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Activity of a newly identified serine protease in CNS demyelination

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Summary

We have identified a novel serine protease, myelencephalon-specific protease (MSP), which is preferentially expressed in the adult CNS, and therein, is abundant in both neurones and oligodendroglia. To determine the potential activity of MSP in CNS demyelination, we examined its expression in multiple sclerosis lesions and in two animal models of multiple sclerosis: Theiler's murine encephalomyelitis virus (TMEV) and myelin oligodendrocyte glycoprotein (MOG)-induced experimental allergic encephalomyelitis (EAE) in marmosets. High levels of MSP were present within infiltrating mononuclear cells, including macrophages and T cells, which characteristically fill sites of demyelination, both in multiple sclerosis lesions and in animal models of this disease. The functional consequence of excess MSP on oligodendroglia was determined *in vitro* by evaluating the effects of recombinant MSP (r-MSP) on oligodendrocyte survival and process number. Application of excess r-MSP resulted in a dramatic loss of processes from differentiated oligodendrocytes, and a parallel decrease in process outgrowth from immature cells. Transfection of oligodendrocyte progenitors with an MSP–green fluorescent protein construct produced similar changes in oligodendrocyte process number. Importantly, r-MSP did not affect oligodendrocyte survival or differentiation towards the sulphatide-positive lineage. We further demonstrate that myelin basic protein, and to a lesser extent myelin oligodendrocyte glycoprotein, can serve as MSP substrates. These studies support the hypothesis that excess MSP, as is present in inflammatory CNS lesions, promotes demyelination.

Keywords: demyelination; glia; inflammation; multiple sclerosis; proteolytic enzyme

Abbreviations: DIG = digoxigenin; EK = enterokinase; GFAP = glial fibrillary acidic protein; GFP = green fluorescent protein; MMP = matrix metalloproteinases; MSP = myelencephalon-specific protease; MSP-IR = MSP immunoreactivity; PLP = proteolipid protein; r-MSP = recombinant MSP; SNK = Student–Newman–Keuls; TMEV = Theiler's murine encephalomyelitis virus

Introduction

Serine proteases are best characterized for their roles in the blood coagulation and fibrinolytic systems, but there is considerable evidence concerning the central functions of proteolytic cascades mediated by these enzymes in the nervous system (Gingrich and Traynelis, 2000). Established or proposed roles of serine proteases and their endogenous serpin inhibitors include: cell migration; neurite outgrowth and pathfinding; synaptic remodelling; cell excitability; and both glial and neuronal cell survival (Moonen *et al*., 1982; Monard, 1988; Seeds *et al*., 1990; Liu *et al*., 1994*a*, *b*; Houenou *et al*., 1995; Tsirka *et al*., 1995, 1997; Davies *et al*., 2001). These events are mediated, in part, by the ability of serine proteases to cleave, thereby activating growth factor precursor proteins, to degrade components of the extracellular matrix, and to bind to cell surface receptors, activating intracellular signalling cascades. The enzymatic activity of serine proteases is tightly regulated, afforded in part by a series of specific endogenous serpin inhibitors. Imbalances between proteases and their inhibitors, due to injury or disease, have been shown to result in CNS pathogenesis, including neuronal degeneration (Tsirka *et al*., 1995, 1997).

Myelencephalon-specific protease (MSP), is a serine protease that has been cloned in our laboratory, which is preferentially expressed in the CNS and predicted to have trypsin-like activity and a broad range of substrate specificity (Scarisbrick *et al*., 1997). The human homologue of MSP has also been identified and has been termed protease M (Anisowicz *et al*., 1996), neurosin (Yamashiro *et al*., 1997), zyme (Little *et al*., 1997) and, most recently, kallikrein 6 (hK6) (Yousef and Diamandis, 2001). Whereas only three kallikrein genes were originally thought to exist in humans, 14 members have now been identified, aligned on chromosome 19q (Yousef and Diamandis, 2001). While several of these newly identified genes have been reported to be expressed in brain, much still needs to be learned regarding their normal physiological roles, and their potential contributions to CNS pathogenesis. For example, changes in both neurosin (hK8) and MSP (hK6) levels, in brain samples, CSF or sera, have been observed in progressive neurodegenerative disorders such as Alzheimer's and Parkinson's disease (Diamandis *et al*., 2000*a*; Okui *et al*., 2001; Shimizu-Okabe *et al*., 2001). hK6 has been shown to cleave amyloid precursor protein *in vitro* (Little *et al*., 1997) and to be elevated in the sera of ovarian cancer patients (Diamandis *et al*., 2000*b*).

We are particularly interested in the potential activity of MSP (hK6) in CNS function and dysfunction, since unlike most other serine proteases identified to date, MSP is robustly expressed in the CNS, but exhibits a more limited distribution in non-neural tissues (Scarisbrick *et al*., 1997; Yamashiro *et al*., 1997). We previously showed that MSP is abundantly expressed by neurones and in a subpopulation of white matter glia in the human and rodent CNS (Scarisbrick *et al*., 1997, 2001). Remarkably, within normal white matter, MSP expression is almost exclusively associated with oligodendroglia (Scarisbrick *et al*., 1997, 2000; Yamanaka *et al*., 1999). Moreover, in response to glutamate receptor-mediated excitotoxic injury in the adult rat spinal cord, a common mediator of CNS injury, we have demonstrated that MSP mRNA is upregulated in both neural and glial elements (Scarisbrick *et al*., 1997).

Given the abundant expression of MSP in oligodendroglia of the adult CNS, and regulation by injury, in this study we set out to determine its potential involvement in CNS demyelinating disease. To accomplish this we examined MSP expression in human multiple sclerosis lesions, and in acute and subacute lesions of experimental allergic encephalomyelitis (EAE) in the common marmoset, *Callithrix jacchus*, and in the spinal cord of mice chronically infected with the Daniel's strain of Theiler's murine encephalomyelitis virus (TMEV). The pathological significance of changes observed has been evaluated by determining the effects of excess of recombinant MSP (r-MSP) on oligodendrocyte differentiation and myelin degradation *in vitro*. Collectively, these studies indicate that MSP is a multifunctional serine protease, which may participate in multiple effector pathways governing demyelination of the CNS.

