Plasminogen activators in multiple sclerosis lesions
Implications for the inflammatory response and axonal damage

Djordje Gveric,1 Roeland Hanemaaijer,2 Jia Newcombe,1 Natascha A. van Lent,2 Cornelis F. M. Sier3 and M. Louise Cuzner1

1Department of Neurochemistry, Institute of Neurology, University College London, UK, 2Gaubius Laboratory, TNO-PG, Leiden, The Netherlands and 3Department of Molecular Medicine, DIBIT, San Raffaele Scientific Institute, Milan, Italy

Summary
Components of the plasminogen activator (PA) and matrix metalloprotease (MMP) cascade have been characterized in multiple sclerosis lesions by immunohistochemistry, enzyme-linked immunosorbent assay and enzyme activity assays in order to establish a functional role for the enzyme sequence in lesion development. Highly significant quantitative increases in urokinase PA (uPA), urokinase receptor (uPAR) and plasminogen activator inhibitor-1 were detected in acute multiple sclerosis lesions (P < 0.0001) and in uPAR in normal-appearing white matter (P < 0.0001) compared with control tissue. All three proteins were immunolocalized to mononuclear cells in perivascular cuffs and to macrophages in the lesion parenchyma. MMP-9 and the tissue inhibitor of metalloprotease-1 also increased during lesion development but the enzyme was present largely in the inactive pro-form. In contrast to uPA, the concentration and activity of tissue PA (tPA), the most abundant plasminogen activator in normal control brain, were reduced in multiple sclerosis specimens. In acute lesions tPA co-localized with fibrin(ogen) on large diameter axons also stained with SMI-32, an immunohistochemical marker of axonal damage. The uPA–uPAR complex, concentrated on inflammatory cells in the perivascular zone of the evolving lesion, may facilitate cellular infiltration into the CNS which is amplified by MMP-mediated degradation of blood vessel matrix. tPA localization on injured axons may be a marker of axonal damage or represent a protective mechanism aimed at removal of fibrin deposits and restoration of axonal function.

Keywords: axon; ELISA; matrix metalloproteases; multiple sclerosis; plasminogen activators

Abbreviations: APMA = aminophenylmercuric acetate; BBB = blood–brain barrier; ECM = extracellular matrix; ELISA = enzyme-linked immunosorbent assay; MMP = metalloprotease; NAWM = normal-appearing white matter; NC = normal control; PA = plasminogen activator; PAI = plasminogen activator inhibitor; TIMP = tissue inhibitor of metalloproteases; tPA = tissue plasminogen activator; uPA = urokinase plasminogen activator; uPAR = urokinase receptor

Introduction
Activation of serine proteases and matrix metalloproteases (MMPs) in inflammatory CNS lesions in multiple sclerosis is considered central to leucocyte entry through a compromised blood–brain barrier (BBB) and demyelination (Gijbels et al., 1993; Cuzner et al., 1996; Anthony et al., 1997; Leppert et al., 1998).

It is significant that one of the earliest detectable signs of inflammation in multiple sclerosis white matter is increased expression of plasminogen activators (PAs) on mononuclear cells in perivascular cuffs (Cuzner et al., 1996) and urokinase receptor (uPAR) on endothelial cells (Dore-Duffy et al., 1994). Urokinase PA (uPA), through binding to its surface receptor uPAR, induces focal pericellular proteolysis in the presence of ubiquitous plasminogen which in turn may initiate downstream activation of the MMP cascade. Co-expression of MMPs and their physiological tissue inhibitors (TIMPs) is prominent in macrophages and to a lesser extent in reactive astrocytes in demyelinating lesions. A striking loss in the immunostaining of tenascins C and R in the hypercellular zone of macrophages at the edge of active plaques is supportive evidence of serine protease and MMP-mediated proteolysis of the extracellular matrix (ECM) (Gutowski

© Oxford University Press 2001
Plasminogen activators in multiple sclerosis

In addition to its proteolytic role, uPAR interacts with integrins and vitronectin to regulate cell adhesion and migration (Chapman, 1997; May et al., 1998). Plasminogen activator inhibitor-1 (PAI-1), on the other hand, is a ‘molecular switch’ which interferes with the uPAR–vitronectin link, leading to detachment of cells from the substrate (Deng et al., 1996). Thus the induction of mononuclear cell and microglial uPA and uPAR in the multiple sclerosis lesion has the potential to constitute a rate-limiting step in the immunopathogenesis of the disease.

