Impaired fibrinolysis in multiple sclerosis: a role for tissue plasminogen activator inhibitors

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Summary

Tissue plasminogen activator (tPA), a neuronal as well as the key fibrinolytic enzyme, is found concentrated on demyelinated axons in multiple sclerosis lesions together with fibrin(ogen) deposits. The decreased tPA activity in normal-appearing white and grey matter and lesions of multiple sclerosis is reflected in diminished fibrinolysis as measured by a clot lysis assay. Nonetheless, peptide products of fibrin, including D-dimer, accumulate on demyelinated axons—the result of fibrinogen entry through a compromised blood-brain barrier (BBB). Analysis of tissue samples on reducing and non-reducing polyacrylamide gels demonstrates complexes of tPA with plasminogen activator inhibitor-1 (PAI-1) but not with neuroserpin, a tPA-specific inhibitor concentrated in grey matter. As total tPA protein remains unchanged in acute lesions and the concentration of PAI-1 rises several fold, complex formation is a probable cause of the impaired fibrinolysis. Although the tPA-plasmin cascade promotes neurodegeneration in excitotoxin-induced neuronal death, in inflammatory conditions with BBB disruption it has been demonstrated to have a protective role in removing fibrin, which exacerbates axonal injury. The impaired fibrinolytic capacity resulting from increased PAI-1 synthesis and complex formation with tPA, which is detectable prior to lesion formation, therefore has the potential to contribute to axonal damage in multiple sclerosis.

Keywords: tPA; fibrinolysis; D-dimer; neuroserpin; multiple sclerosis

Abbreviations: BBB = blood-brain barrier; ELISA = enzyme-linked immunosorbent assay; NAWM = normal-appearing white matter; NAGM = normal-appearing grey matter; NCGM = normal control grey matter; NCWM = normal control white matter; PAI = plasminogen activator inhibitor; PMA = phorbol myristate acetate; SDS–PAGE = sodium dodecyl sulphate–polyacrylamide gel electrophoresis; tPA = tissue plasminogen activator

Introduction

While fibrinogen is largely undetectable in normal CNS tissue, in neuroinflammatory disorders such as multiple sclerosis vascular changes preceding the cellular inflammatory reaction are accompanied by entry into the CNS of plasma components (Lucchinetti et al., 2001). Persistent blood-brain barrier (BBB) damage and fibrin exudation are prominent features of demyelinating lesions (Claudio et al., 1995) and similarly precede the clinical manifestation of experimental allergic encephalomyelitis (EAE), an animal model of multiple sclerosis (Inoue et al., 1996). Studies in peripheral nerve injury have shown that fibrin deposition is a factor impeding axonal regeneration whilst removal of fibrin is associated with restoration of axonal function (Akassoglou et al., 2000). Plasminogen activators are central to degrading fibrin and extracellular matrix proteins in the CNS, dissolving adhesive contacts to promote synaptic contact and axonal outgrowth (Lo et al., 2002).

The proteolytic degradation of the fibrin mesh generates soluble fibrin fragments of which D-dimer, a marker of turnover of cross-linked fibrin, provides the means of detecting and quantifying fibrinolysis (Matsuo et al., 2000). Tissue plasminogen activator (tPA) is the key fibrinolytic enzyme, the activation rate of which is greatly enhanced in the presence of fibrin (Chandler et al., 2000). tPA is also present at a high concentration in neurons, where upon activation, it has been found to have a role in neuronal development and synaptic remodelling (Calabresi et al., 2000). In neurodegenerative diseases, disruption of neuronal cell links with the extracellular matrix by tPA-generated plasmin is a mechanism of neuronal cell death (Strickland,
In the chronic inflammation characteristic of multiple sclerosis, co-localization of tPA to demyelinated axons with non-phosphorylated neurofilament and fibrin(ogen) suggest an association with axonal damage (Gveric et al., 2001). Alternatively, it may represent a protective mechanism to remove fibrin deposits, which exacerbate axonal injury as reported in the model of sciatic nerve damage (Akassoglou et al., 2000) and promote regeneration by activation of growth factors (Siconolfi and Seeds, 2001).

