrease in intact versican in MS lesions is in keeping with the observation of increased versican neoepitope expression in lesional MS in this study. The versican neoepitopes produced are characteristic of ADAMTS glutamyl peptidase cleavage suggesting an increase of their activity in MS lesions.

Generally, western blotting could be a further confirmatory test for the IHC method because western blotting can test the expression of a protein qualitatively or quantitatively. However, in this study western blotting for versican (V0/V2) neoepitopes detection in MS compared to normal controls and NAWM produced different results from the immunohistochemical method. Western blotting is used to investigate the expression of a protein of interest derived from a whole tissue sample and may include normal and lesional tissue, possibly diluting out differences. Immunofluorescence allows specific areas to be investigated, lesional and non-lesional, allowing a better evaluation as to whether neoepitope expression is increased or not. There were no clear differences between V0/V2 neopepitopes detected from MS or normal control tissues by western blotting. Further work is required to examine more samples.

Other bands in addition to the 64 kDa band were observed. Those < 64 kDa may have resulted from further cleavage of 64 kDa species by ADAMTSs or other enzymes.

Bands > 64 kDa were seen in this study and by Westling *et al*, (2004) with the similar antibody though their origin was not discussed. An 86 kDa species could arise from the cleavage of the versican (V0/V2) core protein (see Figure 5.1) (Westling *et al,* (2004) but this is unlikely to be detected by this antibody. These higher molecular weight bands may arise as a result of incomplete deglycosylation. Co-localisation of the versican (V0/V2) neoepitopes with the endothelial marker, vWF, following dual labelled immunofluorescence indicates the localisation of these neoepitopes with blood vessels in both MS and control brain. However MS cases showed also an increased neoepitope in ECM relative to normal control brain. The co-localisation of versican (V0/V2) neoepitope with the blood vessel agrees with an earlier study on versican isoform expression by endothelial cells that showed that isolated endothelial cells express all versican isoforms except the V3. The same study demonstrates that both versican V0 and V2 isoform transcripts are expressed by the healthy human brain tissue (Cattaruzza *et al.*, 2002).

Recently it has been indicated that TLRs are expressed by a variety of peripheral immune cells and resident cells of the CNS. They have a significant role in modulating MS, as well as EAE (Racke and Drew, 2009). In addition to lymphocytes that control adaptive immune responses, dendritic cells and tissue macrophages that regulate innate immune responses also play a role in controlling MS disease pathogenesis. These cells express TLRs that recognize pathogen-associated molecular patterns (PAMPs) present on the surface of pathogens. Following ligand binding to TLRs, innate immune cells produce proinflamatory cytokines and can serve as APCs to prime naïve T cells to recognize antigens (Takeda and Akira, 2005, Hacker *et al.*, 2006). Thus, TLRs play an important role in linking the innate to the adaptive immune response (Bell *et al.* 2005).

TLRs may be activated by fragments of the ECM molecules generated by proteolytic cleavage. The 64 kDa fragment could be a candidate for this. Other ECM molecules may have this effect e.g. short hyaluronan fragments also versican (Piccinini and Midwood, 2010).

Activation of TLRs induces the expression of proinflammtory cytokines such as IL-1, TNF, chemokines and adhesion molecules. TLRs have been found in MS tissues and are thought to be involved in the pathogenesis of MS (Piccinini and Midwood, 2010). Versican breakdown products could be involved in the perpetuation of inflammation in MS.

Chapter 6

General Discussion

Though, it has been demonstrated that ADAMTS expression may be modulated by a number of mediators in a number of different cell systems (Yamanishi *et al.*, 2002, Bevitt *et al.*, 2003, Tsuzaki *et al.*, 2003, Cross *et al.*, 2005), studies of ADAMTSs in neuronal cells are very limited (Sasaki *et al.*, 2001). ADAMTS-1 and -4 expression in astrocytes and neurons has been reported following physical or toxic injury to the CNS and it was suggested that this expression at the site of injury may support neurite outgrowth (Satoh *et al.*, 2000, Lemons *et al.*, 2001, Sasaki *et al.*, 2001, Yuan *et al.*, 2002).

ADAMTS-1, -4 and -5 are known to be expressed in normal adult and MS CNS and have the ability to cleave CSPGs that are present in the brain ECM. ADAMTS-1, -4 and -5 possess aggrecanase activity (Somerville *et al.*, 2003, Vankemmelbeke *et al.*, 2003), ADAMTS-1 and -4 are capable of cleaving versican (Sandy *et al.*, 2001, Somerville *et al.*, 2003, Westling *et al.*, 2004), and brevican is cleaved by ADAMTS-4 (Matthews *et al.*, 2000). It is therefore proposed that these enzymes are intimately involved in brain ECM turnover. ECM alteration due to the activity of the ADAMTSs has not been studied in MS tissue. The aim of this study was to investigate ADAMTS-1, -4 and -5 in neuronal cells and to determine the ADAMTS activity in MS tissue via their versican (V0/V2) cleavage to produce neoepitopes.

6.1 *In Vitro* Studies

6.1.1 Expression and Modulation of ADAMTS-1, -4 and -5 in Human Neuroblastoma Cell Lines

The data in chapter 3 demonstrated that ADAMTS-1, -4 and -5 mRNA are expressed under basal conditions by human neuroblastoma cell lines *in vitro*. The ADAMTS-1 mRNA showed the highest expression levels by both the human neuroblastoma cell lines used in this study SHSY-5Y and SK-N-DZ compared to ADAMTS-4 and ADAMTS-5. Previously, work in our laboratory has shown that these ADAMTSs are

constitutively expressed by human astrocytes and that ADAMTS-1 appeared to show the highest expression level, by ICC, compared to ADAMTS-4 and -5 staining which were much lower (Cross *et al.*, 2006a), in agreement with our finding in the neuronal cells. In this study ADAMTS-1 mRNA showed an upregulation with RetA treatment in SHSY-5Y cell line. RetA plays an essential role in nervous system development, including neuronal survival and neurite outgrowth. It serves as a main regulator of gene expression. RetA exerts its effects on gene transcription by binding to nuclear retinoic acid receptors (RARs) (Clagett-Dame *et al.*, 2006). It has been shown that human neuroblastoma cells decrease their proliferation and exhibit enhanced extension with neurite processes in response to 10^{-5}M treatment of RetA (Encinas *et al.*, 2000).