Tissue plasminogen activator (tPA), a fibrinolytic enzyme implicated in the regulation of neuronal activity and synaptic remodelling (Seeds et al., 1996), is widely expressed in neuronal cells in the adult CNS (Pittman, 1985). In neurodegenerative diseases, disruption of neuronal cell links with supporting ECM by tPA-generated plasmin activity is a mechanism of neuronal cell damage (Tsirka, 1997). In multiple sclerosis, a chronic inflammatory CNS disease, tPA protein and activity are increased in the CSF and are significantly higher that those found in acute inflammatory conditions such as encephalitis and meningitis (Akenami et al., 1996). Furthermore, tPA zymogen has been found in vitro to mediate activation of microglia in a cytokine-like fashion (Rogove et al., 1999). A possible role for tPA in CNS inflammation comes from a model of sciatic nerve injury in which the removal of fibrin deposits by tP/Aplasmin protected axons from inflammatory damage (Akassoglou et al., 2000). Therefore, depending on the stimulus and surrounding microenvironment, tPA has the potential to be either a destructive or a beneficial agent in CNS injury.

The aim of the present study was to identify potential sites for specific enzyme inhibition by immunolocalizing and quantitating the components of the plasminogen activation system and MMP cascade in the evolving multiple sclerosis lesion. The significance of these data in a pathological context was tested by assays of the enzyme activity of PAs and MMPs. containing neutral lipids resulting from myelin degradation were demonstrated with oil red-O staining. On the basis of the number and distribution of oil red-O-positive cells, cellularity and the extent of demyelination, multiple sclerosis lesions were classified into acute, subacute and chronic (Li et al., 1993). In total, 56 snap-frozen blocks (0.5–1 cm3) containing lesions and/or macroscopically normal-appearing white (NAWM) and grey matter from multiple sclerosis cases and 19 blocks of white and grey matter from NC cases were homogenized for protein extraction. Prior to the extraction, 10 µm cryostat sections were cut from each block for histological screening and immunohistochemistry.

Antibodies and immunohistochemistry

Sections were immunoperoxidase stained with antibodies directed against tPA (1 : 100; Monozyme, Hoersholm, Denmark), uPA (1 : 100; Biogenesis, Poole, UK), uPAR (1 : 100; Neomarkers, Fremont, Calif., USA), PAI-1 (1 : 50; Biopool, Ventura, Calif., USA), plasminogen (1 : 10 000; Dako, Glostrup, Denmark), CD18 (integrin β2, 1 : 500; Neomarkers), CD29 (integrin β1, 1 : 1000; Neomarkers), fibrinogen (1 : 20 000; Sigma, Poole, UK) and SMI-32 (non-phosphorylated neurofilament, 1 : 5000; Sternberger Monoclonals, Lutherville, Md., USA). Monoclonal antibodies used to distinguish glial and immune cell populations and to grade multiple sclerosis lesions included: EBM-11 (microglia and macrophages, 1 : 100; Dako), anti-gliaal fibrillary acidic protein (astrocytes, 1 : 1000; Newcombe et al., 1986), 14E (oligodendrocytes and reactive astrocytes, 1 : 20; Newcombe et al., 1992), anti-galactocerebroside (1 : 20; Wolswijk, 1998), anti-myelin basic protein (1 : 200; Serotec, Oxford, UK), SMI-312 (neurofilaments, 1 : 5000; Sternberger Monoclonals), anti-collagen IV (1 : 500; Dako) and anti-Collage1D2 (T-cells, 1 : 500; Dako). Cryostat sections were fixed in methanol (–20°C, 10 min), incubated with primary antibodies overnight (4°C, ~16 h) and stained using a three-step peroxidase method as described by Gveric and colleagues (Gveric et al., 1999). Double immunohistochemical staining was performed using rhodamine-labelled goat anti-mouse IgG (Sigma) for tPA and SMI-32, and biotinylated horse anti-goat IgG (Vector Laboratories, Peterborough, UK) followed by fluorescein isothiocyanate-labelled avidin (Vector) for fibrinogen. Routine immunohistochemical controls included omission of primary antibodies as well as the application of normal rabbit (Dako) and goat (Vector) serum and mouse IgG1 (Sigma) at the same concentrations as primary antibodies.