tPA forms 1:1 stable reversible complexes with the high affinity physiological serine protease inhibitor PAI-1 (plasminogen activator inhibitor 1), which are cleared through internalization by macrophages (Vanmeijer and Pannekoek, 1995). Although the production of PAI-1 increases significantly during lesion development, tPA always remains higher suggesting that a fraction of tPA may remain free from inhibition and thus capable of mediating fibrin degradation (Gveric et al., 2001). In addition to PAIs, the brain-specific inhibitor, neuroserpin, which reacts preferentially with tPA, is concentrated in neuronal cell bodies and axons (Hastings et al., 1997). The co-expression of neuroserpin and tPA in the same regions of the brain suggest it is a likely regulator of tPA activity in vivo, contributing to neuronal plasticity and learning. Furthermore, as a neuronal survival factor, the increased local expression of neuroserpin following ischaemia may represent an innate protective response to elevated tPA levels (Yepes et al., 2000). As the clearance of fibrin from demyelinating axons in multiple sclerosis may have important functional implications, we have investigated the relationship of the tPA inhibitors PAI-1 and neuroserpin to the fibrinolytic potential in lesions and normal-appearing tissue in multiple sclerosis.

### Material and methods

#### Tissue and cell lines

Post-mortem snap-frozen CNS tissue samples from 20 cases diagnosed clinically and histopathologically as multiple sclerosis and 15 normal control cases were obtained from the Neuroresource Tissue Bank, Institute of Neurology, London, UK. All multiple sclerosis cases were classified as secondary progressive multiple sclerosis with a characteristic relapsing-remitting course and increasing disability. The average age, sex ratio, post-mortem time and disease duration for normal control and multiple sclerosis cases are given in Table 1. Cause of death in the normal control category included heart failure (six cases), myocardial infarction (four), bronchopneumonia (two), pulmonary embolism (one), liver carcinoma (one) and renal failure (one), whereas the causes of death for the multiple sclerosis cases were bronchopneumonia (14), septicemia (three), renal failure (one), heart failure (one) and bowel carcinoma (one). Multiple sclerosis lesions were classified according to criteria developed by Li et al., (1993). In total, 58 snap-frozen blocks (0.5–1 cm³) of brain and spinal cord containing lesions and/or macroscopically normal appearing white matter (NAWM) and normal appearing grey matter (NAGM) from multiple sclerosis cases and 19 blocks of white and grey matter from normal control cases were homogenized for protein extraction. Prior to the extraction, 10 μm cryostat sections were cut from each block for histological screening and immunohistochemistry.

The THP-1 cell line was propagated in suspension in RPMI-64 medium supplemented with 10% fetal calf serum and 2 mM glutamine. Cells were treated with 10 ng/ml of phorbol 12-myristate 13-acetate (PMA, Sigma, Poole, UK) for 3 days, which resulted in the occurrence of macrophage-like properties and adherence. Cell lysates were prepared from both PMA-treated and non-treated cells as described for the human CNS samples. Lysates were analysed for neuroserpin content by western blotting.

#### Immunohistochemistry

Sections were immunoperoxidase stained with antibodies directed against tPA (1:100, Monozyme, Glostrup, Denmark), PAI-1 (1:100, Biopool, Umea, Sweden), neuroserpin [1:10 000, (Hastings et al., 1997)], fibrin D-dimer (1:500, Sigma), EBM-11 (microglia and macrophages, 1:100, Dako), anti-collagen IV (1:500, Dako, Glostrup, Denmark) and SMI-32 (neurofilament-H, 1:5000, Sternberger Monoclonals, Lutherville, MA, USA). Cryostat sections were fixed in methanol (−20°C, 10 min), incubated with primary antibodies overnight at 4°C and stained using a three-step peroxidase method (Gveric et al., 1999). Omission of primary antibodies and the application of affinity purified rabbit (Dako), goat (Sigma) and mouse IgG (Sigma) were used as controls at the same protein concentrations as the appropriate primary antibodies.