Methods

Human multiple sclerosis lesions

This study was performed on paraffin-embedded and formalin-fixed archival material from autopsies with clinically (Poser *et al*., 1983) and pathologically confirmed multiple sclerosis. Paraffin-embedded 5-μm sections were stained with routine neuropathological stains including haematoxylin–eosin (H and E), Luxol fast blue/periodic acid Schiff (LFB/PAS), and Bielschowsky silver impregnation axonal stain, as well as immunocytochemistry for the following markers: anti-proteolipid protein (MCA839, Serotec, Raleigh, NC, USA), anti-myelin oligodendrocyte glycoprotein (a gift from Dr Piddlesden, University of Cardiff, Cardiff, UK), and anti-MSP (see below). All cases underwent detailed neuropathological examination and were screened for white matter demyelinating lesions. Demyelinating activity was classified according to recently established criteria (Lassmann *et al*., 1998). Active demyelinating lesions were diffusely infiltrated by macrophages containing myelin proteins as markers of recent and ongoing myelin phagocytosis. Inactive demyelinated lesions were completely demyelinated without signs of remyelination.

TMEV model of multiple sclerosis

Four- to 8-week-old female SJL/J (H-2^s) mice (Jackson Laboratories, Bar Harbor, Mass., USA) were intracerebrally injected with 2×10^6 p.f.u. of the Daniel's strain of TMEV, in a 10 μl volume. Care and handling of mice was in accord with the guidelines of both the NIH and Mayo Clinic Animal Care and Use Committee. At 30, 45, 90, 120 and 180 days post-infection, mice were anaesthetized with pentobarbital (150 mg/kg) and perfused with 4% paraformaldehyde. Spinal cords were blocked transversely at 1 mm, cryoprotected in 25% sucrose, frozen on dry ice and sectioned transversely at 20 μm. Alternatively, blocks were embedded in paraffin and cut at 5 μm. Unfixed spinal cords were obtained at the same time points, snap frozen and stored at –70°C until analysis.

MOG-induced EAE

Marmosets were obtained from Clea, Japan, or the New England Regional Primate Research Center, and housed in the primate colony at the University of California, San Francisco, according to all guidelines of the Institutional Animal Care and Use Committee. EAE was induced by immunization with 100 μg of recombinant rat αMOG (extracellular domain, containing amino acids 1–125), emulsified in complete Freund's adjuvant, followed by intravenous injection of 10^{10} killed *Bordetella pertussis* organisms on the day of immunization and 48 h later (Genain and Hauser, 1997). Clinical signs of EAE developed between 19 and 23 days after immunization. Animals were euthanized with worsening signs during the acute phase (40–42 days after immunization), under deep barbiturate anaesthesia, by

intracardiac perfusion with 4% paraformaldehyde. Slabs of spinal cord were processed for paraffin and 5-μm thick sections were stained with H and E, or processed to localize MSP-immunoreactivity (IR).

Histochemistry

Immunostaining of MSP in mouse, marmoset and human tissue sections was accomplished using purified biotin-conjugated or unconjugated mouse monoclonal MSP antibodies (Scarisbrick *et al*., 2000) or rabbit polyclonal antibodies (Blaber *et al*., 2002), each of which yielded identical staining patterns in the tissues examined. Cell-specific markers used in double labelling studies were: monoclonal anti-glial fibrillary acidic protein (anti-GFAP); Cy3 conjugate (Sigma, St Louis, Mo., USA); rat anti-mouse F480 IgG (Serotec, Raleigh, NC, USA); biotinylated isolectin B_4 (Sigma); rat anti-mouse CD4; or rat biotinylated anti-mouse CD8b.2 (PharMingen, San Diego, Calif., USA). Bound antibodies were detected using mouse adsorbed, fluorochrome-conjugated, species appropriate secondary antibodies (Jackson ImmunoResearch, West Grove, Pa., USA), or with the avidin–biotin immunoperoxidase technique (Vector Laboratories, Burlingame, Calif., USA). In all cases, control for the specificity of immunostaining included staining as above with the omission of primary antibody.

In situ *hybridization*

Examination of MSP and proteolipid protein (PLP) mRNA expression in TMEV-infected mouse spinal cord was accomplished using digoxigenin (DIG)-labelled cRNA probes (Scarisbrick *et al*., 1997, 1999). The MSP-specific probe was prepared by transcription from the MSP cDNA construct pM514, containing 435 base pairs (bp) of rat MSP (nucleotides 220–655), and the PLP probe from construct pGPLP-1, containing 250 bp of mouse PLP (nucleotides 34–285). Hybridization was performed as described previously (Scarisbrick *et al*., 1999), and in some cases hybridized slides were further processed to localize MSP-IR, GFAP-IR or isolectin B_4-IR, as detailed above.

Recombinant MSP

Rat r-MSP was expressed in the baculovirus system, as described in detail elsewhere (Blaber *et al*., 2002). Briefly, the zymogen form of r-MSP with a 44 amino acid synthetic pro-sequence including an enterokinase (EK) recognition sequence and 6× histidine tag, was expressed in baculovirus expression system, purified in a single step utilizing the His-tag fusion and nickel affinity resin and shown to be 98% pure by Coomassie Blue-stained SDS–PAGE. The homogeneity of purified MSP was confirmed using N-terminal sequencing, mass spectrometry and size exclusion high-performance liquid chromatography. After activation by EK (Roche Diagnostics Corp., Indianapolis, Ind., USA), mature r-MSP was further purified by G-50 superfine (Pharmacia Corp., Kalamazoo, Mich., USA) size exclusion chromatography, to eliminate enterokinase and the cleaved propeptide.

Degradation of myelin basic protein and MOG by MSP

Rat myelin basic protein (MBP) was isolated from adult rat brain (Deibler *et al*., 1975) and was incubated with r-MSP in 40 mM phosphate, 150 mM NaCl (pH 7.4) at 100 : 1 mass ratio. The final concentration of r-MSP in the reaction mixture was 35.3 μM. The reaction mixture was incubated at 37°C, time points were taken at 0, 1, 4 and 16 h post-incubation, snap frozen on dry ice and kept at –80°C. The digestion pattern of rat MBP was analysed by loading 10 μl of sample (equivalent to 5 μg of rat MBP) per lane on 16.5% Tricine SDS–PAGE under reducing conditions.

Recombinant rat myelin oligodendrocyte glycoprotein (αMOG), as described above, was incubated with r-MSP in the same conditions as rat MBP, except that the final concentration of αMOG was 31.5 μM. The digested sample was resolved on 16.5% Tricine SDS–PAGE for analysis in the same manner.