Material and methods

Tissue

Post-mortem snap-frozen CNS tissue samples from 14 cases diagnosed clinically and histopathologically as multiple sclerosis and 11 NC (normal control) cases were obtained from the Multiple Sclerosis Society Tissue Bank, London. All multiple sclerosis cases were classified as secondary progressive multiple sclerosis with characteristic relapsing–remitting course and increasing disability. The average age, post-mortem time, disease duration and sex ratio for NC and multiple sclerosis cases are given in Table 1. Causes of death in the NC category included myocardial infarction (six cases), road traffic accident (four) and aortic rupture (one), whereas the causes of death for the multiple sclerosis cases were bronchopneumonia (13) and respiratory failure (one). Inflammatory cuffs in multiple sclerosis lesions were visualized with haematoxylin and eosin, whilst macrophages

Protein extraction

Snap-frozen blocks of brain and spinal cord from multiple sclerosis and NC cases, weighing between 0.5 and 1 g wet weight, were finely cut and resuspended at 1 : 10 g/ml in a Tris–HCl buffer (100 mM Tris, pH 8.1 with 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml...
aprotinin) (Padro et al., 1994). Samples were homogenized in wet ice by sonication (25 s), triturated three times through 19- and 21-gauge syringe needles and incubated on ice for 30 min. The 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.

**Western blotting**

For Western blot analysis of tPA and vitronectin content, 40 µg of supernatant protein was resolved on a 12% sodium dodecyl sulphate–polyacrylamide gel and transferred overnight to an Immobilon-P polyvinylidene difluoride membrane (Kingham and Pocock, 2000). The membrane was blocked with 5% dried milk in Tris-buffered saline (T-TBS: 10 mM Tris–HCl, pH 7.4, 150 mM NaCl and 0.1% Tween-20) for 1 h at room temperature and then incubated with anti-tPA (1 : 500; Monozyme) or anti-vitronectin (1 : 100; Chemicon) antibody for 2 h at room temperature. After washing in T-TBS, the membrane was incubated with goat anti-mouse IgG antibody conjugated to horseradish peroxidase (1 : 1000; Sigma) for 2 h at room temperature. After three final washes, the blots were developed by enhanced chemiluminescence. To ensure equal loading of protein, the membranes were stripped with 100 mM glycine and probed with anti-actin (1 : 1000; Monozyme) antibody conjugated to horseradish peroxidase (1 : 1000; Sigma) for 1 h at room temperature. After washing, the blots were developed by enhanced chemiluminescence. To ensure equal loading of protein, the membranes were stripped with 100 mM glycine and probed with anti-actin antibody (1 : 1000; Sigma). Membranes were scanned with a Bio-Rad densitometer and levels of tPA, vitronectin and actin semiquantified using Molecular Analyst software. Results were expressed as the ratio of relative density units of the vitronectin and actin bands.

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

Commercially available ELISAs for tPA (Biopool, Umea, Sweden), uPAR, PAI-1 (American Diagnostica, Greenwich, USA), TIMP-1, latent MMP-9 and MMP-3 (Amersham Pharmacia, Little Chalfont, UK) and activity assays for tPA and plasminogen (Technoclone, Vienna, Austria) were performed according to the manufacturers’ instructions. To assess the specificity of tPA activity, protein extracts were pre-incubated for 15 min at room temperature with tPA-STOP (American Diagnostica) at a final concentration of 3 nM or with an inhibitory anti-tPA antibody (5 µg; Monozyme; Nielsen et al., 1983).

ELISA for uPA (Koolwijk et al., 1996) and total MMP-9 (Hanemaaijer et al., 1998) and activity assays for MMP-2 and -9 (Hanemaaijer et al., 1998; Capper et al., 1999) were performed as previously reported, and all measurements were made in duplicate. PA and MMP protein concentrations were expressed in ng/mg of protein whilst tPA, MMP-2 and -9 activities were 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 generated plasminogen activity of the standard.

