Differential expression of ADAMTS -1, -4, -5 and TIMP -3 in rat spinal cord at different stages of acute experimental autoimmune encephalomyelitis

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Abstract

Experimental autoimmune encephalomyelitis (EAE) is an animal model of inflammatory demyelination, a pathological event common to multiple sclerosis (MS). During CNS inflammation there are alterations in the extracellular matrix (ECM). A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS) -1, -4 and -5 are proteases present in the CNS, which are able to cleave the aggregating chondroitin sulphate proteoglycans, aggrecan, phosphacan, neurocan and brevican. It is therefore important to investigate changes in their expression in different stages of EAE induction. We have investigated expression of ADAMTS-1, -4, -5 and Tissue inhibitor of metalloproteinase (TIMP) -3, by real-time RT-PCR. We have also examined protein expression of ADAMTS-1, -4 and -5 by western blotting and immunocytochemistry in spinal cord from animals at different stages of disease progression. Our study demonstrated a decrease in ADAMTS-4 mRNA and protein expression. TIMP-3 was decreased at the mRNA level although protein levels were increased in diseased animals compared to controls. Our study identifies changes in ADAMTS expression during the course of CNS inflammation which may contribute to ECM degradation and disease progression.

Key Words: ADAMTS, EAE, extracellular matrix, inflammation, brain.

Running Title: ADAMTS expression in normal and EAE spinal cord.

Introduction

The extracellular matrix (ECM) of the central nervous system (CNS) has both structural and functional roles including anchorage of molecules such as integrins and growth factors. The CNS ECM shares many components found in the ECM of other tissues but differs somewhat in its relatively low collagen and fibronectin content and larger amounts of glycosaminoglycans, either bound to proteins in the form of proteoglycans or unbound as hyaluronan. Proteoglycans of the brain include the chondroitin sulphate proteoglycans (CSPGs) aggrecan, brevican, neurocan, phosphacan, appican and versican [1-5]. These molecules are important to brain structure through maintenance of the correct hydrodynamics and in their interactions with other ECM components. They also contribute to disease processes and their synthesis is modulated by injury. As a group they are known to inhibit neurite outgrowth and axonal regeneration and promote neural cell death [5-9]. However enzymic removal of chondroitin sulphate promotes axonal regeneration [10]. Maintenance of the appropriate quantity and location of CNS CSPGs is thus likely to be of major importance to the correct functioning of the brain and may also influence the balance between neuronal death and survival.

Experimental autoimmune encephalomyelitis (EAE) is a model of neuroinflammation with clinical and histopathological similarities to multiple sclerosis (MS) [11]. Extracellular proteolytic enzymes are implicated in the pathology of both MS and EAE and in particular the role of matrix metalloproteinases (MMPs) is well established [12-16]. The roles of MMPs in neuroinflammation include disruption of the blood-brain barrier by breakdown of the ECM around blood vessels and the use of MMP inhibitors has shown reduced clinical severity of EAE [16]. More recently, some of the ADAM metalloproteinases have been shown to have an important role in CNS inflammation [17]. It is therefore of importance to determine

whether the ADAMTS (A Disintegrin and Metalloproteinase with Thrombospondin motifs) group of metalloproteinases [18,19], known to be expressed in the CNS [20-23], are modulated in CNS inflammatory conditions such as EAE and MS.

The ADAMTSs are a distinct group of phylogenetically related proteins in Family M12 of Clan MA in the *Merops* database (http://merops.sanger.ac.uk/) [24] and are related to the ADAMs and matrix metalloproteinases. A phylogenetic subgroup of the ADAMTS enzymes comprising ADAMTS-1, -4, (EC 3.4.24.82) -5, -8, -9, and -15 possess proteoglycanase and anti-angiogenic activities [25]. ADAMTS-1, -4, -5, -8 [26] and -9 possess aggrecanase activity and have been implicated in cartilage degradation [27],[28]. Molecular modeling has suggested that ADAMTS-15 possesses the same or similar activity [29]. It is known that ADAMTS-1, -4 and -9 are capable of cleaving versican [28], [30], and brevican is cleaved by ADAMTS-4 [31]. The ADAMTS proteoglycanases are inhibited by tissue inhibitor of metalloproteinases (TIMP)-3 [32] which, like the ADAMTSs, is sequestered in the ECM via interactions with sulphated glycosaminoglycans [33-35].