Oligodendrocyte cell culture systems

Two oligodendrocyte culture systems were used; purified oligodendrocyte progenitors and the bipotential CG4 oligodendrocyte cell line (Louis *et al*., 1992). Mixed primary glial cell cultures were prepared from the telencephala of PN-1 Sprague–Dawley rats, and OL progenitors obtained from these, by overnight shaking and differential adhesion as described in detail in McCarthy and De Vellis (1980). Purified OL progenitors were plated onto poly-L-ornithine coated glass coverslips, at a density of $20 \times 10^3/cm^2$ and grown in Dulbecco's minimal essential media (DMEM) containing: 4.5 mg/ml glucose, 2 mM glutamine, N2 supplement (Gibco-BRL, Grand Island, NY, USA), 5 μg/ml insulin, 30 nM T3, 10 ng/ml biotin, 50 U/ml penicillin–streptomycin, 0.1 mg/ml sodium pyruvate (Sigma), and 10 ng/ml each of PDGF-AA (platelet derived growth factor AA) and bFGF (basic fibroblast growth factor) (R & D Systems, Minneapolis, Minn., USA). Undifferentiated CG4 cells were grown in Ham's DMEM F12 containing the same supplements.

To examine the effects of excess exogenous r-MSP, telencephalon-derived or CG4 O2A progenitor cells were differentiated toward the oligodendrocyte lineage by replacement of mitogenic factors, PDGF and FGF, with 0.05% bovine serum albumin (BSA). The effect of r-MSP on differentiated oligodendrocytes was examined by exposing cells differentiated for 72 h, to 1 or 10 μg/ml (40 or 400 nM) of r-MSP for an additional 72 h, with media changes containing fresh r-MSP every 24 h. To evaluate the effect of r-MSP on oligodendrocyte differentiation, progenitors

were plated in differentiation media as above, but media were supplemented with 1 or 10 μg/ml of r-MSP after a 30 min culture period, allowing for cellular attachment. As above, cells were then allowed to differentiate for a further 72 h before analysis. To distinguish between cell surface or substrate effects, in a third paradigm, CG4 O2A cells were treated in one of three ways: (i) cells were plated and media changed to differentiation media containing 1 or 10 μg/ml of r-MSP 30 min after plating; (ii) cells were resuspended in, and incubated for 30 min with, 1 or 10 μg/ml of r-MSP, spun down and resuspended in protease-free differentiation media before plating; or (iii) prior to plating, the polyornithine coated coverslip was incubated for 1 h at 37°C with 1 or 10 μg/ml of r-MSP. Cells were differentiated for a further 24 h prior to analysis. Control wells were supplemented with an equal volume of vehicle (40 mM NaOAc, 100 mM NaCl, pH 4.5), alone. To visualize oligodendrocyte processes, coverslips were briefly rinsed in HEPES-buffered saline solution (HBSS), and stained live in HBSS containing 1% BSA, for the presence of cell surface sulphatide, using the monoclonal antibody (mAb) O4 (Sommer and Schachner, 1981), and fluorescein (FITC)-conjugated secondary antibodies (Jackson ImmunoResearch). Labelled cells were fixed in 2% paraformaldehyde and coverslipped with 90% glycerol (pH 8.0), containing 10 μg/ml of the nuclear stain bisbenzamide (Sigma).

In each cell culture paradigm, process outgrowth and cell number were evaluated from six (165 mm^2) fields per coverslip, which were imaged digitally (40× objective) using an Olympus AX70 microscope, fitted with a SPOT colour digital camera (Diagnostic Instruments, Inc., Sterling Heights, Mich., USA). The number of oligodendrocyte O4-IR processes that crossed horizontal lines of a 0.25 inch grid superimposed on each image were counted for each field. Additionally, in each field counts were also made of the total number of O4-positive cells and all cells stained with bisbenzamide. On average, 120 cells were counted per culture condition in each experiment. The mean and standard error of counts from triplicate wells were calculated and analysed by one-way analysis of variance and the Student–Newman–Keuls (SNK) *post hoc* test. All experiments were performed in triplicate and repeated at least twice using independent cell culture preparations.

Overexpression of MSP in CG4 cells

To prepare the green fluorescent protein (GFP)–rat MSP construct, the full-length MSP clone, without stop codon, was amplified by polymerase chain reaction from vector SB12–42B, and subcloned in-frame with the cycle 3 GFP protein of pcDNA3.1/CT-GFP-TOPO® (Invitrogen, Carlsbad, Calif., USA). For cellular transfection, vectors containing the MSP–GFP construct, or GFP alone, were digested with *Bgl*II, ethanol precipitated and resuspended in sterile water. Proliferating CG4 cells grown on polyornithine-coated 60 mm dishes at a density of 2×10^5 per 35 mm well were transfected with 2 μg of DNA, using FuGENE 6 reagent (Roche Diagnostics). Cells successfully transfected were identified by expression of Cycle 3 GFP when viewed with a 40× objective on an inverted Olympus IX70 microscope, fitted with a FITC filter set. GFP-positive cells were imaged digitally, using both fluorescence and phase microscopy, at 24, 48 and 96 h post-transfection, and the number of processes evaluated by counting those that crossed a superimposed 0.25 inch grid. At each time point the number of processes associated with cells transfected with MSP–GFP or GFP alone were compared using the Mann–Whitney rank sum test.

Results

Expression of MSP in human multiple sclerosis lesions

To determine the potential involvement of MSP in the development of multiple sclerosis lesions, we examined the appearance of MSP-IR in actively demyelinating CNS plaques. Multiple sclerosis lesions demonstrated prominent MSP-IR within inflammatory cells in areas with on-going demyelination, and within the central demyelinated core of plaques in association with reactive astrocytes (Fig. 1A–D). In three actively demyelinating lesions from a chronic autopsy case of multiple sclerosis, MSP-IR was abundant along the plaque border between the actively demyelinating plaque edge and the surrounding periplaque white matter. Alignment of sequential sections stained for LFB/PAS or MSP revealed that the intense rim of MSP-IR was located on the lesion side, bordering normal white matter (Fig. 1B and D). This pattern of MSP-IR created the appearance of 'rings' around actively demyelinating lesions. Within these rings, MSP-IR was associated with inflammatory cells and reactive astrocytes (Fig. 1D).

Regulated expression of MSP in animal models of multiple sclerosis

To profile the activity of MSP in CNS demyelination, we have examined its expression in two previously characterized animal models of multiple sclerosis. *Callithrix jacchus* is an outbred new-world primate that is susceptible to immunization with myelin antigens, producing a form of EAE with close clinical and neuropathological similarities to human multiple sclerosis (Genain and Hauser, 1997). TMEV is a mouse model of virus-induced immune-mediated demyelination characterized by CNS mononuclear cell infiltration and widespread spinal cord demyelination (Lindsley and Rodriguez, 1989; Miller and Gerety, 1990). Consistent with previous experience with MOG-induced EAE in *C. jacchus*, all animals examined herein displayed multifocal, perivascular inflammatory infiltrates of mononuclear cells and macrophages, accompanied by prominent concentric demyelination throughout the CNS (Genain and Hauser, 1997).