In this study, no modulations in the expression of ADAMTS mRNA were observed in SHSY-5Y cells with proinflammatory cytokines. However, the SK-N-DZ cell line showed an increase in ADAMTS-1 mRNA with IL-1β (10 ng/ml) and ADAMTS-4 mRNA with TNF (10 and 100 ng/ml). A previous *in vitro* study showed modulation of ADAMTS-1 and -4 mRNA expression by TNF in astrocytes (Cross *et al.*, 2006a). This study also showed that IL-1β treatment did not significantly alter mRNA expression levels of ADAMTS-1, -5 and TIMP-3 in astrocytes, although there was a significant increase in ADAMTS-4 at the low concentration of 1 ng/ml IL-1β. In the same study, no changes were seen, on IL-1β treatment, in ADAMTS-1, -4, -5 or TIMP-3 protein levels compared with control. However, IL-1β has been shown to increase ADAMTS-1 expression in other systems (Kuno *et al.*, 1997). ADAMTS-5 mRNA is expressed at low levels in both neuronal cell lines in this study, which concurs with the study of Haddock *et al*, (2006) which showed ADAMTS-5 mRNA expression to be low in brain tissue (Haddock *et al.*, 2006). Modulation of ADAMTSs with proinflammatory cytokines in neuronal cells and astrocytic cells (Cross *et al.*, 2006a, Haddock *et al.*, 2006) is summarized in Figure 6.1.

6.1.2 Expression and Modulation of ADAM-17 and TIMP-3 in Human Neuroblastoma Cell Lines

ADAM-17 is an ADAM metalloproteinase, a well known sheddase which is involved in releasing soluble TNF (17 kDa extracellular domain) from its membrane-bound precursor (pro-TNF) (Moss *et al.*, 1997, Black *et al.*, 1997). ADAM-17 is also responsible for the cleavage of a wide variety of substrates involved in inflammation including transforming growth factor-alpha, L-selectin, p75 (Peschon *et al.*, 1998) and p55 TNF receptors (Reddy *et al.*, 2000), the chemokine (fractalkine) (Garton *et al.*, 2001) and amyloid precursor protein (Garton *et al.*, 2001). TNF is involved in the adaptive immune response to injury or infection and is upregulated after ischaemic events (Cardenas *et al.*, 2002).

ADAM-17 expression has been shown in the CNS (Goddard *et al.*, 2001). Plumb *et al* (2006) observed expression of ADAM-17 in activated macrophage/microglia and astrocytes in MS white matter (Plumb *et al.*, 2005, Plumb *et al.*, 2006). Its expression is upregulated in active MS lesions (Plumb *et al.*, 2006). It has also been studied in spinal cords of rats with EAE where there was an upregulation of ADAM-17 mRNA expression with a decrease in mRNA levels of its inhibitor TIMP-3, suggesting a role for ADAM-17 in EAE pathogenesis (Plumb *et al.*, 2005, Plumb *et al.*, 2006).

As previous findings indicate, ADAM-17 can be implicated in the pathogenesis of MS. Neuronal ADAM-17 has been investigated in this study to further elucidate its role in MS. This *in vitro* study showed no modulation in the expression levels of neuronal ADAM-17 with proinflammatory cytokines in both differentiated and undifferentiated SHSY-5Y cells. It suggests any involvement of ADAM-17 in MS pathogenesis may be via other cell types.

TIMP-3 has been shown to be the only member of the TIMP family able to effectively inhibit the actions of ADAM-17 (Amour *et al.*, 1998). It is also known as the main

inhibitor of ADAMTSs. This study showed no modulation in TIMP-3 expression *in vitro* in SHSY-5Y cells. SK-N-DZ cells did not express TIMP-3 at the mRNA level. This contrasts with previous studies which demonstrated a significant decrease in TIMP-3 mRNA in MS human brain tissue compared to controls (Haddock *et al.*, 2006) and in EAE (Plumb *et al.*, 2005).

6.2 Antibody Optimisation and ADAMTS-1 Expression (Western Blotting)

The aim of chapter 4 was to verify the specificity of the commercial ADAMTS-1 antibody for further use in this study. Data in chapter 4 demonstrated, using a number of commercial antibodies from different sources (Triple Point Biologics, Abcam and Santa Cruz Biotechnology), that the most suitable antibody for ADAMTS-1 protein detection was from Santa Cruz Biotechnology. This was used in further investigations. It was the only antibody that produced reproducible results from SHSY-5Y extracts and had an available blocking peptide. It produced a 37 kDa band which indicates the expression of a fragment of ADAMTS-1 and was reduced in intensity on ADAMTS-1 siRNA knockdown and blocked on pre-absorption of the antibody with the immunizing peptide. The peptide blocking and the siRNA knockdown methods were able to confirm the specificity of the antibody to ADAMTS-1 in this study. It is suggested that the 37 kDa band could represent a fragment of ADAMTS-1 produced by C-terminal proteolytic processing or may represent protein splicing variants. Miguel *et al*, (2005) observed a similar sized band in other neurodegenerative diseases in agreement with the present work. Furthermore, Miguel *et al* (2005), showed another band at 50 kDa band. Interestingly, both bands were fivefold over-expressed in neurodegenerative diseases (Miguel *et al.*, 2005). The findings of Miguel *et al* (2005), suggest that this fragment could be involved in neurodegenerative disease. Further studies on ADAMTS-1 are needed to determine the functional role of this fragment in processes occurring MS. This Santa Cruz Biotechnology ADAMTS-1 antibody used here has been used previously by Pockert *et al*, (2009) in a study of intervertebral disc degeneration.

ADAMTS-1 was detected immunohistochemically in degenerative discs compared with non degenerative discs. Also their results showed that ADAMTS-1, -4, -5, and -15 at the gene level were significantly increased in degenerated tissue compared with nondegenerated tissue. However, the immunostaining of ADAMTS -4, -5, -9, and -15 were significantly increased in degenerated tissue compared with nondegenerated tissue.