To our knowledge this is the first study of ADAMTS expression in EAE. We have investigated the expression of the proteoglycanases ADAMTS-1, -4, and -5, the original ADAMTSs categorized as aggrecanases and for which antibodies were available, as well as their inhibitor TIMP-3, in animals at different stages of EAE. We demonstrate differential expression of proteases and ECM components in an inflammatory environment *in vivo*.

Materials and Methods

Spinal cord tissue

Spinal cord tissue from Lewis rats following EAE induction by immunisation with spinal cord homogenate plus adjuvant was kindly provided by Dr T Smith, Eisai Research Laboratories, London, UK. Clinical disability was scored: 0, no detectable change in muscle tone and motor behaviour; 1, flaccid tail; 2, impairment of righting reflex and/or loss of muscle tone in hindlimbs; 3, complete hindlimb paralysis; 4, paraplegia; and 5, death [36]. Tissue was snap frozen in liquid nitrogen cooled isopentane and included: 5 naïve controls, 4 animals which were pre-disease (sacrificed on day 10 post-immunisation, prior to the onset of disease symptoms, disability score of 0), 5 animals at the peak of disease (sacrificed on day 12 post-immunisation, disability score of 3-3.25) and 6 recovered animals (sacrificed 4 days after their disability score had returned to 0).

RNA and protein extraction

Spinal cord white matter was excised and homogenised in Tri-reagent (Sigma, UK), followed by vortex mixing and centrifugation at 12000 x *g* for 15 minutes at 4°C to remove lipids. The supernatant was then transferred to a clean microfuge tubes and the RNA and protein were extracted following the manufacturer's instructions. Protein was stored at 4°C and RNA at -80°C prior to use.

Real-time (q) RT-PCR

RNA was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen, Paisley, UK) and the resulting cDNA was used as a template for qRT-PCR using the ABI PRISM 7900HT sequence detection system and 2xSYBR Green mastermix (Applied Biosystems, Warrington, UK) for primers designed using Primer Express software (Applied Biosystems).

Primers were synthesised by MWG Biotech (Ebbersberg, Germany). In order to preclude the amplification of nuclear DNA, all primers crossed an exon-exon boundary. Primer sequences and accession numbers are shown in Table 1. Expression of ADAMTS-1, -4, -5 and TIMP-3, were analysed, as well as the housekeeping gene GAPDH to normalise expression between different samples. Sequences were confirmed as unique by a BLAST search (www.ncbi.nlm.nih.gov/BLAST).

Each primer pair generated a single product of the appropriate size when visualised by agarose gel electrophoresis, and by melt curve analysis following qRT-PCR. Primer pair efficiency was determined by plotting log cDNA dilution against cycle threshold (CT), the slope of which was -3.3 +/- 0.1 for each primer pair, indicating maximal efficiency. Relative mRNA levels of ADAMTS-1, -4, -5, and TIMP-3, were determined using the formula $2^{-\Delta CT}$ where ΔCT = CT (target gene) – CT (GAPDH).

Western blotting

Western blot analyses of ADAMTS-1, -4 and -5 and TIMP-3 were carried out using rabbit polyclonal antibodies for C-terminal epitopes of ADAMTS-1, -4 and -5 (Triple Point Biologics, USA) and a monoclonal antibody for TIMP-3 (Oncogene, Germany). Protein concentrations were determined using the bicinchoninic acid assay [37]. 6µg of protein per well was separated by electrophoresis on 12% NuPAGE gels (Invitrogen, Paisley, UK) under reducing conditions. The proteins in the gel were electroblotted onto a nitrocellulose membrane (Hybond-C, Amersham, UK) at 150V for 1 hour, on ice. Blots were blocked for 2 hours at ambient temperature with 5% non-fat milk in Tris-buffered saline containing 0.05% Tween 20 (Sigma, UK) (TBST). The blots were washed 3 x 10 min in TBST and the primary antibody was added at 1: 5000 dilution or in the case of TIMP-3 blots, 1: 1000 in TBST