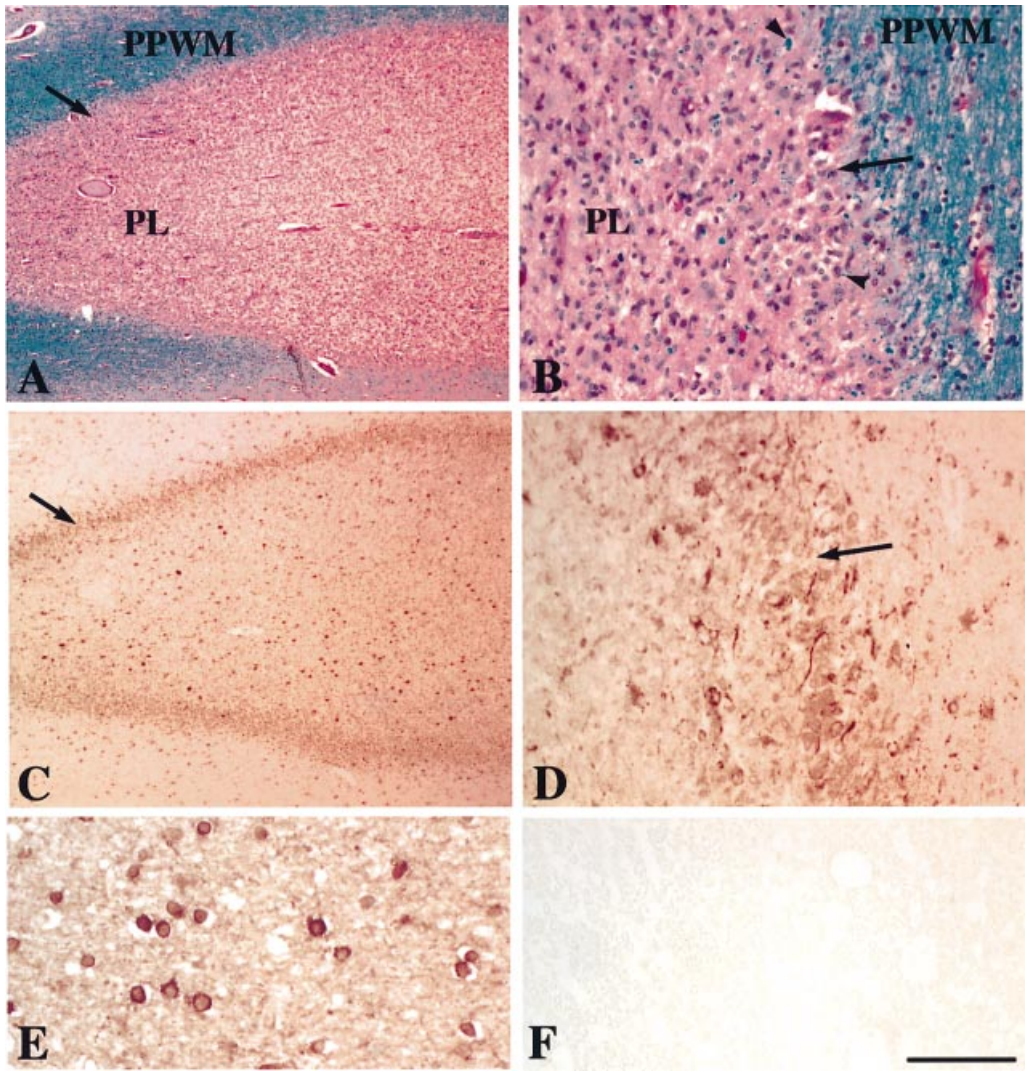

Fig. 1 Distribution of MSP in human multiple sclerosis lesions. **A** illustrates a well demarcated multiple sclerosis lesion, located in the subcortical white matter (LBF/PAS myelin stain). The plaque (PL) shows complete loss of myelin compared with the periplaque white matter (PPWM). (**B**) Higher power view of **A** demonstrates myelin debris (LFB^+ blue granules at arrowheads), present within macrophages along the PL edge, corresponding to an area of active demyelination. **C** and **D** show sequential sections to **A** and **B**, stained for MSP-IR. Levels of MSP-IR were elevated at the border between the PL and PPWM (arrows, **A**–**D**), and within reactive astrocytes in the fully demyelinated plaque core. A high magnification view (**D**) shows the elevated levels of MSP-IR to be located on the lesion side, at the border between the PPWM and the demyelinated PL. In this area of active demyelination, dense MSP-IR was associated with inflammatory cells and reactive astrocytes (**D**). (**E**) In normal CNS white matter, MSP-IR is largely confined to oligodendrocytes and associated white matter. **F** illustrates background levels of immunostaining in a multiple sclerosis lesion processed in the absence of primary antibody. Scale bar is 200 μm in **A** and **C**, and 50 μm in **B** and **D**–**F**.

We previously demonstrated in rat and human that MSP in normal white matter is largely confined to oligodendroglia (Fig. 1E), with significant levels of expression also in CNS grey matter; this pattern was conserved in both the marmoset and mouse spinal cord (Fig. 2A–D) (Scarisbrick *et al*., 2000, 2001). Parallel to observations in human multiple sclerosis lesions, sites of demyelination in spinal cord and brain of the marmoset were infiltrated with MSP-IR inflammatory cells (Fig. 2A and B). Similarly, in areas of demyelination in TMEV-infected mice, examined from 30 to 180 days post-infection, a striking increase in the number of cells associated with MSP-IR and MSP mRNA was observed (Figs 2C and D, and 3A–I). To determine the identity of MSP producing cells within inflammatory lesions, immunohistochemical double labelling techniques were used to examine TMEV-induced lesions (Fig. 3A–F). These experiments demonstrated MSP-IR associated with F480- and isolectin B_4-positive macrophages, in addition to CD4 and CD8 immunoreactive T cells.