For determination of total MMP-2 in tissue extracts, microtitre plates (COSTAR 2588, High Wycombe, UK) were coated with 100 µl per well of sheep anti-MMP-2 antibody (5 µg/ml, The Binding Site, Birmingham, UK) in PBS (phosphate-buffered saline) overnight at 4°C. After washing four times with PBS containing 0.05% Tween-20 and 10 mM EDTA (ethylenediamine tetraacetic acid) (PBS-T/EDTA), tissue extracts were diluted 1 : 2 in PBS-T/EDTA containing 0.1% casein (PBS-T/EDTA/C) and incubated overnight at 4°C. Plates were then washed four times with PBS containing 0.05% Tween-20 (PBS-T) and incubated for 1 h at 37°C with 100 µl of rabbit anti-MMP-2 antibody (1 mg/ml; TNO, Leiden, The Netherlands) diluted in PBS-T/EDTA/C. A further four washes were carried out in PBS-T and the plates were incubated for 1 h at 37°C with 100 µl of biotin-labelled goat anti-rabbit antibody (1 : 25 000 in PBS-T/EDTA/C; Dako), followed by incubation for 1 h at 37°C with avidin-labelled horseradish peroxidase (1 : 10 000 in PBS-T/EDTA/C; Pierce, Rockford, Ill., USA). Non-bound conjugate was washed away and the chromogenic substrate 3,3′,5,5′-tetramethyl benzidine in the presence of H2O2 was added. The reaction was stopped at 30 min by addition of 2 M H2SO4 and the absorbance was measured at 450 nm.

**Statistical analysis**

Statistical analysis was carried out using Fisher’s exact test suitable for small sample size with a significance level set at P < 0.05 (SAS software, SAS Institute, 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 included, and the r value of the Spearman rank test given where appropriate. All results are presented as mean ng/mg or µg/mg of...
Results

uPA, uPAR and PAI-1

uPA and its receptor uPAR were largely undetectable in NC white matter whilst in NAWM both antigens were immunolocalized to mononuclear cells in perivascular inflammatory infiltrates (Fig. 1A and C). In the borders and centre of acute multiple sclerosis lesions, uPA and uPAR (Fig. 2C) were found on foamy macrophages. Using an assay which recognizes free and a proportion of PAI-complexed uPA, a highly significant rise in enzyme levels was observed in acute multiple sclerosis lesions compared with NC white matter (Fig. 3), whilst uPAR was significantly increased in both NAWM and acute multiple sclerosis lesions (Fig. 3). The Spearman rank test established a strong correlation between uPAR–uPA (r = 0.527, P = 0.0023) and uPAR–PAI-1 (r = 0.355, P = 0.049) but not between uPA and PAI-1 antigen levels in multiple sclerosis samples. The integrin subunit β2 (CD18), which co-clusters with uPAR on migrating cells, was expressed on mononuclear cells in perivascular inflammatory infiltrates (Fig. 1D) and membranes of foamy macrophages in active lesions (Fig. 2D). However, the β2 subunit was also visualized on resting and activated microglia in NC and NAWM, respectively. On the other hand, the integrin β1 subunit (CD29) was visualized predominantly on blood vessel walls in both NC and multiple sclerosis (data not shown). Vitronectin, a uPAR ligand, was found in blood vessel walls and occasionally in the form of fibrils in the perivascular space and inclusions in macrophages, but was absent from the lesion parenchyma (Fig. 1G). Western blotting revealed a decrease in intact vitronectin and a rise in amounts of lower molecular weight fragments in acute and subacute lesions compared with NC white matter. Although limited numbers of representative samples were assayed, the appearance of breakdown product was not a reflection of longer post-mortem times (data not shown).

PAI-1, the main inhibitor of PAs, was expressed at low levels in NC white matter and at the same location as uPA and uPAR on perivascular mononuclear cells in NAWM. In acute and subacute multiple sclerosis lesions, expression was more widespread and localized to cytoplasm of foamy macrophages and hypertrophic astrocytes (Fig. 2E). There was a good correlation between the immunohistochemical detection and ELISA measurements of PAI-1. A highly statistically significant increase in PAI-1 was found in acute lesions compared with NC white matter (Fig. 3). Furthermore, PAI-1 concentrations were several orders of magnitude higher than those of uPA and uPAR, but never reached levels of tPA in the same type of sample, with the tPA:PAI-1 ratio varying from as high as 30 : 1 in NC white matter to 2 : 1 in acute lesions. Expression of plasminogen was confined to blood vessel walls in both NC and NAWM (Fig. 2B). In contrast, in acute multiple sclerosis lesions, the plasma membrane of foamy macrophages was positively stained, while staining of blood vessels diminished (Fig. 2B).