#### Western blotting and protein extraction

Snap-frozen blocks of brain and spinal cord from multiple sclerosis and normal control cases, weighing between 0.5 and

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Mean age in years (range)</th>
<th>Post-mortem time in hours (range)</th>
<th>Multiple sclerosis duration in years (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control (n = 15; male:female = 12:3)</td>
<td>64 (34–80)</td>
<td>26 (10–52)</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Multiple sclerosis (n = 20; male:female = 9:11)</td>
<td>57 (29–77)</td>
<td>23 (4–64)</td>
<td>23 (8–43)</td>
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Table 1: Summary of clinical data for normal control and multiple sclerosis cases
1 g wet weight, were finely cut and homogenized in a Tris–HCl buffer (100 mM Tris, pH 8.1 with 1% Triton X-100) (Padro et al., 1994) by sonication. Tissue suspensions were spun at 20 000 g for 45 min at 4°C and the supernatants collected and stored at −70°C. Protein concentrations were determined by the Lowry method.

For western blot analysis of tPA, PAI-1, neuroserpin and D-dimer content, 40 μg of supernatant protein was resolved by electrophoresis on a 10% sodium dodecyl sulphate-polyacrylamide gel (SDS–PAGE) with recombinant tPA, PAI-1 (Technoclone, Vienna, Austria) and neuroserpin (E.coli extract, Hastings et al., 1997) as protein standards. Gels were transferred overnight to Immobilon-P polyvinylidene difluoride membranes (Kingham et al., 1999). The membranes were stripped with 100 mM glycine and probed with anti-actin antibody (1:1000, Sigma). Specificity of immunostaining was ensured by omitting or replacing the primary antibodies with appropriate species-specific affinity purified IgG fractions. Non-reducing SDS–PAGE was employed to visualize tPA/PAI-1 and tPA/neuroserpin complexes as well as D-dimer (Hastings et al., 1997) in normal control and multiple sclerosis samples. tPA and PAI-1 standards were mixed in equimolar amounts and pre-incubated for 60 min at 37°C in order to obtain complexes (Simon et al., 1995), which were used as standards for non-reducing western blots.

**Enzyme-linked immunosorbent assays (ELISA)**

Commercially available ELISA for D-dimer (Biopool), tPA (Biopool) and PAI-1 (American Diagnostica, Greenwich, CN, USA) and activity assays for PAI-1 (Biopool) and plasminogen (Technoclone) were performed according to the manufacturers’ instructions. Protein concentrations of D-dimer, tPA and PAI-1 were expressed in ng/mg of protein, whilst the activity of PAI-1 was expressed in U/mg of protein. The plasminogen activity assay measures the amount of activatable plasminogen and the results are given as a percentage of activatable plasminogen in the plasma standard.

**Clot lysis assay**

A clot lysis assay was used to evaluate fibrinolytic activity in the CNS tissue extracts (Urano et al., 1996). The turbidity assay relies on the degradation of an in vitro formed fibrin clot in the presence of plasminogen activators and plasminogen, resulting in a decrease in absorbance. Tissue extracts were premixed at 1:10 with 50 mM Tris–HCl buffer, pH7.4, containing 7.3 μM human fibrinogen (Sigma), 0.25 μM human lys-plasminogen (Chromogenix, Milan, Italy), 1.7 mM CaCl2, 0.7 mM MgCl2, and 12.5 mM NaCl. Human tPA (2 mg/ml, Technoclone) was used as a positive control, whilst omission of sample or plasminogen in the buffer was used as a negative control. In addition, a selection of samples were pre-incubated for 30 min at room temperature with the serine protease inhibitor Pefablock SC (1 mM, Roche Diagnostics, Mannheim, Germany). Samples were added in duplicates to 96-well microtitre plates with 20 μl of human thrombin (100 U/ml, Sigma) and incubated at 37°C. Absorbance was measured at 405 nm after the turbidity had reached a stable level, in 30 min intervals over 360 min. The absorbance data were plotted against the reaction time, and the clot lysis time (t1/2) was determined as the average of the maximum and minimum absorbance values (Urano et al., 1998). In addition, data are presented as a percentage of decrease in sample opacity at the midpoint of incubation (180 min), which is proportional to the amount of degraded clot.