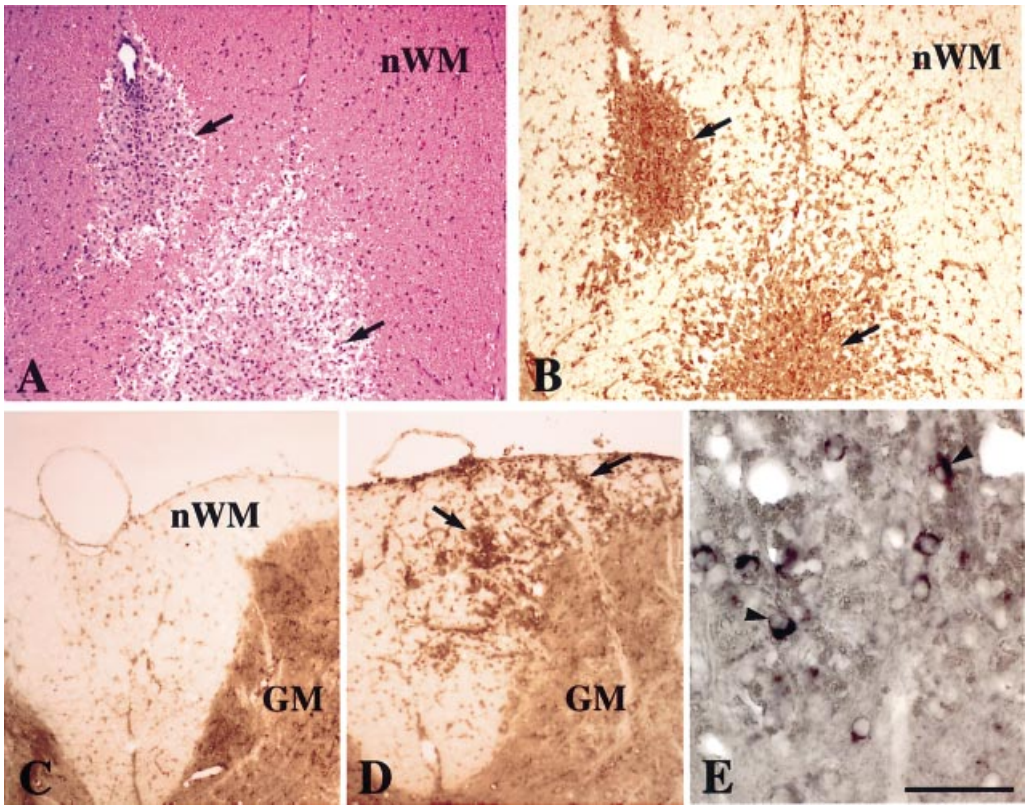

Fig. 2 Changes in MSP within MOG- and TMEV-induced demyelinating lesions. In **A**, a section of spinal cord from a marmoset with MOG-induced EAE shows foci of inflammatory cells, seen as areas of hypercellularity (arrows) in H and E counterstained sections. In an adjacent section (**B**), MSP-IR was associated with both oligodendroglia in normal white matter (nWM), and with inflammatory cells at sites of active demyelination (arrows). **C** and **D** show the typical appearance of MSP-IR in the adult mouse spinal cord of a control animal (**C**), and at 180 days following TMEV infection (**D**). As in the marmoset model, MSP-IR was dense in oligodendrocytes in areas of nWM, and in association with inflammatory cells at sites of active demyelination (arrows). Dorsal funiculi of the spinal cord are depicted in **A**–**D**. **E** demonstrates expression of PLP mRNA by oligodendroglia (arrowheads) in an area of demyelination in the mouse cord 180 days post-TMEV infection, suggesting that even in chronic disease, oligodendrocytes are available to effect repair. Note the high levels of MSP-IR in the grey matter (GM) of the normal and TMEV-infected mouse spinal cord. Scale bar = 200 μm in **A**–**D**, and 50 μm in **E**.

To confirm MSP production by phagocytic macrophages, MSP mRNA was co-localized within isolectin B_4-positive cells (Fig. 3G–I). While observed infrequently in the normal cord (Scarisbrick *et al*., 2000), the presence of MSP-IR in GFAP-positive astrocytes was also seen (data not shown). In active lesions, it was difficult to delineate MSP-IR oligodendroglia, due to the high levels of MSP expression by inflammatory cells. However, even within chronic TMEV lesions, PLP mRNA-producing oligodendroglia were present (Fig. 2E). Together, these findings underscore the potential pathogenic importance of elevated MSP expression by inflammatory cells in CNS immune-mediated demyelination.

Degradation of myelin-specific proteins by MSP

The potential involvement of MSP in myelin turnover was assessed by determining the ability of r-MSP to degrade the myelin-associated proteins, rat MBP and rat myelin oligodendrocyte glycoprotein (αMOG, amino acids 1–125). Although the aqueous digestion conditions used in this *in vitro* analysis were not identical to the physiological conditions in which these proteins are naturally located, these studies did demonstrate that both rat MBP and αMOG are subject to degradation upon incubation with r-MSP at physiological pH. The degree of digestion of each protein by r-MSP, however, was different (Fig. 4A and B). While rat MBP was readily fragmented, degradation of αMOG was much slower. The digestion of rat MBP was so extensive that no intact rat MBP remained within 1 h of incubation with r-MSP, and no fragments with more than ~6 kDa molecular weight could be resolved on 16.5% Tricine SDS–PAGE after overnight digestion (Fig. 4A, lanes 3 and 5). There was a distinct pattern of four lower molecular weight rat MBP fragments clearly seen 1 h post r-MSP digestion (Fig. 4A, lane 3). N-terminal sequencing of these fragments revealed that cleavage occurred after an arginine residue in each case

