ADAMTS -1 and -4 are up-regulated following transient middle cerebral artery occlusion in the rat and their expression is modulated by TNF in cultured astrocytes

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Abstract

ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) enzymes are a recently described group of metalloproteinases. The substrates degraded by ADAMTS-1, -4 and -5 suggests that they play a role in turnover of extracellular matrix in the central nervous system (CNS). ADAMTS-1 is also known to exhibit anti-angiogenic activity. Their main endogenous inhibitor is tissue inhibitor of metalloproteinases (TIMP)-3.

The present study was designed to investigate ADAMTS-1, -4 and -5 and TIMP-3 expression after experimental cerebral ischaemia and to examine whether cytokines known to be up-regulated in stroke could alter their expression by astrocytes *in vitro*. Focal cerebral ischaemia was induced by transient middle cerebral artery occlusion in the rat using the filament method.

Our results demonstrate a significant increase in expression of ADAMTS-1 and -4 in the occluded hemisphere but no significant change in TIMP-3. This was accompanied by an increase in mRNA levels for interleukin (IL)-1β, IL-1 receptor antagonist (IL-1ra) and tumour necrosis factor (TNF). ADAMTS-4 mRNA and protein was up-regulated by TNF in primary human astrocyte cultures. The increased ADAMTS-1 and -4 in experimental stroke, together with no change in TIMP-3, may promote ECM breakdown after stroke, enabling infiltration of inflammatory cells and contribute to brain injury. *In vitro* studies suggest that the *in vivo* modulation of ADAMTS-1 and -4 may be controlled in part by TNF.

1. Introduction

Focal brain ischaemia induces a complex cascade of events leading to extracellular matrix remodelling, gliosis and neovascularisation. An increase in the expression of matrix metalloproteinases (MMPs) correlates to stroke pathology [6], [34], [35] and the use of metalloproteinase inhibitors in animal models has demonstrated effects such as a reduction in infarct size [13] and limitation of early blood brain barrier (BBB) opening [34]. ADAM-17, a member of the ADAMs family of metalloproteinases, is a key sheddase that releases TNF from its membrane bound form and is up-regulated after an ischaemic event [5]. Selective antagonists of ADAM-17 systemically administered in a rat model of transient middle cerebral artery occlusion (tMCAo) also reduced infarct size and neurological deficit [45].

A more recently described and distinct group of metalloproteinases, the ADAMTSs (A Disintegrin and Metalloproteinase with Thrombospondin motifs) [31], [41] are part of Family M12 of Clan MA in the *Merops* database [32] and are related to the ADAMs and MMPs. A phylogenetic subgroup of the ADAMTS enzymes comprising ADAMTS-1, -4, -5, -8, -9, and -15 possess proteoglycanase and anti-angiogenic activities [44]. ADAMTS-1, -4, -5 and -9 have aggrecanase activity and are implicated in cartilage degradation [8], [40], [43]. The ADAMTS proteoglycanases are inhibited by tissue inhibitor of metalloproteinases (TIMP)-3 which, like the ADAMTSs, is sequestered in the ECM via interactions with sulphated glycosaminoglycans [11], [18], [47]. ADAMTS expression has been detected in the CNS [1], [17], [37] and is known to be altered in disease states [10], [22], [23].

The chondroitin sulphate proteoglycans (CSPGs), aggrecan, brevican, neurocan, phosphacan, appican and versican are expressed in the brain [3], [12], [26], [28], [46] and are potential substrates for ADAMTSs. These CSPGs 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 [14]. As a group, CSPGs are known to inhibit neurite outgrowth and axonal regeneration and promote neural cell death [2], [12], [15], [19], [39]. Therefore alterations in levels of CSPGs are relevant to neuronal survival and regeneration after an ischaemic event. The ADAMTSs are likely to be involved in modulation of CSPGs and angiogenesis in stroke pathology due to their substrate specificities and anti-angiogenic properties.

Astrocytes are activated in response to a range of CNS pathologies including trauma, neurodegenerative diseases and stroke [29]. Astrocytes have many roles in cerebral ischaemia such as scavenging of free radicals and release of growth factors as well as astrogliosis which is inhibitory to axonal regeneration [25]. Recent research has demonstrated that the expression of CSPGs is increased by treatment of astrocytes with cytokines such as transforming growth factor (TGF) β1 and epidermal growth factor (EGF) [38].