Other workers, in studies of ADAMTS-1 protein by western blotting, have observed species with different molecular sizes depending on the antibody source and the source of cell or tissue extract. In a study of CNS tissue after induction of experimental cerebral ischaemia by transient middle cerebral artery occlusion in the rat, using western blotting, ADAMTS-1 protein produced a band at 97 kDa. In the same study a band for ADAMTS-1 was also observed at 51 kDa in protein extracts from primary human astrocytes (Cross *et al.*, 2006a). Other studies demonstrated bands for ADAMTS-1 at 64 kDa and 50 kDa in EAE (Cross *et al.*, 2006b) and in human brain tissue, ADAMTS-1 bands were seen at 62 kDa and a doublet at 53 and 50 kDa which corresponds to the mature active form and two proteolytic cleaved forms, respectively. This suggests these antibodies detect different processed forms of ADAMTS-1 depending on the cell or tissue type.

6.3 Post mortem Studies

6.3.1 Versican (V0/V2) Neoepitopes in Normal and MS Human Brain Tissue

The CSPGs are widely expressed throughout the developing and adult CNS and have a role in guiding or limiting neurite outgrowth and cell migration. Alterations in the synthesis or breakdown of the ECM may contribute to disease processes (Jones *et al.*, 2003, Medina-Flores *et al.*, 2004, Miguel *et al.*, 2005, Haddock *et al.*, 2006). Thus, ADAMTS-mediated cleavage of lecticans, aggrecan, versican and brevican in the CNS ECM, via their GEP activity, could have a potential role in normal ECM turnover and ECM breakdown in MS. To support this hypothesis this study demonstrated CSPG

breakdown by ADAMTSs via the production of versican (V0/V2) neoepitopes as an indicator of enzyme activity. This provides evidence for a potential role of these ADAMTSs in MS pathogenesis.

Detection of neoepitopes of versican (V0/V2) used an antibody specific for the new C-terminal sequence (NIVSFE405) generated by cleavage of versican V0 or V2 at the Glu405-Gln406 bond by ADAMTS glutamyl endopeptidase activity. IHC results showed increased versican (V0/V2) neoepitopes in lesional MS compared to normal controls. Western blotting results showed bands for versican (V0/V2) neoepitopes in both normal and MS samples at 64 kDa. This is in agreement with Westling *et al*, (2004) who observed a similar sized fragment with this antibody on versican V0/V2 cleavage by ADAMTSs. The V2 isoform of versican is the predominant form in the brain. It is composed of the G1 domain, GAG-alpha domain, and the G3 domain. Westling *et al*, 2004 found that the cleavage of versican V2 at the Glu405-Gln406 bond generates a versican (64 kDa) fragment on ADAMTS-4 cleavage (Westling *et al.*, 2004).

In contrast to the IHC method western blotting did not show an increase in neoepitopes in MS human brain tissues. Western blot procedure is often conducted to complement and support IHC studies. Inconsistent results between western blot and IHC studies may reflect differences in the experimental conditions. IHC can distinguish lesional and non-lesional tissue so can determine more specifically the increase of versican (V0/V2) neoepitopes in MS lesions. For western blotting sections (5 X 30 μm) of human brain tissue were collected to obtain a sufficient amount of protein for western blotting. This tissue contains lesions and NAWM in the same sections and may cause 'diluting out' of any differences making them more difficult to determine. This is particularly so if the lesion constitutes a small proportion of the tissue extracted for western blotting. Selection of tissue from more specific regions, lesion or NAWM would be required to obtain more meaningful results.

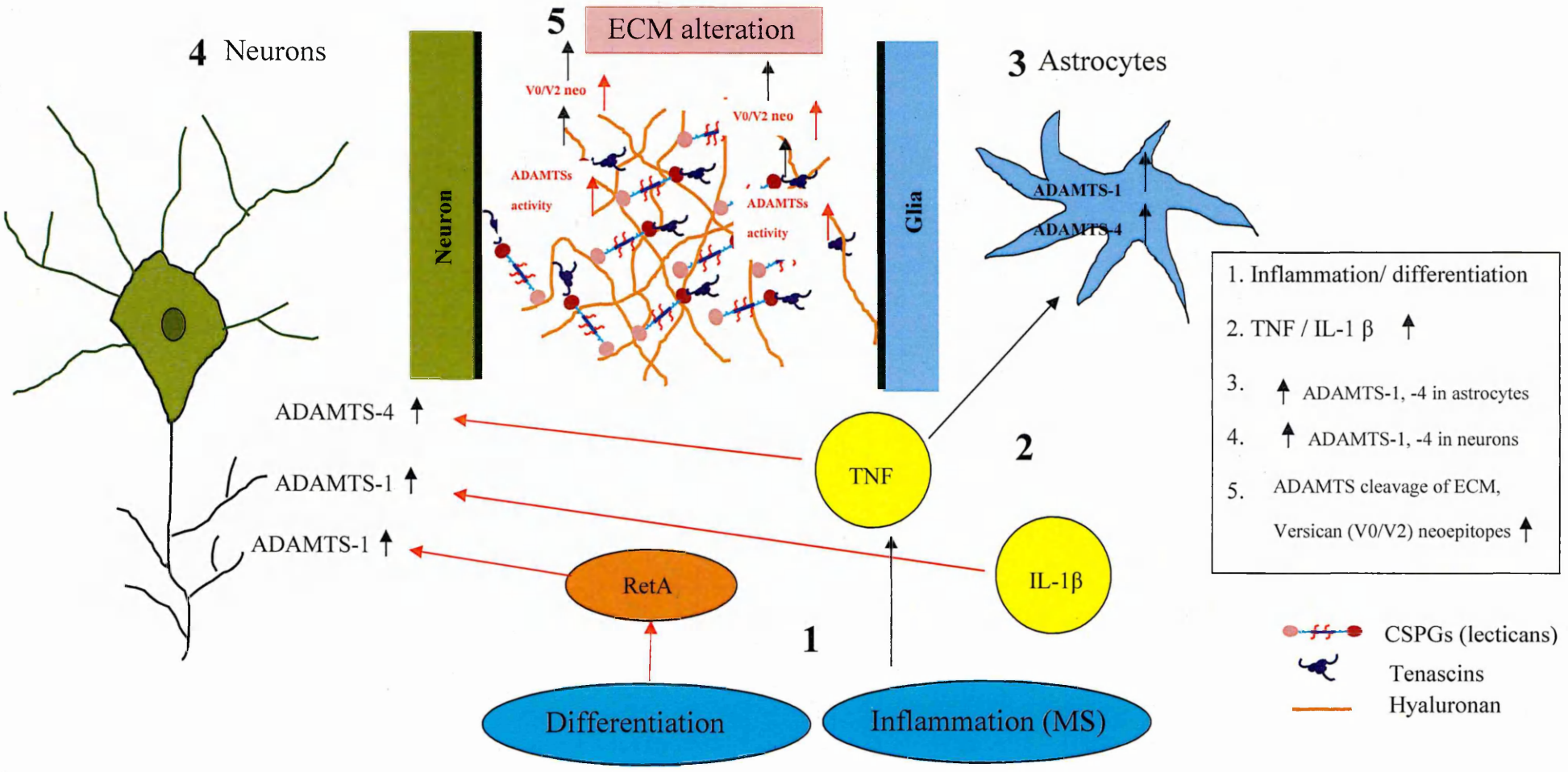
It has been reported that ADAMTS-4 may be involved in the loss of ECM by cleaving proteoglycans, as demonstrated in other tissues (Matthews *et al.*, 2000, Sandy *et al.*, 2001, Tortorella *et al.*, 2002, Vankemmelbeke *et al.*, 2003). Sobel and Ahmed