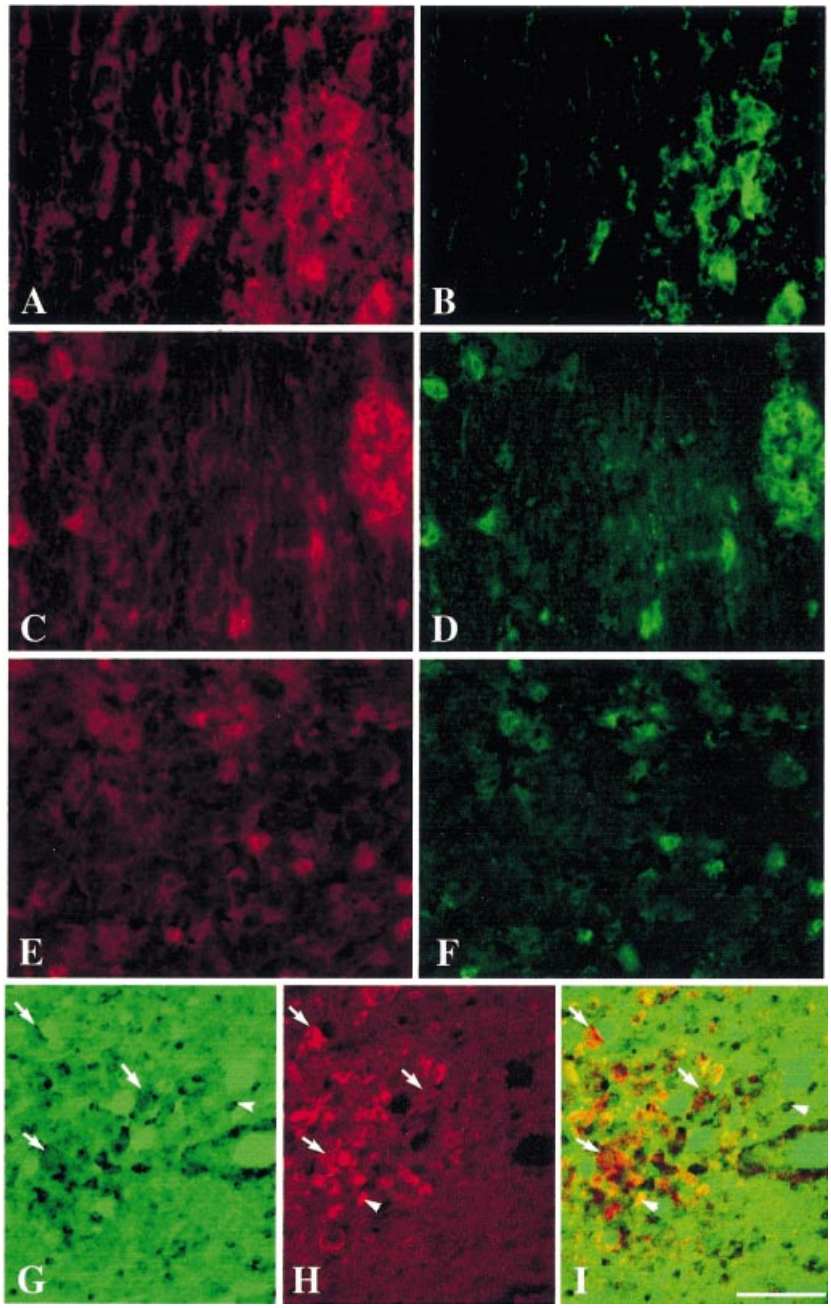

Fig. 3 Expression of MSP by inflammatory cell subsets in TMEV-induced demyelinating lesions. In **A**, **C** and **E**, MSP-IR (red) was localized by immunofluoresence at sites of demyelination in mouse spinal cord white matter, 90 days post-TMEV. **B**, **D** and **F** show immunofluoresence of inflammatory cell markers (green). **B** is the same section as **A**, stained for F480; **D** is the same section as **C**, stained for CD4; and **F** is the same section as **E**, stained for CD8. This double-labelling technique demonstrated high levels of MSP-IR associated with macrophages (F480, **B**), as well as CD4 (**D**) and CD8 (**F**) T cells. In **G**–**I**, the expression of MSP mRNA (**G**) by macrophages (**H**) in the spinal cord at 180 days post-TMEV infection was demonstrated by localizing MSP mRNA using a DIG-labelled MSP riboprobe and alkaline phosphatase histochemistry (producing a purple-black reaction product at sites of MSP mRNA hybridization (**G**), and staining for isolectin B_4 (a macrophage marker, red; **H**), in the same tissue section. The image in **I** shows the overlap of images **G** and **H**, revealing that many isolectin B_4-positive macrophages contain MSP mRNA (arrows). Cells labelled only with isolectin B_4, or with the MSP cRNA probe, were also observed (arrowheads). Controls for hybridization included the sense strand riboprobe in each case, or hybridization without added DIG-labelled riboprobe, each of which produced no reaction product. Scale bar is 50 μm in **A**–**F**, and 25 μm in **G**–**I**.

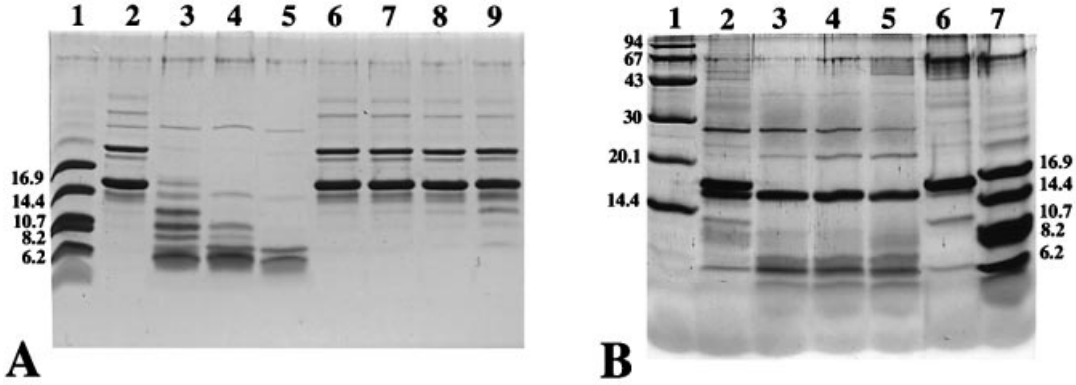

Fig. 4 Cleavage of myelin-specific proteins by MSP. SDS–PAGE (**A**) shows the cleavage of rat MBP by r-MSP. Rat MBP was cleaved into four major products after 1 h incubation with r-MSP (lane 3). These cleavage products were further degraded at 4 h (lane 4), and were almost completely digested by overnight (16 h) incubation with r-MSP (lane 5). Incubation of rat MBP with r-MSP at the 0 h time point (lane 2), or incubation in the absence of MSP, at 0 h (lane 6), 1 h (lane 7), 4 h (lane 8) or 16 h (lane 9), showed no significant rat MBP digestion. Lane 1, low molecular weight peptide markers. SDS–PAGE (**B**) shows cleavage of rat αMOG, amino acids 1–125, by r-MSP. Lane 1, high molecular weight markers; lane 2, αMOG + r-MSP, immediately after r-MSP addition; lane 3, αMOG + r-MSP, 1 h incubation; lane 4, αMOG + r-MSP, 4 h incubation; lane 5, αMOG + r-MSP, 16 h incubation; lane 6, αMOG, no r-MSP, 24 h incubation; lane 7, low molecular weight peptide markers.

(Blaber *et al*., 2002). It should be noted that αMOG showed a rapid molecular weight reduction from 15.5 to 14.7 kDa immediately after addition of r-MSP (Fig. 4B, lane 2). N-terminal sequencing showed this to be the result of digestion after an arginine residue within an N-terminal leader sequence originating from the cloning vector. The internal sites of αMOG were more resistant to digestion by r-MSP; however, slow hydrolysis was apparent.