The purpose of this study was to identify any changes in the expression of ADAMTS-1, 4 or -5 or their endogenous inhibitor, TIMP-3, in cytokine treated astrocytes and in CNS tissue of rats at various time points following transient focal cerebral ischaemia, compared with sham operated controls. Interleukin (IL)-1β, IL-1 receptor antagonist (IL-1RA) and tumour necrosis factor (TNF) have been previously demonstrated to be

upregulated following MCAo in the rat model [4], [20] and we have therefore included these genes in the study to confirm the extent of ischaemia and to allow comparison with ADAMTS and TIMP-3 expression.

2. Materials and methods

Surgical procedure

All experiments were performed on male Sprague–Dawley rats (290–320 g; Charles River, U.K.) under halothane anaesthesia [Fluothane, Zeneca, U.K., 1–2% in a mixture of oxygen and nitrous oxide (1:2 ratio)]. Body temperature was maintained (37.0±0.5°C) throughout anaesthesia by means of a homeothermic blanket (Harvard Apparatus, U.K.). All surgical procedures were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act (1986).

Transient focal cerebral ischaemia was induced in rats by intraluminal thread occlusion of the middle cerebral artery (tMCAo), as described previously [24]. The thread was withdrawn to allow reperfusion at 90 mins post-occlusion. Sham-operated animals were exposed to the same surgical procedure, with the exception that the thread was inserted only briefly and not to its full extent. The animals were then killed at different time points following tMCAo and the brains removed and frozen on dry ice, prior to subsequent analysis. Numbers of animals included in each group were as follows: 2 animals for 6 hour sham operated and 4 animals for 6 hour post tMCAo, 4 animals for each of 24 hour sham operated and 24 hours post tMCAo, and 3 animals for each of 5 day sham operated and 5 days post tMCAo groups.

Tissue preparation and analysis

Brains were dissected into ipsilateral and contralateral hemispheres and each hemisphere was cut approximately in half again coronally. Sections of each hemisphere (10µm) were cut and stained with Toluidine blue to identify the ischemic areas by microscopical examination.

Astrocyte culture

All cell culture reagents were from Invitrogen, Paisley, Scotland, unless otherwise stated. Human primary astrocyte cells (a kind gift from I. Romero, Milton Keynes, UK) were cultured in Hams F10/MEM 1:1 supplemented with 10% FCS, 1% human serum, 1% amphotericin B and 0.1% plasmocin (prophylactic) (Autogen Bioclear, Wiltshire UK). All cell culture media contained 100U/ml penicillin and 100μg/ml streptomycin and were maintained at 37°C in 5% CO_2/95% air in a humid environment. Cells were trypsinised and seeded into 24-well plates at a density of $1x10^5$/well (1ml medium/ well) and allowed to adhere for 24 hours. The cells were treated with either IL-1β or TNF (recombinant cytokines; Peprotech, UK) in triplicate at 0, 1, 10, or 100ng/ml for 24 hours in serum-free medium. Each experiment was repeated 5 times.

RNA/ protein extraction

5 x 10μm sections of each quarter of brain tissue from each animal were taken and homogenised in 1ml of Tri-reagent (Sigma, UK), followed by vortex mixing and centrifugation at 12,000 x g for 15 minutes at 4°C to remove lipids. The supernatant was transferred to a clean microfuge tube and the RNA and protein were extracted following the instructions provided by the manufacturer. RNA and protein was extracted from triplicate wells of astrocytes into 1ml of Tri-reagent following the manufacturer's instructions. Samples were coded and stored at -80°C prior to use, when they were analysed in blinded experiments.

Real time PCR (qRT-PCR)

RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Paisley, Scotland) 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). PCR primers for ADAMTS-1, -4, -5, TIMP-3 and GAPDH were designed using Primer Express software (Applied Biosystems) and obtained from 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 for ADAMTS-1, -4, -5, TIMP-3 and GAPDH are shown in Table 1. 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 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. Primers and FAM labelled probes were used for determination of IL-1β, IL-1ra and TNF expression levels (Applied Biosystems, assay numbers: Rn00580432-m1, Rn00573488-m1, Rn00562055 respectively). Expression of ADAMTS-1, -4, -5, TIMP-3, IL-1β, IL-1RA and TNF was normalised against expression of the housekeeping gene GAPDH. Relative mRNA levels of ADAMTS-1, -4, -5, TIMP-3, IL-1β, TNF, and IL-1ra were determined using the formula $2^{-\Delta CT}$ where ΔCT = CT (target gene) – CT (GAPDH). Relative quantification of targets from cytokine stimulated cells compared to untreated cells was calculated by the comparative cycle threshold method, using the formula $2^{-\Delta\Delta CT}$ where $\Delta\Delta CT = \Delta CT$ (stimulated cells) – ΔCT (unstimulated cells) (Applied Biosystems recommendations).