**Statistical analysis**

Statistical analysis was carried out using Fisher’s exact test suitable for a small sample size with a significance level set at P < 0.05 (SPSS software, SPSS Inc., Chicago, IL, USA). The non-parametric Spearman rank correlation test was used for the regression analysis and the effects of age and post-mortem time were checked for all data. Partial correlation correcting for these effects was carried out when indicated and the r value of the Spearman rank test given where appropriate. All results are presented as mean ng or U per mg of protein ± SD.

**Results**

**Fibrinolyis in multiple sclerosis lesions**

The clot lysis assay was used to measure the net fibrinolytic activity in control and multiple sclerosis samples (Fig. 1A–C). The highest fibrinolytic activity was detected in normal control white matter (NCWM) and normal control grey matter (NCGM) with t1/2 of 99.82 ± 30.87 min and 70.80 ± 10.31 min, respectively (Fig. 1B). The percentage decrease in sample opacity, which reflected the amount of degraded fibrin clot, was 51 ± 18% for NCWM and 49 ± 23% for NCGM (Fig. 1C). In contrast, all multiple sclerosis tissue samples were characterized by markedly decreased fibrinolytic activity (Fig. 1A–C). The lowest fibrinolytic activity was observed in acute multiple sclerosis lesions with t1/2 of 255.8 ± 79.54 min and only 13 ± 17% of the clot degraded. Addition of tPA or plasmin to sample buffer resulted in rapid clot degradation within the first hour of incubation, while omission of plasminogen in the sample buffer or pre-treatment of samples with serine protease inhibitor Pefablock inhibited clot lysis (Fig. 1A).

**Fibrin D-dimer**

In the white matter tissue sections from control and multiple sclerosis cases, D-dimer was confined to the lumen and walls of blood vessels, whilst in acute multiple sclerosis lesions, it was immunolocalized on foamy macrophages and large diameter axons (Fig. 2A–D). Immunostaining of serial
sections with antibody directed against neurofilament protein (SMI-32) confirmed the axonal localization of fibrin D-dimer (Fig. 2E and F). Non-reducing western blots of control and multiple sclerosis samples revealed an increase in the 260 kD complex that corresponds to plasmin-generated D-dimer complex (Walker and Nesheim, 1999) and smaller fibrin degradation products in multiple sclerosis tissue samples (Fig. 2G). Increased amounts of D-dimer and fibrin degradation products were also detected by ELISA (Dempfle et al., 2001) in multiple sclerosis tissue, although the difference between controls and multiple sclerosis was not statistically significant (Fig. 3A). These results appear to be at variance with impaired fibrinolysis. However, the clot lysis activity reflects end-point fibrinolytic capacity in samples while the

Fig. 1 Clot lysis assay on normal control and multiple sclerosis tissue samples. (A) Line graphs showing clot lysis curve for three representative samples for each type of tissue and a graph showing clot lysis curve for tPA, normal control sample after omission of plasminogen in sample buffer and normal control sample pre-treated with Pefablock. (B) Clot lysis time ($t_{1/2}$) and (C) percentage of clot degraded for control and multiple sclerosis samples. $n = 6$ for all samples except for AL, SAL and CL where $n = 12$. Bars represent mean $\pm$ 1SD. AL = acute lesion; CL = chronic lesion; SAL = subacute lesion.
D-dimer represents accumulation of fibrin and its degradation products at lesion sites during the chronic inflammatory process.