Matrix metalloproteases

The enzyme activity of MMP-2 and -9 in homogenates was determined using modified uPA as the enzyme substrate (Hanemaaijer et al., 1998). A statistically significant increase of p-aminophenylmercuric acetate (APMA)-activated MMP-9 zymogen was found in NAWM compared with NC white matter, with a further increase in acute multiple sclerosis lesions (Table 2). However, free active enzyme was detected in only a small number of tissue extracts for which there was no pathological correlation. The observation that ELISA values for total MMP-9 (latent plus TIMP-complexed enzyme) were not increased above those of latent enzyme confirmed that it was present largely in the pro-form. Similarly, gelatin zymography demonstrated the absence of complexed active MMP-9, with only molecular bands corresponding to latent enzyme found in the gel (D. Gveric, unpublished observations). As enzyme levels of MMP-9 in multiple sclerosis but not NC samples were significantly affected by post-mortem time (r = -0.68, P = 0.0001 for latent and r = -0.45, P = 0.0119 for total MMP-9), the results may not represent the situation in vivo accurately. Total MMP-2 was expressed at constitutive levels, with little variation between NC (1.27 ± 0.53 ng/mg protein), NAWM (1.58 ± 0.59) and acute multiple sclerosis lesions (1.59 ± 0.75). Furthermore, APMA-activated MMP-2, which indicates the amount of pro-enzyme, showed the same expression pattern. TIMP-1 values reflected the inflammatory activity, with between 5- and 12-fold higher amounts than that of MMP-2 and -9 being detected in NAWM and multiple sclerosis lesions (Table 2). A highly significant increase in TIMP-1 paralleled an increase in activatable MMP-9 in acute multiple sclerosis lesions.

tPA

tPA, found to be the most abundant plasminogen activator in normal human brain and spinal cord, was immunolocalized on fine axons (Fig. 4A) and blood vessels walls. In NAWM, mononuclear cells in perivascular cuffs expressed tPA (Fig. 1B), while in the borders of acute multiple sclerosis lesions tPA staining was intensified on predominantly large diameter axons and axonal ovoids (Fig. 4D and E), as well as on macrophages. On cross-sections of spinal cord, tPA was distributed over the whole cut surface of individual axons, indicating intra-axonal localization of this enzyme as well as membrane staining representing tPA released by...
Fig. 1 A perivascular inflammatory infiltrate in NAWM from a multiple sclerosis patient. Mononuclear cells in the perivascular space stained in serial sections with (A) anti-uPA, (B) anti-tPA, (C) anti-uPAR, (D) anti-β2 integrin and (E) CD2 antibodies. Distribution of (F) collagen IV and (G) vitronectin in the blood vessel wall and perivascular space. Bar = 40 μm as in (C) except for (G) where bar = 20 μm.
Fig. 2 Expression of uPA, uPAR and PAI-1 in an acute multiple sclerosis lesion. Low-power photomicrograph of lesion parenchyma and adjacent NAWM stained with (A) EBM-11, a microglial/macrophage marker, and (B) anti-plasminogen antibody showing positive macrophages in lesion. A high-power insert shows plasminogen-positive macrophages in the lesion border. WM = normal-appearing white matter; AL = acute lesion parenchyma. Foamy macrophages in the lesion stained with (C) anti-uPAR and (D) anti-β2 integrin antibodies. (E) Cytoplasm of astrocytes and macrophages stained with anti-PAI-1 antibody. Bar = 20 µm in (C) except for (A) and (B) where bar = 320 µm.