Western blotting

Western blot analyses of ADAMTS-1, -4 and -5 were carried out as previously described [10] using polyclonal antibodies for C-terminal epitopes of ADAMTS-1, -4 and -5 (Santa Cruz Biotechnologies Inc., USA). Anti -ADAMTS-1 and ADAMTS-4 are goat polyclonal antibodies raised against a peptide mapping near the C terminus of the human protein.

Briefly, 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, Blots were blocked with 5% non-fat milk in Tris-buffered saline containing 0.05% Tween 20 (Sigma, UK) (TBST), washed 3 x 10 min in TBST and the primary antibody was added at 1: 5000 dilution in TBST containing 5% non-fat milk. The blots were incubated overnight at 4^{o}C and washed as described above, then incubated with rabbit anti-goat IgG horseradish peroxidase (HRP) conjugate (Sigma, UK), 1:80000 in TBST for 2 hours at ambient temperature with gentle shaking. Blots were then washed again and developed using the ECL Plus chemiluminescence substrate (Amersham, UK). Peptide blocking experiments were performed on the antibodies using the commercially available peptides to ensure no non-specific binding of the secondary antibody. Detection of actin using an anti-actin antibody (Abcam, UK) was included as loading control in all the gels. 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.

Data Analysis

Comparison of the mRNA levels of ADAMTSs, TIMP-3, IL-1β, IL-1ra and TNF between contralateral and ipsilateral hemispheres was performed using a paired Students t-test. For the cell culture experiments, statistical significance of any increase or decrease in expression of a particular target was determined by a one-sample t-test of the mean. $P<0.05$ was considered to be significant. All bands from western blots were compared to the sham operated contralateral hemisphere which was normalised to 1. Mean optical density values of repeated experiments were calculated and statistical significance of any observed differences were calculated using t- tests.

Immunocytochemistry

Primary astrocytes were plated out into chamber slides at a density of 2×10^5 cells per well and were allowed to adhere for 24 hours. The slides were fixed in ice-cold acetone for 10 minutes, air-dried and incubated in the primary rabbit polyconal antibody against ADAMTS -1, -4, -5 (Triple Point Biologics, USA, 1:50) or glial fibrillary acidic protein (GFAP) as the control (Chemicon International, UK) (1:1000) overnight at 4°C. Cells were then washed in PBS, and incubated with goat anti-rabbit IgG alexa 488 (Molecular Probes, 1:500) for 1 hour at room temperature. Non-specific staining was determined by omission of the primary antibody and no staining of the tissue was observed. The nuclei were counterstained with propidium iodide (Dako, UK). Immunofluorescent images were captured using a Zeiss 510 confocal scanning laser microscope equipped with a krypton/argon laser. Fluorophores were excited at wavelengths of 488nm and 568nm.

3. Results

ADAMTS-1, -4 -5 and TIMP-3 mRNA were all constitutively expressed in normal and stroke tissue (Figure 1). ADAMTS-1 and -4 was found to be constitutively expressed by western blotting in sham operated and MCAo tissue however using a number of commercially antibodies available we were unable to detect ADAMTS-5 by western blotting (Figure 2). Basal expression of ADAMTS-4 mRNA was higher than that of ADAMTS-1 and -5 whereas TIMP-3 was expressed higher levels than the ADAMTSs. ADAMTS-1 mRNA expression was significantly increased in the ipsilateral hemisphere of animals 6 and 24 hours post tMCAo (Figure 1a) compared to the contralateral hemisphere (mean 4.2 and 6.6 - fold increase respectively compared to contralateral hemisphere). The level of ADAMTS-1 mRNA expression showed no significant difference between hemispheres of sham operated animals and contralateral hemispheres of experimental animals. Protein levels of ADAMTS-1 were unchanged in tMCAo tissue compared to sham operated at 6 and 24 hours (data not shown). At 5 days post tMCAo, ADAMTS-1 mRNA levels were not significantly different to the sham operated or contralateral hemisphere; however, protein levels of ADAMTS-1 were significantly increased in both the ipsilateral and contralateral hemispheres of tMCAo animals compared to sham operated animals (Figure 2a and d). ADAMTS-1 western blots showed bands at approximately 95 and 100kDa.

ADAMTS-4 mRNA was also significantly increased in the ipsilateral hemisphere (Figure 1b), at 6 and 24 hours following tMCAo, (mean 1.8 and 2.7-fold increase respectively, compared to the contralateral hemisphere). ADAMTS-4 expression was similar in sham operated and contralateral hemispheres of experimental animals. Protein levels of ADAMTS-4 were unchanged in tMCAo tissue compared to sham

operated at 6 and 24 hours (data not shown). At 5 days post MCAo, ADAMTS-4 mRNA levels were not significantly different to the sham operated or contralateral hemisphere whereas ADAMTS-4 protein levels were significantly increased in the ipsilateral hemisphere of the tMCAo brains compared with contralateral hemisphere and sham operated animals (Figure 2b and e). Bands on western blots were observed at approximately 51, 65-70, 85 and 100-110 kDa for the different processed forms of the enzyme.