**PAI-1 and neuroserpin**

In contrast to present (Fig. 3B) and previously reported ELISA data (Gveric et al., 2001), western blotting revealed there was no decrease in tPA protein levels in multiple sclerosis tissue samples nor was there was any evidence of tPA degradation (Fig. 4A). According to ELISA data, however, PAI-1 was increased in multiple sclerosis tissue (Fig. 3B) although, on immunoblots, this appeared to be largely attributable to lower molecular weight PAI-1 peptides (~29–36 kD, Fig. 4A). Non-reducing immunoblots indicated that tPA is complexed with PAI-1 but not with neuroserpin (Fig. 4B). Electrophoresis of tPA and PAI-1 standards alone and as complexes followed by immunostaining of gel blots with the relevant antibodies provided confirmation of complex formation in the appropriate molecular weight range. No PAI-1 inhibitory activity was detected in multiple sclerosis samples, providing further evidence that it is bound to tPA or present as inactive proteolytic fragments.
Neuroserpin immunoreactivity was present predominantly in neuronal cells in control (Fig. 5C) and multiple sclerosis grey matter while, in NCWM (insert in Fig. 5C), some glial cells were positively stained. Activated microglia in NAWM and foamy macrophages in acute multiple sclerosis lesions were positive (Fig. 5D), but there was no evidence of axonal staining in any of the samples regardless of pathology. Both native (52 kD) and cleaved (49 kD) neuroserpin were identified by SDS-PAGE in THP-1 cell extracts (Fig. 5G), in line with the detection of the inhibitor in macrophages in acute multiple sclerosis lesions (Hastings et al., 1997). Furthermore, cytospins of both PMA treated and non-treated THP-1 cells were immunostained positively with anti-neuroserpin antibody (data not shown).

In tissue samples, the highest level of neuroserpin was found in NCGM—predominantly in a native form which appeared to be reduced in NAGM (Fig. 4A). Similar levels of native neuroserpin were found in control and NAWM, but multiple sclerosis lesions were characterized by the absence of native neuroserpin and an increase in smaller molecular weight fragments (Fig. 4A). Non-reducing western blots revealed negligible proteinase-complexed neuroserpin in any of the samples (Fig. 4B).

**Statistical analysis**
Statistically significant correlations were found between the protein concentration of fibrinolytic enzymes and parameters of fibrinolysis in control and multiple sclerosis tissue samples. After correcting for age, a direct correlation was found between D-dimer, the only variable influenced by age or post-mortem time, and plasminogen in control ($r = 0.60, P < 0.05$) and multiple sclerosis samples ($r = 0.46, P < 0.001$) (Fig. 3A). Statistical analysis also revealed a significant negative correlation between clot lysis time ($t_{1/2}$) and tPA protein levels in both control ($r = -0.93, P < 0.001$) and multiple sclerosis tissue samples ($r = -0.73, P < 0.001$). The percentage of clot degradation by tissue samples correlated positively with tPA (control: $r = 0.72, P < 0.001$, multiple sclerosis: $r = 0.72, P < 0.001$), but negatively with PAI-1 (control: $r = -0.83, P < 0.001$, multiple sclerosis: $r = -0.30, P < 0.05$) protein levels.

**Discussion**
The data reported in this study demonstrate the fibrinolytic potential in demyelinating multiple sclerosis lesions to be...
markedly diminished—the result of a documented reduction in tPA enzyme activity and increase in PAI-1 inhibitor levels (Gveric et al., 2001). A statistically significant correlation between t-PA, clot lysis time, plasminogen and D-dimer in multiple sclerosis tissue samples strongly suggests that fibrinolysis is dependent on the plasminolytic activity of tPA. In line with a marked increase in PAI-1, gel analysis of enzyme-inhibitor complex formation suggests that PAI-1, but not neuroserpin, inhibits tPA activity and subsequently the fibrinolytic potential in chronic neuroinflammatory disorders. The clot lysis assay demonstrates a loss of functional fibrinolytic potential in both NAWM and demyelinating lesions—a feature of all inflammatory pathologies with disruption of vascular integrity, in which an imbalance in the tPA:PAI-1 ratio reduces the proportion of active tPA enzyme (Kopeikina et al., 1997). There is MRI evidence of fibrin deposition prior to clinical signs of multiple sclerosis (Kermode et al., 1990) and, immunohistochemically, it precedes the cerebral parenchymal reaction and demyelination (Wakefield et al., 1994). In experimental models of peripheral nerve damage, mice deficient for tPA or plasminogen display aggravated axonal degeneration and delayed functional regeneration, which can be rescued by fibrinogen deficiency (Akassoglou and Strickland, 2002). Fibrin is colocalized on the denuded multiple sclerosis axon with tPA and there is accumulation of high molecular weight fibrin peptides, products of tPA proteolysis. This may represent pockets of residual activity of tPA, as no PAI-1 inhibitory capacity was detected at these sites. In the chronic inflammatory state, this could be seen as an attempt to reduce fibrin...
accumulating on the demyelinated axon and interfering with axonal function. However, the decrease in fibrinolytic potential in multiple sclerosis tissue is not due to a decrease in tPA, but to formation of complexes with inhibitors, most likely PAI-1, as observed in samples and in preformed complexes of tPA and PAI-1 standards electrophoresed without reduction. This was borne out by western blot analysis under reducing conditions, which showed that tPA is a stable enzyme expressed at similar levels in both control and multiple sclerosis tissue. ELISA assays measure only non-complexed active tPA (Gveric et al., 2001)—hence the apparent decrease.