endothelial or mononuclear cells. However, in chronic multiple sclerosis lesion tPA staining was found predominantly on blood vessel walls whilst the staining of axons in demyelinated lesions parenchyma was absent. A focal increase in immunohistochemical staining contrasted to ELISA which showed a decrease in tPA antigen in NAWM and multiple sclerosis lesions compared with NC white and grey matter (Table 2). A decrease in tPA antigen was also observed with Western blotting which further confirmed the specificity of the anti-tPA antibody used for immunohistochemical staining (data not shown). The Spearman rank test found the concentration of tPA antigen to correlate directly with the age of multiple sclerosis cases ($r = 0.27, P = 0.044$), the only component of the PA system to be significantly affected by age or post-mortem time of NC or multiple sclerosis cases. The highest tPA activity was measured in NC grey and white matter which, like the antigen, was diminished in multiple sclerosis samples (Table 2). A significant correlation was observed between tPA antigen and activity ($r = 0.55, P = 0.005$, age adjusted) in multiple sclerosis tissue. To test the specificity of tPA activity, protein extracts from NC and multiple sclerosis tissue samples were pre-incubated with the synthetic tPA inhibitor tPA-STOP or Monozyme anti-tPA antibody (Nielsen et al., 1983), which resulted in ~70% inhibition of activity.

Fibrinogen, a tPA/plasmin substrate, was also visualized on the subset of large diameter axons in acute multiple sclerosis lesions (Fig. 4F and G) whilst in NC tissue (Fig. 4B), NAWM and lesions at the later stage of development blood vessel walls and occasionally macrophages were
stained. Co-localization of tPA and fibrin(ogen) on axons was shown with double immunofluorescence staining. Although individual axons were seen which expressed only tPA or only fibrin(ogen), the majority of large diameter axons were double positive (Fig. 4F). Axons highlighted with anti-tPA and anti-fibrin(ogen) antibodies also expressed the non-phosphorylated neurofilament-H fraction as shown by staining with SMI-32 antibody (Fig. 4G).

Immunohistochemical controls, including omission of primary antibodies or substitution of monoclonal antibodies with mouse IgG1 or anti-plasminogen antibody with normal rabbit serum, were negative. Normal goat serum, used as a negative control for anti-fibrin(ogen) antibody, weakly stained glial cell bodies but no axons were visualized.

Discussion

The data reported in this study demonstrate distinct expression profiles and functions for uPA and tPA in the pathogenesis of multiple sclerosis lesions. tPA was the most abundant PA in control brains, with antigen concentration and enzyme activity several orders of magnitude higher than those of uPA. However, it was the highly significant increases in uPA, uPAR and PAI-1, immunolocalized on perivascular mononuclear cells and macrophages, which characterized developing lesions. Membrane-bound uPAR, significantly increased in NAWM, was found co-localized with β2 integrins on macrophages and mononuclear cells, an interaction which reinforces cell adhesion. Quantitative increases in MMP-9 and TIMP-1 in acute multiple sclerosis lesions were in
and PAI-1, but not PAs and PAI-1, suggests that the role of fi
with the uPAR predominance of PAI-1, which detaches cells by interfering
mechanism crucial for both the formation and the progression
ponents constitutes a rate-limiting pericellular proteolytic
focal uPA activity (Chavakis
samples
Table 2
Protein concentration and activity of tPA and MMP-9 in extracts from normal control and multiple sclerosis tissue
Antigen  NCWM  NAWM  AL  SAL  CL
TPA antigen (ng/mg)  38.87 ± 28.97  19.37 ± 7.12  19.09 ± 5.94  18.69 ± 6.65  27.93 ± 10.90
TPA activity (U/mg)  10.48 ± 8.32  4.70 ± 0.84  4.50 ± 1.31  6.63 ± 1.17  5.85 ± 3.23
Latent MMP-9  0.65 ± 0.96  1.40 ± 1.07  1.88 ± 1.29  1.55 ± 1.40  0.94 ± 0.67
Total MMP-9  0.55 ± 0.42  0.87 ± 0.44  1.70 ± 0.88  1.28 ± 0.77  1.50 ± 0.81
APMA-activated MMP-9  1.97 ± 1.23  4.87 ± 2.89*  7.57 ± 3.62**  7.55 ± 4.86  5.58 ± 4.99
TIMP-1  6.44 ± 1.78  9.62 ± 4.73  19.11 ± 4.69***  15.80 ± 8.50  11.70 ± 4.59