ADAMTS-5 mRNA showed a small increase in the ipsilateral hemisphere 6 hours post MCAo (1.4 fold compared to contralateral hemisphere) but was not significantly different from sham operated animals (Figure 1c). There was also no significant difference between ipsilateral and contralateral hemispheres, 24 hours post stroke or compared to the sham operated group at this time point. However, at 5 days post MCAo there was a small but significant increase in the ipsilateral hemispheres compared to the sham operated animals but no difference between ipsilateral and contralateral hemispheres (Figure 1c).

CNS TIMP-3 expression levels were not significantly different between MCAo and control animals at any of the time points tested (Figure 1d).

IL-1β, IL-1RA and TNF have all previously been shown to be increased in stroke [4], [16], [20]. We therefore examined the expression of these genes as evidence of the ischaemic event. Increased expression of IL-1β, (mean 34-fold increase) IL-1RA (mean 15-fold increase) and TNF (mean 3-fold increase) was observed in the

ipsilateral tissue compared to contralateral stroke tissue at 24 hours after tMCAo (Figure 3).

Using immunocytochemistry, primary human astrocytes *in vitro* were shown to constitutively express ADAMTS-1, -4 and -5 proteins. ADAMTS-1 appeared to show the highest expression levels and ADAMTS-4 and -5 staining was much weaker (Figure 4). Real time PCR experiments showed that IL-1β treatment did not significantly alter expression levels of ADAMTS-1, -5 or TIMP-3 in astrocytes, although significantly increased ADAMTS-4 expression at 1ng/ml (mean of 3.5 fold increase) (Figure 5a). No significant changes were seen at 10 and 100ng/ml IL-1β. Western blotting experiments showed no significant differences in ADAMTS-1, -4, -5 or TIMP-3 between control and IL-1β stimulated cells (data not shown). ADAMTS-1 (mean of 3-fold increase at 100ng/ml TNF) and ADAMTS-4 (mean of 9-fold increase at 100ng/ml TNF) expression levels however, were significantly increased following TNF treatment, with no effect on the levels of ADAMTS-5 or TIMP-3 (Figure 5b). The increase in ADAMTS-4 mRNA in TNF treated astrocytes was mirrored in western blotting experiments, although a significant increase in ADAMTS-1 was not observed at the protein level. ADAMTS-5 protein was significantly increased following TNF stimulation and TIMP-3 levels remained unchanged (Figures 6 and 7).

4. Discussion

CNS trauma and stroke lead to a reactive gliosis characterised by increased GFAP expression and hypertrophy of astrocytes [29]. This is accompanied by alterations to many brain ECM components including an increase in CSPGs [9], [38]. ADAMTSs specifically degrade CSPGs and an increase in degradation of ECM components may aid recovery by removal of the CSPGs that are inhibitory to neurite outgrowth. Conversely, increased ADAMTS mediated CSPG degradation may potentiate brain injury and enable infiltration of inflammatory cells [14], [33].

These results demonstrate that ADAMTS-1 and -4 mRNA expression is significantly increased in the ipsilateral hemisphere compared to either the contralateral hemisphere or to brain tissue from sham operated rats at 6 hours and is further increased at 24 hours post tMCAo. This increase then returns to control levels by 5 days post tMCAo. TIMP-3 mRNA expression levels remained unchanged at each of the time points tested. Protein levels of ADAMTS-1 and -4 were also upregulated in tMCAo although this was only observed at 5 days post procedure. Interestingly, ADAMTS-1 protein was upregulated in both hemispheres of the tMCAo brains at 5 days post procedure whereas ADAMTS-4 was only upregulated in the ipsilateral hemisphere. This indicates that there may be different control mechanisms between different ADAMTSs. The unchanged TIMP-3 levels following stroke is in contrast to experimental autoimmune encephalomyelitis where we demonstrated a significant decrease in TIMP-3 mRNA at the peak of the disease [30].