There are several mechanisms that may contribute to a protease-inhibitor imbalance and reduced fibrinolysis in the inflammatory multiple sclerosis lesion. Influx of serum albumin and low density lipoprotein (LDL) (de Sain-van der Velden et al., 2000) and broad spectrum protease inhibitors such as α2-macroglobulin and α1-antitrypsin through a damaged BBB may directly interfere with fibrinolysis. In contrast, the plasminogen activator specific inhibitor PAI-1 is present only as a trace protein in plasma, the biosynthesis of which is rapidly stimulated by pro-inflammatory cytokines such as IL-1β and TNFα (Deng et al., 1996). The interaction of tPA with fibrin-bound PAI-1 causes a dissociation of PAI-1 and the formation of complexes (Vanmeijer and Pannekoek, 1995), which are rapidly internalized by macrophages. Lack of measurable PAI-1 activity and an increase in lower molecular weight fragments in multiple sclerosis tissue confirms uptake of tPA/PAI-1 complexes and intracellular degradation of PAI-1. However, as the tissue level of tPA remains unchanged and that of PAI-1 is increased in multiple sclerosis lesions, turnover of complexes must be balanced by the production of enzyme and inhibitor by locally inflammatory macrophages.

A second candidate for inhibition of tPA in the demyelinating multiple sclerosis lesion is neuroserpin, which is constitutively co-expressed with tPA in grey matter neurons and axons and is a regulator of tPA activity in vivo (Hastings et al., 1997). Predictably, in our study the highest levels were found in NCGM, but expression in white matter may be attributed to axons as well as microglia. In contrast to published data in stroke (Yepes et al., 2000) and dementia (Yazaki et al., 2001), in which increased local production of neuroserpin appears to represent an innate protective response to elevated tPA levels, the expression of neuroserpin in multiple sclerosis white matter and active lesions is reduced—possibly a reflection of axonal damage in the ongoing chronic inflammatory reaction. Furthermore, there is little evidence of neuroserpin–tPA complex formation in either control or multiple sclerosis tissue. This is in agreement with a recent report that, although tPA forms complexes with neuroserpin in vitro, the interactions are short lived, rapidly progressing to cleavage and regeneration of active tPA (Barker-Carlson et al., 2002). In this study, we have also for the first time described expression and upregulation of neuroserpin in vitro in cells of the macrophage lineage and neuroserpin-positive macrophages are demonstrated in acute multiple sclerosis lesions. The latter observation may represent neuroserpin produced by macrophages or alternatively phagocytosed neuroserpin of axonal origin. Nevertheless, there was no evidence of complex formation with tPA in the lesion, indicating that binding sites for PAI-1 in the inflammatory loci, e.g. vitronectin and fibrin, provide the optimum conditions for formation of tPA–PAI-1 complexes (Vanmeijer and Pannekoek, 1995).

The detection of an apparent increase in tPA–PAI-1 complex formation and fibrin degradation peptides in both NAWM and NAGM is the most striking observation of the study. With increasing evidence of axonal damage at the earliest stages of the inflammatory demyelinating reaction in multiple sclerosis, deposition of fibrin and an imbalance in the tPA–PAI-1 ratio in apparently normal tissue would produce a situation in which axonal integrity is compromised. Downstream damaged axons would be restricted in their ability to penetrate the altered extracellular matrix environment and adherent inflammatory cells to attain synaptic contact. The evident dependence of peripheral nerve regeneration on the plasminogen activator system has important implications for neuroprotection in the CNS and specifically for therapeutic approaches in multiple sclerosis to minimize fibrin deposition in the earliest stages of lesion formation.

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