containing 5% non-fat milk. The blots were incubated overnight at 4°C with gentle shaking and washed as described above, then incubated with goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate (Sigma, UK), 1:80000 in TBST for the anti-ADAMTS and TIMP-3 antibodies, and rabbit anti-mouse IgG HRP conjugate (Dako, UK), 1:1000 in TBST containing 5% non-fat milk for 2 hours at ambient temperature with gentle shaking. The blots were washed 3 x 5 min in TBST followed by three washes in Tris-buffered saline for 5 min. Development of the blots was carried out using the ECL Plus chemiluminescence substrate (Amersham, UK). Images were captured using a UVP bioimaging system (Biorad, Hertfordshire, UK). Semi-quantitation of the bands was carried out by densitometry. Western blot experiments were repeated three times.

Immunohistochemistry

Immunohistochemistry was carried out on acetone-fixed cryostat sections (15μm) of spinal cord. For single staining, ADAMTS–4 was detected using the antibody against the C-terminal epitope of ADAMTS-4 (1:50) described above, followed by incubation with Alexa 488 (green) goat anti-rabbit IgG (1:500) (Molecular Probes). Dual immunofluorescence labelling was carried out using the ADAMTS-4 antibody followed by Alexa 568 (red) -conjugated goat anti-rabbit IgG (1:500) with detection of glial fibrillary acidic protein (GFAP) (astrocyte marker) (1:100) or a macrophage marker, ED-1 (1:50) (Serotec, UK) using a primary monoclonal antibody followed by FITC-labelled (green) rabbit anti-mouse IgG (1:50). Slides were examined using a Zeiss LSM 510 confocal microscope.

Data Analysis

Comparison of the expression levels of ADAMTSs and TIMP-3 between different groups was performed using the Mann-Whitney test. $P<0.05$ was considered to be significant.

Results

Rat spinal cord white matter, from all normal animals was found to express mRNA for ADAMTS-1, -4, -5 and TIMP-3 (Figure 1). ADAMTS-1 being the lowest expressed of the ADAMTSs. ADAMTS-4 was the highest expressed gene amongst the control group and ADAMTS-5 demonstrated the most variable levels between naive animals (Figure 1).

Although ADAMTS-1 was the lowest expressed gene tested, this was the only ADAMTS which showed a statistically significant increase in mRNA expression levels following induction of EAE, although this was only in the animals in the pre-disease group (Figure 2a). At the peak of disease and in recovery animals, the ADAMTS-1 levels were not significantly different from controls. In contrast, protein levels of ADAMTS-1 showed a decrease in the predominant form (64kDa) and in a 50kDa truncated form (assessed by western blotting and densitometry) ($P<0.05$), in all EAE animals compared to controls (Figure 3a and f).

In contrast, ADAMTS-4 mRNA levels were significantly decreased in the group of rats at the peak of clinical symptoms compared to the control group and levels had returned to normal in the recovery group of animals (Figure 2b). Protein levels of ADAMTS-4 (65, 55, 50kDa forms) were also lower in EAE animals compared to control animals (Figure 3b and g), although the recovery group showed a slight (non-significant) increase in ADAMTS-4 compared to the animals at peak of disease, whilst still being significantly lower than control animals.

ADAMTS-5 mRNA levels were not significantly different in any of the groups of animals (Figure 2c) although protein levels of ADAMTS-5 were significantly lower in recovery animals compared to controls, pre-disease and peak disease groups (Figure 3c and h).