demonstrated that in active plaque centres and active lesions, versican, aggrecan and neurocan were decreased in the ECM (Sobel and Ahmed, 2001). The present study is consistent with this and suggests that the decrease of versican in lesional MS is due to the excess of ADAMTS cleavage. Furthermore Haddock *et al*, (2006) showed that high levels of ADAMTS-4 were observed in active MS lesions (Haddock *et al.*, 2006).

Little is known about ADAMTS-1 in CNS or its function. This study investigated ADAMTS-1 in human brain tissue since it may have a potential role in MS, as it has been shown to be the most abundant ADAMTS with an upregulation with RetA treatment in the *in vitro* study. It is expressed in MS and normal human brain tissue as demonstrated by western blot method, in agreement with Haddock *et al*, 2006. However, the ADAMTS-1 IHC staining did not produce consistent results and required further investigation in human brain.

In other tissue types, ADAMTS-1 is involved in the development and function of many organs, which is attributable to the proteoglycan processing capacity of this protease (Shindo *et al.*, 2000, Cross *et al.*, 2005). It has been previously shown that ADAMTS-1 is important for ECM remodeling around growing ovarian follicles (Brown *et al.*, 2006). Brown *et al*, (2010) demonstrated that ADAMTS-1 is critical in both ovulation and fertilization processes *in vivo* (Brown *et al.*, 2010). The protease activity of ADAMTS-1 mediates neomorphogenesis of the ovulating follicle wall and cumulus-oocyte matrix necessary for successful ovulation and fertilization, as well as subsequent catabolism of versican required for degradation of cumulus-oocyte matrix after fertilization. Furthermore, cardiogenesis, remodeling of the myocardium, requires cleavage of versican by ADAMTS-1 (Stankunas *et al.*, 2008), with the cleaved versican acting to reduce cell-cell adhesion and weaken tissue structure (Kern *et al.*, 2007).

Figure 6.1: Expression and Modulation of ADAMTS-1, -4 and -5 in MS. Data in this study has shown that ADAMTS-1, -4, and -5 are expressed by neuronal cell lines SHSY-5Y cells and SK-N-DZ *in vitro*. Neuronal expression (SK-N-DZ cells) of ADAMTSs is modified by IL-1β or TNF. Also, neuronal ADAMTS-1 demonstrated an upregulation on RetA treatment (red arrows). However, previous studies by Cross *et al*, (2006) showed an increase in ADAMTS-1 and ADAMTS-4 mRNA by TNF in astrocytes (black arrows). As pro-inflammatory cytokines and RetA all are potent mediators in CNS inflammation and have been shown to modulate ADAMTSs *in vitro* this suggests that ADAMTSs could be implicated in the pathogenesis of MS. To support this implication and show the activity of these ADAMTS and to determine their functional role an *ex vivo* study was preformed. Data showed an upregulation of versican (V0/V2) neoepitopes as an index of the ADAMTSs activity suggesting an ECM breakdown in MS as shown in Figure 6.1. This could be detrimental as ADAMTS degradation of CSPGs produced by neuronal cells or other cells may increase access of inflammatory cells promoting axonal damage. In addition it may be speculated that ECM breakdown products may activate TLRs, perpetuating inflammation. Conversely it may be beneficial as CSPG breakdown by ADAMTSs produced by CNS cells may enable axonal regeneration which is inhibited by CSPGs.


4 Neurons
5 ECM alteration
3 Astrocytes
Neuron
Glia
V0/V2 neo
V0/V2 neo
ADAMTSs activity
ADAMTSs activity
ADAMTS-1
ADAMTS-4
ADAMTS-4
ADAMTS-1
ADAMTS-1
TNF
2
RetA
IL-1β
1
Differentiation
Inflammation (MS)
1. Inflammation/ differentiation
2. TNF / IL-1 β
3. ADAMTS-1, -4 in astrocytes
4. ADAMTS-1, -4 in neurons
5. ADAMTS cleavage of ECM, Versican (V0/V2) neoepitopes
CSPGs (lecticans)
Tenascins
Hyaluronan

6.4 Future Work

To enhance understanding of the functional role of ADAMTSs in MS, the investigations in this study could be consolidated and extended.

ADAMTS-1 siRNA was transfected into SHSY-5Y cells in this study. Knockdown was assessed at the mRNA level by qRT-PCR and at the protein level by western blotting. ADAMTS-1 knockdown experiments in animal models (EAE) could be performed to provide more understanding of the functional role of ADAMTS-1 in the pathogenesis and the progression of the disease in different stages of MS.