Effect of r-MSP on oligodendrocyte process outgrowth

To determine the significance of elevated MSP at sites of active demyelination to oligodendrocyte survival and function, oligodendroglia cultured from the post-natal Day 1 (PN-1) rat brain were exposed, at different stages of differentiation, to excess r-MSP *in vitro*, over a 72 h culture period. Exposure of differentiated oligodendrocytes to 1 or 10 μg/ml (40 or 400 nM) of active r-MSP resulted in a 2-fold decrease in the number of sulphatide (O4)-positive processes per cell, compared with control wells exposed to vehicle alone ($P \leqslant 0.005$; SNK *post hoc* test) (Fig. 5A). A parallel 2-fold decrease in process outgrowth was observed when oligodendrocyte progenitors were differentiated in the presence of r-MSP ($P < 0.005$; SNK, data not shown). Notably, neither of these treatments had a significant effect on the total number of cells stained by the nuclear stain or bisbenzamide, or the percentage of those immunoreactive for O4 (Fig. 5B and C). The effects of excess MSP in these experiments was identical in the case of both telencephalon-derived O2A cells and for CG4 oligodendrocytes.

The effect of r-MSP on process stability and outgrowth may have been mediated by activity of the enzyme at the cellular surface, on the polyornithine-coated substratum or both. To distinguish between these possibilities, CG4 oligodendrocyte precursors were exposed to 1 or 10 μg/ml of r-MSP shortly after plating as above, were resuspended and pre-incubated in media containing r-MSP before plating, or were plated in r-MSP free media onto polyornithine-coated coverslips that had been pre-treated with r-MSP. Assessment of O4-positive processes, O4-positive cells and total cell number after a 24 h period revealed that exposure of cells to excess r-MSP shortly after plating, or before plating, decreased process outgrowth by ~2-fold (SNK $P < 0.05$) (Fig. 6A). In contrast, pre-treatment of the substratum had no significant effect on the number of O4-positive processes. Again, none of the treatment protocols affected total cell number or the percentage of O4-positive cells (Fig. 6B and C). We conclude from these experiments that the primary activity of excess r-MSP on immature oligodendrocytes was to affect their ability to extend processes, a result already apparent by 24 h and which was likely mediated primarily on the cellular side, not on the substratum.

Effects of MSP overexpression on oligodendrocyte differentiation

To determine whether excess endogenous MSP had similar effects to excess protease applied exogenously, the full-length clone for MSP was inserted into a vector containing a C-terminal GFP fusion protein. CG4 oligodendrocyte precursors were transfected with the MSP–GFP construct, or vector containing GFP alone, and the number of processes in cells expressing GFP assayed at 12, 48 or 96 h post-transfection (Fig. 7). While neither construct affected oligodendrocyte survival over the period examined, MSP-overexpressing cells had significantly fewer processes from the 48 h time point onwards ($P < 0.001$, 48 h; $P = 0.005$, 96 h;

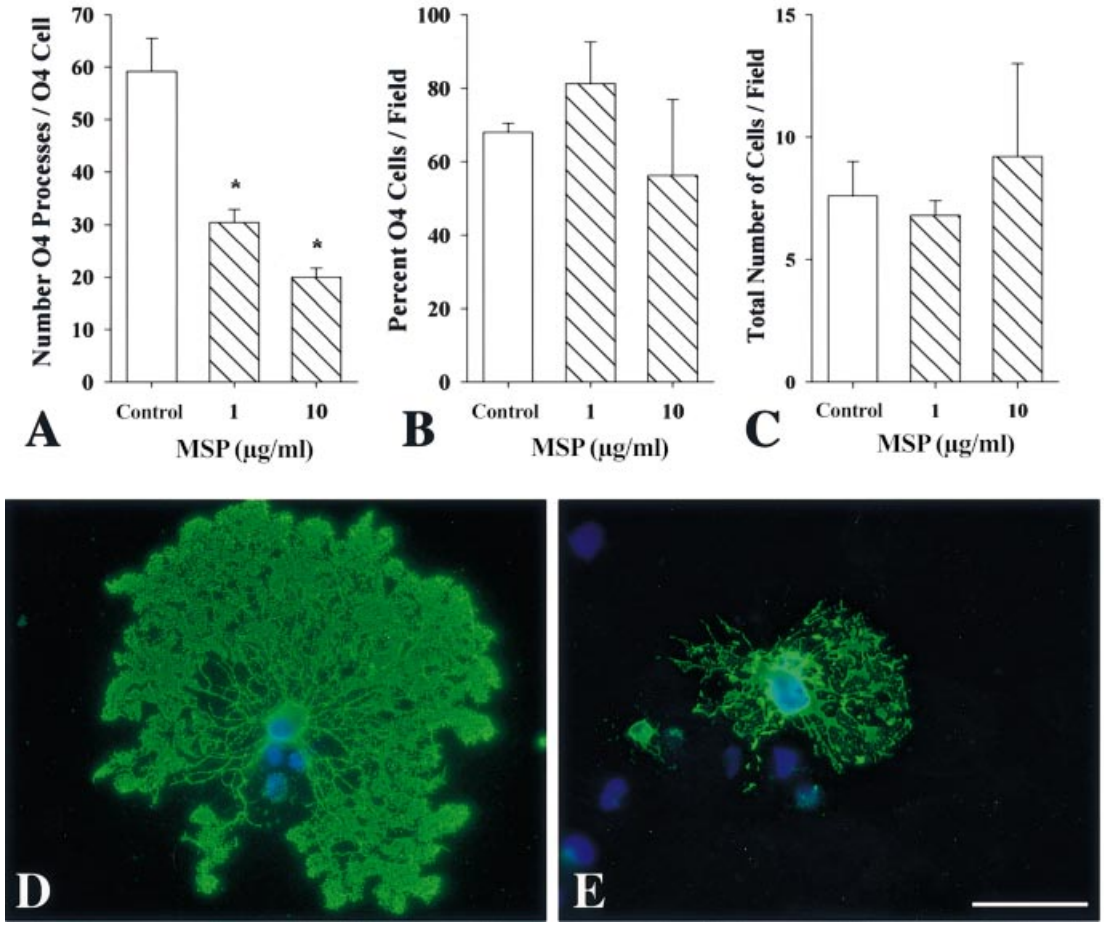

Fig. 5 Effect of excess recombinant MSP on differentiated oligodendroglia *in vitro*. In **A**–**C**, the quantitative assessment of the effects of exposure of telencephalon-derived oligodendrocytes, differentiated for 72 h and then exposed to 1 or 10 μg/ml of r-MSP for an additional 72 h is shown. In **A**, a two-fold decrease in the number of O4-positive processes ($^*P \leqslant 0.005$) was seen, but no significant changes were observed in the percentage of cells in each dish associated with O4 immunoreactivity (**B**), or the total number of cells stained with the nuclear marker bisbenzamide (**C**; blue in **D** and **E**). **D** shows the typical expanse of O4-positive (green) oligodendrocyte processes after a 144-h culture period. **E** illustrates the dramatic reduction in O4-positive oligodendrocyte processes (green) after incubation of differentiated oligodendrocytes with 10 μg/ml r-MSP for an additional 72 h. Data shown represent the mean ± SEM of triplicate wells from a single experiment, but similar results were obtained from two independent experiments, using different cell culture preparations. Scale bar is 50 μm.