IL-1β has previously been shown to increase ADAMTS expression in other systems such as a murine colon carcinoma cell line [17] and in human tendon cells [42]. The increase in cytokine expression following stroke has previously been documented by

others [4], [20], [27], [36]. Levels of IL-1β mRNA were 34 times higher in ipsilateral hemisphere compared to the contralateral hemisphere in the present study. Clausen et al, [7] have also reported a similar fold increase in IL-1β mRNA levels 24 hours after MCAo in a mouse model and further demonstrated a dramatic decline in the relative levels of IL-1β mRNA in the ischaemic hemisphere after 5 days post MCAo. Our results also demonstrated a 15-fold increase in IL-1RA mRNA levels in ipsilateral compared to contralateral stroke tissue which was previously demonstrated to be increased by Legos *et al*, [20] following focal ischaemia in the rat. IL-1β has been shown to play an important role in ischaemic brain injury, where there is a reduction in ischemic cell death in rodents when IL-1ra is administered [24]. We have also shown a significant (3-fold) increase in TNF in ipsilateral tissue compared to contralateral, 24 hours after MCAo.

We have demonstrated the expression of ADAMTS-1, -4 and -5 by primary human astrocytes *in vitro* and have shown an increase in ADAMTS-4 mRNA and protein expression in response to TNF treatment. In contrast, IL-1 did not significantly alter expression of ADAMTS-1 mRNA or protein by astrocytes and only increased ADAMTS-4 mRNA expression at 1ng/ml, the lowest concentration used. IL-1 has been shown to increase ADAMTS-1 expression in other systems [17], suggesting a differential cell type specific response. These findings support evidence for TNF induced up-regulation of ADAMTS-4 expression by astrocytes *in vivo* following stroke.

It remains to be determined whether the increase in ADAMTS-1 and -4 is beneficial or detrimental to the process of regeneration of function following stroke but as this becomes more clearly defined, new therapeutic targets may emerge.

Figure legends

Figure 1. Relative mRNA expression of ADAMTS-1 (a), ADAMTS-4 (b), ADAMTS-5 (c) and TIMP-3 (d) at 6, 24 and 120 hours post tMCAo and in sham operated rat brain measured by real time PCR. Data are represented as mean +/- standard error of the mean from contralateral (CL), ipsilateral (IL) and sham operated hemispheres. * denotes significant increase ($P<0.05$) ** ($P<0.01$) between data using a paired T test.

Figure 2. Protein samples from rat brain (6µg/well) were analysed using ADAMTS-1 and -4 antibodies. A representative western blot of sham operated rat brain and MCAo, 5 days post procedure for ADAMTS-1 (a) and -4 (b). Blots show two animals for each contralateral (CL) and ipsilateral (IL) hemispheres in sham operated and MCAo brain. The actin control for the representative samples is also shown (c). Densitometric analysis is shown for the mean of 3 animals in each group +/- SEM (pooled analysis of molecular weight bands) for ADAMTS-1 (d) and -4 (e) expression. * represents a significant increase in expression $P<0.05$.

Figure 3. Relative mRNA expression of IL-1β (a), IL-1RA (b) and TNF (c) in sham operated rat brain and 24 hours after tMCAo measured by real time PCR. Data are represented as mean +/- standard error of the mean of 4 repeat experiments. CNS tissue from sham operated rats is numbered 1-4 and tissue from tMCAo, numbered 5-8, left side (L) and right (occluded) side (R). * denotes significant increase ($P<0.05$) compared to contralateral hemisphere using a paired T test.

Figure 4 Immunocytochemistry on primary human astrocytes. Cells were plated into chamber slides at a density of $2x10^5$ cells per well and allowed to adhere for 24 hours. Cells were incubated with primary antibodies against GFAP (a), ADAMTS-1 (b), ADAMTS-4 (c) or ADAMTS-5 (d) followed by goat anti-rabbit IgG alexa 488 secondary antibody (green). Nuclei were counterstained using propidium Iodide (red). Bar represents 50μM.

Figure 5 mRNA expression of ADAMTS-1, -4, -5 and TIMP-3 in primary human astrocytes by real time PCR following treatment with either IL-1β (a) or TNF (b) for 24 hours. Data is expressed as fold increase compared to control unstimulated mRNA levels. * denotes significant increase ($P<0.05$) between control and cytokine treated cells using a one sample t test.

Figure 6 Protein expression of ADAMTS-1 (a), -4 (b), -5 (c) and TIMP-3 (d) in TNF stimulated primary human astrocytes by western blotting. A representative blot is shown for each. Lanes 1: Control, 2: TNF 1ng/ml, 3: TNF 10 ng/ml, 4: TNF 100ng/ml.

Figure 7 Densitometry on western blots from TNF stimulated primary human astrocytes for ADAMTS-1 (a), -4 (b), -5 (c) and TIMP-3 (d). * indicates significant difference compared to control ($P<0.05$).

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