More investigations could be made on the effects of ADAMTS knockdown by using cells plated on native ECM substrates and determine the production of neoepitopes by western blotting of concentrated culture medium or looking at the breakdown of quench fluorescent ECM substrates, if available, which may produce fluorescence on cleavage. This could add to the understanding of the functional roles of these ADAMTSs in breakdown of the ECM. Also the expression and modulation of ADAMTSs in animal or human primary neuronal cells (supplied from Neuromics) could be studied.

This study has provided evidence of a functional role of ADAMTSs via the increase in the expression of versican (V0/V2) neoepitope in MS compared to normal tissue which relates to its cleavage by ADAMTSs and possibly increased activity. Previous studies and the current data suggest that ADAMTS-4, as well as other described 'GEPs' (ADAMTS-1, -5 or -9), may play important roles in the processing of this molecule and other lecticans, such as aggrecan and brevican, in the CNS *in vivo* (Westling *et al.,* 2004, Haddock *et al.,*2006). To support this hypothesis, further investigations are required to study the co-localisation in MS brain tissue (fresh samples), of ADAMTSs, with versican (V0/V2) neoepitopes produced as a result of ADAMTS specific cleavage of versican. This will determine which ADAMTS enzyme correlates with the versican breakdown in MS. The cellular association of ADAMTSs and their relationship to neoepitope production, especially with neuronal cells, could also be studied.

Studying cleaved fragments of lecticans in the brain using their specific neoepitope antibodies would increase our knowledge of CNS ECM breakdown in MS. Brevican is a specific CNS PG with a high molecular weight (145 kDa), and is secreted by astrocytes and neurons (Seidenbecher *et al.*, 1998, Jaworski *et al.*, 1999). The anti-EAVESE antibody recognizes the neoepitope sequence generated by ADAMTS cleavage at the C-termini which is associated with a 55 kDa fragment of brevican (Matthews *et al.*, 2000). Aggrecan is another member of the lectican family of proteoglycans which is found in cartilage and brain. It is also cleaved by ADAMTSs and forms a C-terminus neoepitope NITEGE recognized by an antibody (Wilson *et al.*, 2009, Voigt *et al.*, 2009). These neoepitopes could be detected with specific antibodies in further investigations in human brain tissue. The cleavage of versican and other lecticans e.g. aggrecan and brevican by the ADAMTSs may result in a loosening of the lectican–hyaluronan–tenascin matrix and promote the access of inflammatory cells and cytokines increasing axonal damage (Figure 6.1).

An alteration of ECM was identified in this study and a versican V2 fragment similar to GHAP is produced (Westling *et al.*, 2004). The function of this fragment is still not known, however the breakdown of ECM components and the production of GHAP may result in the activation of TLRs in the same way as hyaluronan fragments or other endogenous ligands of TLR do (Piccinini and Midwood, 2010). These ECM fragments could be important playing a key role in MS pathogenesis, inducing inflammatory responses by TLRs induction and or stimulation. TLR expression and the effects of synthetic GHAP could be studied in CNS-derived cells on parameters such as cytokine and protease production.

6.5 Conclusion

The main aim of this study was to elucidate a role for ADAMTS-1, -4 and -5 in the pathogenesis of MS. This study has shown that ADAMTS-1, -4 and -5 are constitutively expressed in SHSY-5Y and SK-N-DZ neuronal cells. It also provides evidence that ADAMTS-1 has the potential to be implicated in MS pathogenesis, showing an

upregulation after the RetA treatment. In the SK-N-DZ cells ADAMTS-1 mRNA increased with IL-1β. Further investigations on primary neuronal cells which could further elucidate the role of neuronal ADAMTSs are required. Post mortem studies provided evidence that ADAMTSs may be implicated in the pathogenesis of MS by the increased versican (V0/V2) neoepitope expression generated due the cleavage of versican by these enzymes. This suggests ADAMTSs do appear to have a potential role in the pathogenesis of MS particularly in lesion development and in ECM breakdown, which may be detrimental or beneficial. Further investigations are required to determine the importance of the versican fragment(s) generated which could be ligands of TLRs and may have a potential link with processes occurring in MS. The GEPs such as ADAMTS-1 and -4, which are produced in the CNS may be a possible future target for MS therapy.

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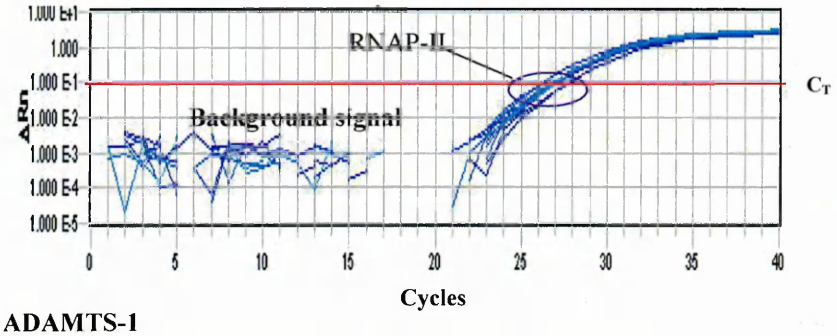
Appendix I

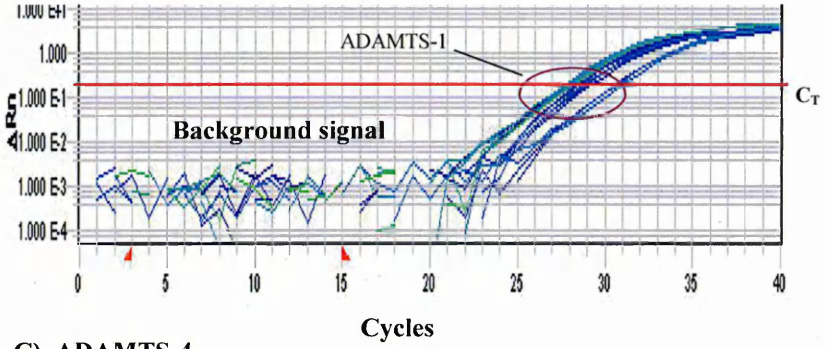
Amplification Plots for cDNA from SHSY-5Y Cells