Levels of mRNA expression of TIMP-3 were significantly decreased in the animals at the peak of disease compared to the normal animal group (Figure 2d). In the recovery group of animals, TIMP-3 levels were not significantly different from the mRNA levels detected in normal control animals. The protein levels of TIMP-3 did not mirror the mRNA levels, with an increase in TIMP-3 detected as a 24kDa species in all EAE groups compared to the control animals (Figure 3d and i). An additional higher molecular weight species (48kDa) was detected in all samples.

Immunohistochemical analysis of näive and EAE spinal cord demonstrated an overall decrease in the amount of ADAMTS-4 staining associated with astrocytes (Figure 4a-f). A greater amount of staining for ADAMTS-4 in an area of inflammation in diseased tissue was observed (Figure 4g-i). Some of the ADAMTS-4 staining in the inflamed area was colocalised to astrocytes although some was not. This ADAMTS-4 was subsequently colocalised with ED-1 positive infiltrating macrophages (Figure 4j-l). Not all ED-1 positive cells were colocalised with ADAMTS-4 suggesting that these cells may be the resident macrophages. In both control and EAE tissue there was colocalisation of GFAP positive cells with ADAMTS-4 positive cells and this appeared to be strongest in the astrocyte processes (Figures 4m-o). There was also staining of the ECM, particularly in the diseased tissue. Only very weak staining was achieved using ADAMTS-1 and -5 antibodies with no differences in distribution between normal and EAE tissue (data not shown).

Discussion

The brain ECM components are altered during injury and inflammation when the normal balance between proteases and their inhibitors is changed. Proteolytic activity in the CNS may lead to breakdown of the blood-brain barrier, or to loss of myelin, whereas an over-expression of protease inhibitors may cause fibrosis [38].

Little is known about the role of ADAMTSs in CNS inflammation and injury. A small number of studies [20-23] have reported the presence of ADAMTSs in the CNS but few have quantitatively examined ADAMTS expression in diseased CNS.

We have demonstrated the presence of ADAMTS-1, -4 and -5 in normal and EAE spinal cord and shown changes in expression of mRNA and protein levels during the course 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. An increase in ADAMTS-1 mRNA has also been demonstrated by Sasaki et al [39], 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. In our study the increase in ADAMTS-1 mRNA in pre-disease animals was not mirrored by protein levels. The predominant ADAMTS-1 species of 64kDa and the 50kDa truncated species were decreased in pre-, peak and recovered EAE animals compared to controls. This may suggest that ADAMTS-1 itself is under attack from other proteases, such as MMPs, which are likely to be upregulated in an inflammatory condition [16].

Our study revealed that ADAMTS-4 was the most highly expressed RNA of the ADAMTSs studied. It is likely to be important in CNS CSPG turnover and the reduction in its expression in EAE may decrease proteolytic degradation of CSPG and, depending on the degree of C-terminal processing of ADAMTS-4, other substrates such as fibromodulin and decorin [40]. Although ADAMTS-4 expression was found to be reduced in EAE compared with naive tissue, immunohistochemical analysis of näive and EAE spinal cord also demonstrated a greater amount of staining for ADAMTS-4 in an area of inflammation. There was, in both cases colocalisation with GFAP which demonstrated that some of the ADAMTS-4 was associated with astrocytes, possibly via interaction of cell surface proteins with the disintegrin-like region of ADAMTS-4, or through binding of the thrombospondin motif to cell-surface proteoglycans such as syndecans. Some of the ADAMTS-4 immunoreactivity in the inflammatory cuff was colocalised with macrophages as well as astrocytes although not all macrophages were ADAMTS-4 positive. There was also some staining of the ECM, particularly in the diseased tissue, which may be mediated by the thrombospondin motif on the enzyme. Such a store of sequestered enzyme may represent a pool of proteolytic activity in the remodelling matrix. Pericellular and ECM localisation has been observed previously for ADAMTS-5 in diseased synovium [41].