All data are given as mean ng/mg protein ± standard deviation except for activity assays where data are given as mean U/mg protein ± standard deviation. *P values indicate a statistically significant difference between multiple sclerosis samples and the appropriate normal control and are designated as follows: *P < 0.05, **P < 0.01 and ***P < 0.001 (Fisher’s exact test). Highest tPA antigen (71.87 ± 26.38 ng/mg) and activity (25.57 ± 11.93 U/mg) were measured in NC grey matter and were both markedly lower in multiple sclerosis grey matter (31.9 ± 8.49 ng/mg and 13.10 ± 8.44 U/mg). MMP-2 was expressed at constitutive levels whilst antigen levels of MMP-3 and free enzyme activity of MMP-2 and -9 were negligible in all samples except for trace amounts in some acute and chronic lesions. APMA = p-aminophenylmercuric acetate; NCWM = normal control white matter; NAWM = normal-appearing white matter; AL = acute lesion; SAL = subacute lesion; CL = chronic lesion.

concert with those of the uPA complex, although ELISA data showed that the enzyme was present largely in the pro-form. Enzyme activity assays bore this out as only APMA-activated pro-enzyme could be measured. Although quantitatively decreased in multiple sclerosis white matter and lesions, tPA was concentrated on large diameter axons containing non-phosphorylated neurofilaments in the vicinity of axonal fibrin(ogen) deposits in demyelinated zones.

The significant increase in all three components of the uPA complex in macrophages in the acute lesion delineates the focal proteolysis which promotes adhesion and migration of activated microglia and inflammatory cells through CNS parenchyma. Transmigration of the BBB involves sequential steps of chemoattraction and proteolysis, and the uPA complex is unique in its ability to fulfill both functions (Blasi, 1997). The increase in uPAR in perivascular mononuclear cells in NAWM may initiate adhesion of mononuclear cells to blood vessel walls and transmigration during the formation of primary multiple sclerosis lesions. Accordingly, in tumour tissue uPAR has been found associated mainly with initial tumour invasion (Gong et al., 2000). The co-localization of uPAR with β2 integrins (Simon et al., 1996; May et al., 1998) may further promote adhesion of inflammatory cells to the vitronectin meshwork detected in the blood vessel walls in the active lesion (Sobel et al., 1995) amplifying focal uPA activity (Chavakis et al., 1998). The quantitative predominance of PAI-1, which detaches cells by interfering with the uPAR–vitronectin link, may be a factor promoting cell infiltration in later stages of multiple sclerosis lesion development. A significant positive correlation between uPAR and PAI-1, but not PAs and PAI-1, suggests that the role of uPAR and PAI-1 in cellular migration may be more significant than that of PAI-1 in inhibition of PA-mediated proteolysis. Therefore, uPA/uPAR interaction with PAI-1 and ECM components constitutes a rate-limiting pericellular proteolytic mechanism crucial for both the formation and the progression of multiple sclerosis lesions through facilitated infiltration of mononuclear cells and migration of activated microglia.

It is puzzling that despite a significant increase in MMP-9 in acute multiple sclerosis lesions there was no detectable active enzyme. Furthermore, there was no indication of complexed active MMP-9 as total values were not increased above those of latent enzyme. However, a high CSF level of MMP-9 in multiple sclerosis patients has been reported in disease exacerbation with MRI evidence of BBB damage, although there was also a concurrent increase in TIMP levels (Rosenberg et al., 1996; Leppert et al., 1998). The increase in activatable MMP-9 in perivascular mononuclear cells in the vicinity of collagen IV and vitronectin implicates MMPs in BBB breakdown. The detection of vitronectin breakdown products in demyelinating multiple sclerosis lesions provides further support for this hypothesis in line with the previous immunohistochemical demonstration of loss of tenascins C and R in macrophage-rich borders of active lesions (Gutowski et al., 1999). It may be the case that MMP-9 is more affected by the post-mortem time of samples than the upstream PAs, sensitivity to breakdown of the active enzyme accounting for the loss of measurable MMP activity. Our data may represent the situation in chronic conditions such as rheumatoid arthritis where activity is transient, localized and under stringent regulation by associated TIMPs, leading to rapid turnover of TIMP–MMP-9 complexes following activation (Hanemaaijer et al., 1998).