SHSY-5Y

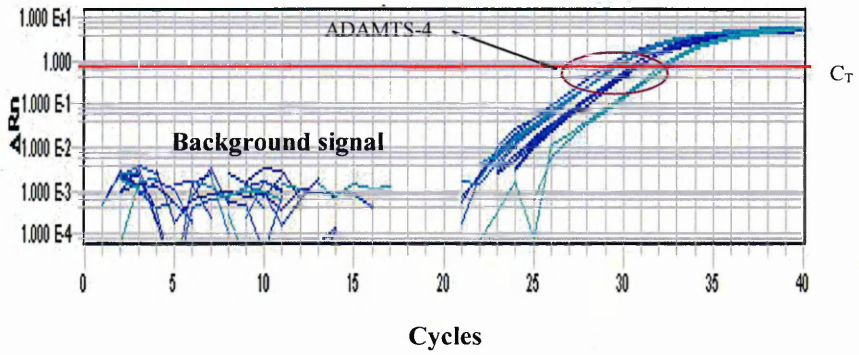
A) RNAP-II



B) ADAMTS-1

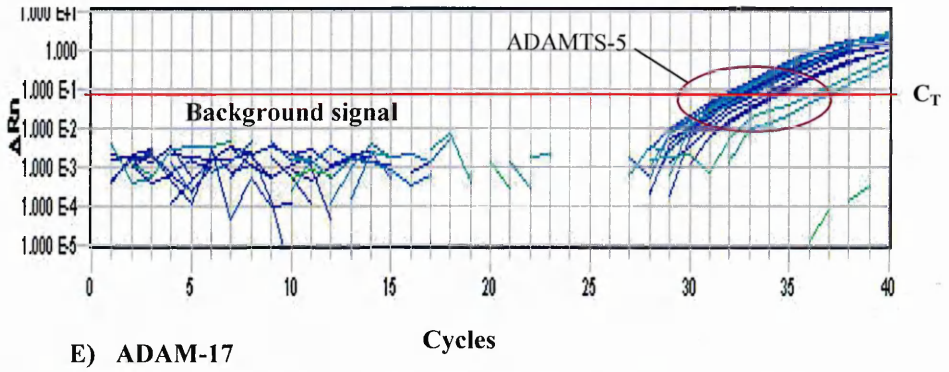


C) ADAMTS-4

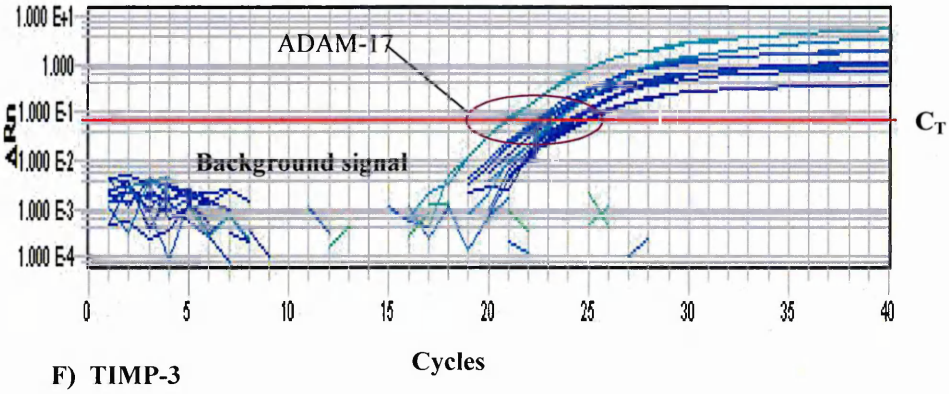


Amplification plots of qPCR for RNAP-II (A), ADAMTS-1 (B) and -4 (C). RNAP-II showed constant expression following treatment (C_T values 26.9-27.6) with cytokine. Both ADAMTS-1 and ADAMTS-4 C_T values were between 29-32.

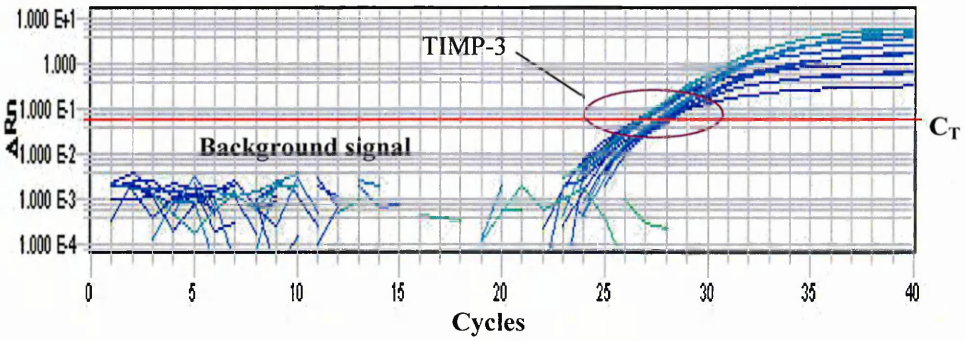
D) ADAMTS-5



E) ADAM-17



F) TIMP-3



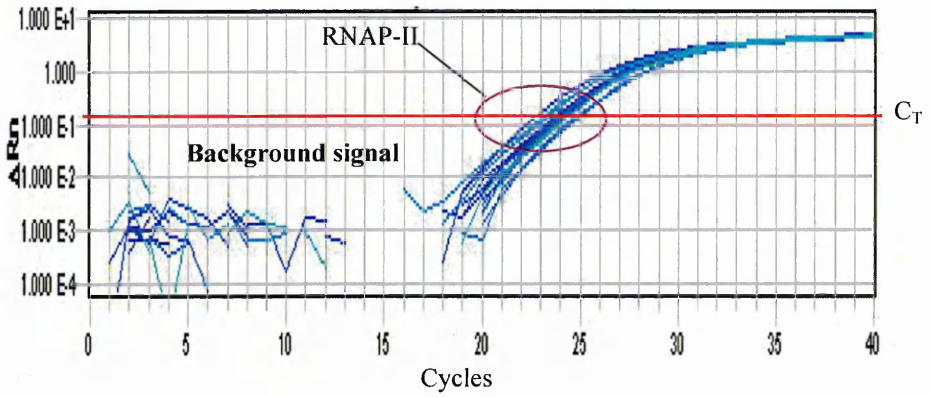
Amplification plots of qPCR for ADAMTS-5 (D), ADAM-17 (E) and TIMP-3 (F). ADAMTS-5 expression following treatment C_T values was > 30, ADAM-17 and TIMP-3 C_T values variable numbers.