We have recently demonstrated that cytokines involved in CNS inflammation alter the processing of ADAMTS-4 in primary human astrocytes and astrocytoma cells (Cross *et al*, in preparation). Changes in the processing of the enzyme *in vivo* may depend on the cytokine microenvironment whereby an overall decrease in levels of ADAMTS-4 and changes in the processing of the enzyme, are likely to be important factors impacting upon the composition of the CNS ECM during EAE. A reduction in ADAMTS activity in EAE may lead to an increase in CSPG deposition and glial scarring creating an inhibitory environment for neurite

outgrowth and repair. It must also be noted that other metalloproteinases, such as MMP-3 (stromelysin) are able to cleave CSPGs [42]. MMP-3 is expressed in the CNS and is also inhibited by TIMP-3 [16].

In the present study, TIMP-3 mRNA levels were significantly reduced in peak EAE compared to control animals, although TIMP-3 protein levels appeared to be increased in EAE. Toft-Hansen et al, [43], also reported a decrease in TIMP-3 expression at the mRNA level EAE spinal cord in a mouse model. The increase in TIMP-3 protein could reflect an increase in translation of its mRNA transcript, or could be due to increased stability (longer half-life) of the protein. It was noticeable that the 24kDa form of TIMP-3 was only seen during and after the disease process and that a 48kDa species was seen throughout. TIMP-3 is known to be bound to the ECM unlike TIMP-1 and TIMP-2 [44],[45] and the higher molecular weight species have been proposed to be stable complexes of TIMP-3 with ECM ligands [46]. An alternative explanation is that the 48kDa species may be an as yet un-described brain specific dimer of TIMP-3. An increase in TIMP-3 protein levels in peak EAE indicates inhibition and control of metalloprotease activity during and after the peak of the inflammatory response.

In conclusion, we have demonstrated changes in the expression levels of ADAMTSs and TIMP-3. These changes may be important in tissue damage during inflammation as well as tissue remodelling and repair. Due to the increasing number of known substrates for the ADAMTSs, changes in expression levels could be either beneficial or detrimental. A decrease in ADAMTSs, could lead to decreased degradation of CSPGs allowing accumulation of matrix proteins and formation of glial scars. The outcome of the response to the CNS inflammation will depend in part on the relative levels of the full-length and truncated forms of ADAMTSs and TIMP-3.

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Figure legends

Figure 1. Real-time PCR was carried out on spinal cord from normal animals to show relative levels (compared to GAPDH, calculated using the formula $2^{-\Delta CT}$ where ΔCT is CT of target gene - CT of GAPDH) of ADAMTSs and TIMP3 in normal rat spinal cord. Data represents median (-) and relative levels compared to GAPDH for individual animals.

Figure 2. Real time PCR was carried out on spinal cord from control animals and those with EAE at different stages of disease progression. Data shows real time PCR results for ADAMTS-1 (a), ADAMTS-4 (b), ADAMTS-5 (c) and TIMP-3 (d). The median value is shown as (-) and data are calculated as $2^{-\Delta CT}$ where ΔCT is CT of target gene - CT of GAPDH (housekeeping gene). * indicates significant difference from control ($P<0.05$).

Figure 3 Western blot analysis and densitometry for ADAMTS and TIMP-3 expression.
Western blots of ADAMTS-1 (a), ADAMTS-4 (b), ADAMTS-5 (c) TIMP-3 (d) and β-actin loading control (e). Lanes: M = molecular weight marker, 1-2 normal, 3-4 pre-disease, 5-6 peak disease, 7-8 recovered.
Densitometry for ADAMTS-1 (f) ADAMTS-4 (g), ADAMTS-5 (h) and TIMP-3 (i). Each bar on the graph represents mean integrated optical density (IOD) +/- standard error of the mean of each group of animals (normal, pre-disease, peak disease and recovered) repeated 3 times. IOD values were pooled from all detected forms. * indicates significantly different from control ($P<0.05$).

Figure 4 Immunohistochemistry on control (a-c) and EAE (d-o) tissue. Figures 4a, d,

g, j, and m show ADAMTS-4 staining. Figures 4b, e, h and n show GFAP staining. Figure 4k shows ED-1 staining. True colocalised pixels are represented as white in the merged images (Figures 4c, f, i, l and o). Bar represents 50μM on all except for m, n and o where it represents 20μM.