The presence of constitutive tPA antigen and activity in NC grey matter is in agreement with its role in neuronal activity and synaptic remodelling (Seeds et al., 1996). The activity detected in control brain is in all probability compartmentalized but in contrast to MMPs, less stringently regulated and therefore detectable in detergent extracts of control tissue. The enzyme activity not inhibited by tPA-STOP may represent other serine proteases including locally produced uPA. Although an overall reduction in antigen concentration,
Fig. 4 Immunohistochemical analysis of tPA in NC and multiple sclerosis samples. Serial sections of NCWM stained with (A) anti-tPA, (B) anti-fibrinogen) and (C) SMI-32 antibodies. (D) Low-power photomicrograph and (E) high-power detail of an acute lesion stained with anti-tPA antibody. Double immunofluorescence staining of serial sections of this lesion with (F) anti-tPA (red) and anti-fibrinogen) (green) and (G) with SMI-32 (green) and anti-fibrinogen) (red) antibody pairs showing thick axons. Yellow colour indicates double-stained structures. Bar = 20 μm as in (G) except for (D) where bar = 320 μm.
confirmed by immunoblotting and enzyme activity, signifies that tPA is unlikely to be as closely associated with focal pericellular proteolysis as the uPA–MMP cascade, macrophage-associated tPA is observed in active lesions and could be contributory to BBB breakdown. The loss of tPA during lesion development is reflected in a significant and relatively specific tPA increase in the CSF of multiple sclerosis patients in contrast to acute inflammatory CNS conditions (Akenami et al., 1996). This phenomenon is also described in other pathological conditions including rheumatoid arthritis (Ronday et al., 1996) and tumors (Arai et al., 1998), in which it is associated with greater disease severity, exacerbation or poorer outcome (Brommer et al., 1992; Bindal et al., 1994; Busso et al., 1997).

Localization of tPA to denuded axons stained with SMI-32 antibody, an immunohistochemical marker of non-phosphorylated neurofilament (Trapp et al., 1998), in actively demyelinating multiple sclerosis lesions suggests that the enzyme is associated with axonal damage. Plasminogen and tPA are present in sufficient amounts on foamy macrophages in active lesions in the vicinity of tPA-positive large diameter axons, creating a potential for periaxonal plasmin generation which may remove laminin and other axon-supporting ECM molecules (Chen and Strickland, 1997). Alternatively, the co-localization of tPA with fibrinogen, which stimulates the enzyme activity several fold, on the same population of axons in demyelinating lesions may be a protective measure to remove fibrin deposits. In a recently reported study of sciatic nerve damage in tPA-deficient animals, fibrin deposition was found to exacerbate axonal injury (Akassoglou et al., 2000). As tPA production is increased in multiple sclerosis neurons (Akenami et al., 1999) and the enzyme is subject to anterograde axonal transport (Lochner et al., 1998), it may be targeted actively to damaged axons in response to fibrin deposition. Therefore, tPA has the potential to be a detrimental and/or beneficial agent in axonal injury and may represent an axonal response to a CNS microenvironment non-permissive to axonal regeneration (Fawcett and Asher, 1999).

In conclusion, the increase in uPA, uPAR and PAI-1 in multiple sclerosis tissue may be a trigger for focal plasmin generation facilitating cellular infiltration into the brain parenchyma which can be complemented by short-lived MMP activity during opening of the BBB. MMP-9 which co-localizes with PAs and plasminogen may be involved primarily in ECM remodelling. On the other hand, findings in the lesion parenchyma and CSF point towards tPA involvement in axonal damage and/or repair. Thus therapeutic strategies targeting the PA system could interrupt both the causes and the consequences of an inflammatory process in the CNS of multiple sclerosis patients.

Acknowledgements
We wish to thank Miss Anne Doward for excellent technical assistance, Dr Guus Wolswijk for the gift of anti-GC antibody and Dr Axel Pen Zold for his valuable assistance with statistical analysis of data. The work in the Neuroinflammation Group at the Institute of Neurology is supported by the Wellcome Trust.

References
Cuzner ML, Gveric D, Strand C, Loughlin AJ, Paemen L, Opdenakker G et al. The expression of tissue-type plasminogen activator, matrix metalloproteases and endogenous inhibitors in the


Received February 12, 2001. Revised May 4, 2001. Accepted May 23, 2001