Appendix II

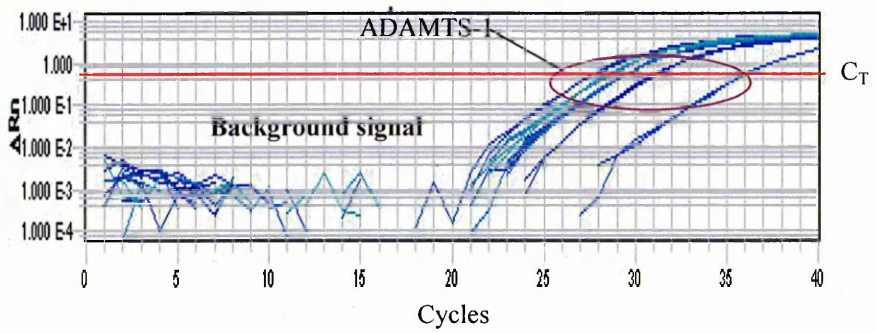
Amplification Plots for cDNA from SK-N-DZ Cells

SK-N-DZ

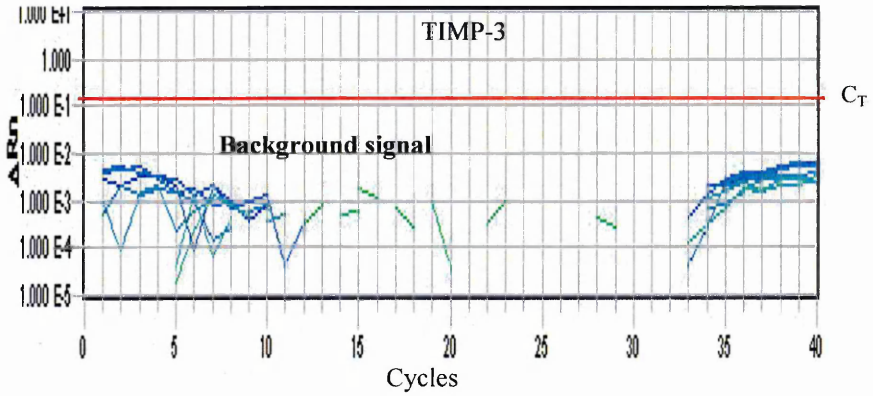
A) RNAP-II



B) ADAMTS-1



C) TIMP-3



Amplification plots of qPCR for RNAP-II (A), ADAMTS-1 (B) and TIMP-3 (C). RNAP-II showed constant expression following treatment (C_T values ~ 24-25) with cytokine. ADAMTS-1 showed variable C_T values and no TIMP-3 gene expression.

Appendix III

Modules, Publications and

Presentations

Modules

Research Studies (15M Credits): During the first year (2007-2008)

Research Methods (15M Credits): 10-12th December 2007

Published Abstracts

GIBREL, G., CROSS, A.K., HADDOCK, G., BUTTLE, D.J. and BUNNING, R.A.D. (2011)
The Role of ADAMTSs and Versican Cleavage in Multiple Sclerosis. Poster presented at BSMB Meeting 'Matrix Signals-Cell Matrix Interactions in Health and Disease' 8-9th September 2011, Newcastle, UK. International Journal of Experimental Pathology.

GIBREL , G., CROSS, A.K., HADDOCK, G., BUTTLE, D.J. and BUNNING, R.A.D. (2010)
The Functional Role of ADAMTSs in the Pathogenesis of Multiple Sclerosis. Poster presented at 10th International Congress of Neuroimmunology-ISNI, Sitges (Barcelona, Spain) 26-30th October 2010, Journal of Neuroimmunology, 15 November 2010; 228, ISSUES 1-2).

GIBREL , G., CROSS, A.K., HADDOCK, G., BUTTLE, D.J. and BUNNING, R.A.D. (2010)
The Expression and Modulation of ADAMTS-1 and TIMP-3 in Neuronal SHSY-5Y Cells and CNS Tissue. Poster presented at BSMB Autumn Meeting 'Inflammation Meets Matrix Biology' 6-7th September 2010, Norwich, UK. International Journal of Experimental Pathology.

Departmental Oral Presentations (BMRC)

Final year presentation: 29th March 2011

Third year presentation: 17th February 2009

First year presentation (PhD confirmation): 22nd October 2008

Introductory oral presentation: 31st October 2007

Posters Presentations

BMRC/MERI Winter Poster Session: 16th December 2011, Sheffield Hallam University, Sheffield, UK.

British Society for Matrix Biology (BSMB) Meeting: 'Matrix Signals-cell Matrix Interactions in Health and Disease' 8-9th September 2011, Newcastle, UK.

MS Frontiers Research Event: 21-22nd May 2009; 23-24 th June 2011, London, UK.

10th International Congress of Neuroimmunology-ISNI: 26-30th October 2010, Sitges (Barcelona, Spain).

British Society for Matrix Biology (BSMB) Autumn Meeting: 'Inflammation Meets Matrix Biology' 6-7th September 2010, Norwich, UK.

Faculty of Health and Wellbeing Research Day: 17th December 2008; 17th June 2010, Sheffield, UK.

The 3rd Symposium for Libyan Students in the UK: 12th June 2010, Sheffield Hallam University, Sheffield, UK.

Conference Attendance

British Society for Matrix Biology (BSMB) Meeting: 'Matrix Signals-cell Matrix Interactions in Health and Disease' 8-9th September 2011, Newcastle, UK.

10th International Congress of Neuroimmunology-ISNI: 26-30th October 2010, Sitges (Barcelona, Spain)

British Society for Matrix Biology (BSMB) Autumn Meeting: 'Inflammation Meets Matrix Biology' 6-7th September 2010, Norwich, UK

The University of Sheffield Neuroscience Away Day: 19 th September 2009, Sheffield, UK.

The Northern Neuro-Immunology Interest Group 8th Annual Meeting: 2nd November 2007, Nottingham, UK.

Professional Activities

Four years (2008-2011) member of a Learned Society: British Society of Matrix Biology (BSMB).

Appendix IV

Ethical Approval

NHS
Central Office for Research Ethics Committees
(COREC)

CONDITIONS OF ETHICAL APPROVAL

Research Ethics Committee:	MREC FOR WALES
Research Tissue Bank:	THE UK MULTIPLE SCLEROSIS TISSUE BANK
REC reference number:	08/MRE09/31
Name of applicant:	PROFESSOR RICHARD REYNOLDS (DESIGNATED INDIVIDUAL : MR G. ROPER)
Date of approval:	08 MAY 2008

Ethical approval is given to the Research Tissue Bank ("the Bank") by the Research Ethics Committee ("the Committee") subject to the following conditions.

1. Further communications with the Committee

1.1 Further communications with the Committee are the personal responsibility of the applicant.

2. Duration of approval

2.1 Approval is given for a period of 5 years, which may be renewed on consideration of a new application by the Committee, taking account of developments in legislation, policy and guidance in the interim. New applications should include relevant changes of policy or practice made by the Bank since the original approval together with any proposed new developments.

3. Licensing

3.1 A copy of the Licence from the Human Tissue Authority (HTA) should be provided when available (if not already submitted).

3.2 The Committee should be notified if the Authority renews the licence, varies the licensing conditions or revokes the Licence, or of any change of Designated Individual. If the Licence is revoked, ethical approval would be terminated.

4. Generic ethical approval for projects receiving tissue

4.1 Samples of human tissue or other biological material may be supplied and used in research projects to be conducted within the establishment responsible for the Bank *and/or* by researchers and research institutions external to the Bank within the UK and in other countries in accordance with the following conditions.

4.1.1 The research project should be within the fields of medical or biomedical research described in the approved application form.

4.1.2 The Bank should be satisfied that the research has been subject to scientific critique, is appropriately designed in relation to its objectives and (with the exception of student research below doctoral level) is likely to add something useful to existing knowledge.

4.1.3 Where tissue samples have been donated with informed consent for use in future research ("broad consent"), the Bank should be satisfied that the use of the samples complies with the terms of the donor consent.

4.1.4 All samples and any associated clinical information must be non-identifiable to the researcher at the point of release (i.e. anonymised or linked anonymised).

4.1.5 Samples will not be released to any project requiring further data or tissue from donors or involving any other research procedures. Any contact with donors must be confined to ethically approved arrangements for the feedback of clinically significant information.

4.1.6 A supply agreement must be in place with the researcher to ensure storage, use and disposal of the samples in accordance with the HTA Codes of Practice, the terms of the ethical approval and any other conditions required by the Bank.

4.2 A research project in the UK using tissue provided by a Bank in accordance with these conditions will be considered to have ethical approval from the Committee under the terms of this approval. In England, Wales and Northern Ireland this means that the researcher will not require a licence from the Human Tissue Authority for storage of the tissue for use in relation to this project.

4.3 The Bank may require any researcher to seek specific ethical approval for their project. Such applications should normally be made to the Committee and booked via the COREC Central Allocation System.

4.4 A Notice of Amendment form should be submitted to seek the Committee's agreement to change the conditions of generic approval.

5. Records

5.1 The Bank should maintain a record of all research projects to which tissue has been supplied. The record should contain at least the full title of the project, a summary of its purpose, the name of the Chief Investigator, the sponsor, the location of the research, the date on which the project was approved by the Bank, details of the tissue released and any relevant reference numbers.

5.2 The Committee may request access to these records at any time.

6. Annual reports

6.1 An annual report should be provided to the Committee listing all projects for which tissue has been released in the previous year. The list should give the full title of each project, the name of the Chief Investigator, the sponsor, the location of the research and the date of approval by the Bank. The report is due on the anniversary of the date on which ethical approval for the Bank was given.

6.2 The Committee may request additional reports on the management of the Bank at any time.

7. Substantial amendments

7.1 Substantial amendments should be notified to the Committee and ethical approval sought before implementing the amendment. A substantial amendment generally means any significant change to the arrangements for the management of the Bank as described in the application to the Committee and supporting documentation.

7.2 The COREC Notice of Amendment form should be used to seek approval. The form is available at http://www.corec.org.uk/applicants/apply/amendments.htm#other.

7.3 The following changes should always be notified as substantial amendments:

7.3.1 Any significant change to the policy for use of the tissue in research, including changes to the types of research to be undertaken or supported by the Bank.

7.3.2 Any significant change to the types of biological material to be collected and stored, or the circumstances of collection.

7.3.3 Any significant change to informed consent arrangements, including new/modified information sheets and consent forms.

7.3.4 A change to the conditions of generic approval.

7.3.5 Any other significant change to the governance of the RTB.

8. Serious adverse events

8.1 The Committee should be notified as soon as possible of any serious adverse event or reaction, any serious breach of security or confidentiality, or any other incident that could undermine public confidence in the ethical management of the tissue. The criteria for notifying the Committee will be the same as those for notifying the Human Tissue Authority in the case of research tissue banks in England, Wales and Northern Ireland.

9. Other information to be notified

9.1 The Committee should be notified of any change in the contact details for the applicant or where the applicant hands over responsibility for communication with the Committee to another person at the establishment.

10. Closure of the Bank

10.1 Any plans to close the Bank should be notified to the Committee as early as possible and at least two months before closure. The Committee should be informed what arrangements are to be made for disposal of the tissue or transfer to another research tissue bank.

10.2 Where tissue is transferred to another research tissue bank, the ethical approval for the Bank is not transferable. Where the second bank is ethically approved, it should notify the responsible Research Ethics Committee. The terms of its own ethical approval would apply to any tissue it receives.

11. Breaches of approval conditions

11.1 The Committee should be notified as soon as possible of any breach of these approval conditions.

11.2 Where serious breaches occur, the Committee may review its ethical approval and may, exceptionally, suspend or terminate the approval.