Mann–Whitney rank sum test). Thus, overexpression of MSP within the cell had the same effect as applying excess protease exogenously, i.e. a decrease in oligodendrocyte processes.

Discussion

This study shows for the first time the potential involvement of the newly identified serine protease MSP in the pathogenesis of demyelinating disease, and the biological activity of r-MSP in an oligodendrocyte cell culture system. We demonstrate that MSP, now known as hK6 in humans (Yousef and Diamandis, 2001), is expressed at high levels in inflammatory cells at sites of active demyelination in human multiple sclerosis lesions, and in both autoimmune-mediated disease in the common marmoset and TMEV-induced demyelination in the mouse. The localization of MSP within both macrophages and T cell subsets at sites of demyelination and its demonstrated ability to degrade myelin-specific proteins, coupled with the profound negative effect of an excess on oligodendrocyte process outgrowth and integrity, support the hypothesis that MSP may be a key effector molecule contributing to inflammatory cell-mediated demyelination.

Multiple sclerosis is an inflammatory demyelinating disease of the CNS and represents the most common cause of neurological disorder in young adults in North America and Europe. While considerable progress has been made in understanding genetic susceptibility and pathogenesis of this disease (Noseworthy *et al*., 2000), there is still no known uniformly effective treatment strategy. The enzymatic digestion of both barriers to the CNS and myelin, by serine

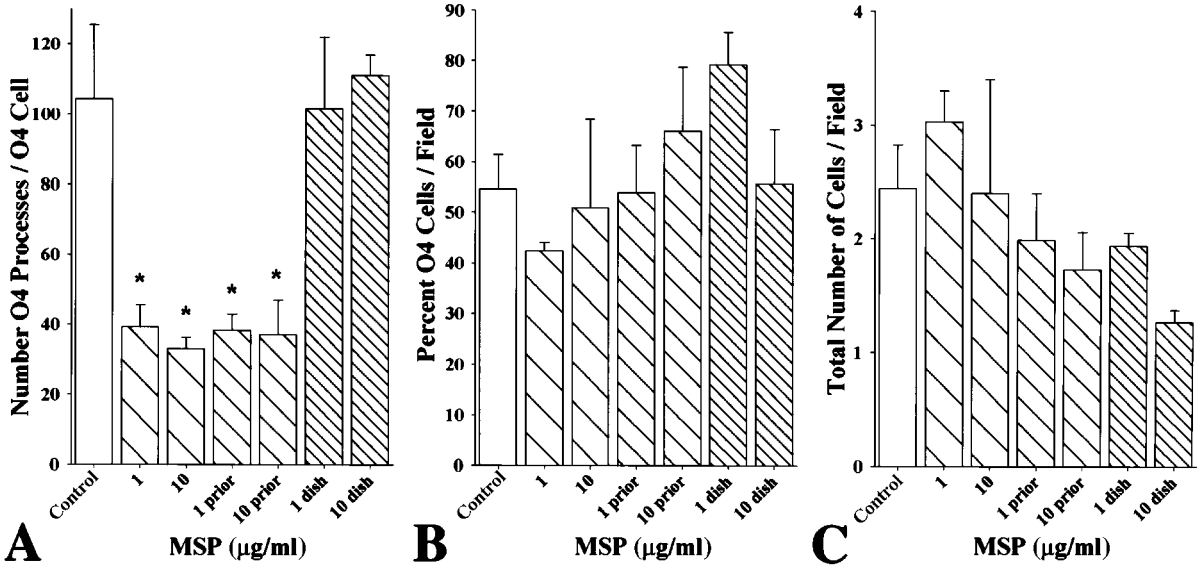

Fig. 6 Effect of recombinant MSP on oligodendrocyte process outgrowth. In **A–C**, the quantitative assessment of the effects of excess r-MSP at the cellular surface, or on the substratum, is shown. CG4 oligodendrocyte progenitors were either: plated in differentiation media containing 1 or 10 μg/ml r-MSP; pre-treated with 1 or 10 μg/ml of r-MSP prior to culture in protease-free differentiation media (prior); or the polyornithine-coated coverslip was pre-treated with 1 or 10 μg/ml of r-MSP, and cells plated in protease-free media (dish). CG4 oligodendroglia were allowed to differentiate for a further 24 h, and stained for sulphatide using the O4 mAB. Counts of O4-positive processes (**A**) indicated that treatment of the cells at the time of plating, or prior to plating, both significantly reduced the number of O4-positive processes (*$P < 0.05$, SNK), but treatment of the polyornithine coated substratum had no effect. None of the treatments produced a significant change in the percentage of O4-positive cells (**B**), or the total number of cells stained by bisbenzamide (**C**).

proteases, is known to contribute to the development and progression of multiple sclerosis (Cuzner *et al*., 1978; Alvord *et al*., 1979; Cammer *et al*., 1986; Gijbels *et al*., 1993; Proost *et al*., 1993; Norga *et al*., 1995). The identification and characterization of key enzymatic players, therefore, may suggest new therapeutic targets to reduce lesion load and promote remyelination, even in cases of chronic disease.

Role of inflammatory cell MSP

The functional consequence of robust MSP expression by inflammatory cells at sites of inflammation and demyelination in multiple sclerosis may be several-fold. The signal peptide within MSP (Scarisbrick *et al*., 1997), the demonstration of high levels in human CSF and serum (Diamandis *et al*., 2000*b*, *c*; Okui *et al*., 2001) and what is known about other serine proteases all suggest MSP is secreted by cells, exerting its activity, at least in part, in the extracellular space. A direct outcome of inflammatory cell MSP production therefore may be its secretion to facilitate transendothelial migration of inflammatory cells into, and within, the CNS. This possibility is corroborated by the demonstration herein of dense MSP expression by all inflammatory cell subsets examined, including macrophages, as well as CD4 and CD8 T cells, not only within perivascular cuffs, but also within the parenchyma of the CNS. A role for secreted MSP in facilitating cell migration is further supported by our previous work, demonstrating that MSP cleaves components of the extracellular matrix, including laminin, fibronectin and collagen, each a component of the blood–brain barrier basal lamina (Blaber *et al*., 2002). It remains to be determined whether MSP is upregulated in activated inflammatory cells or constitutively expressed therein. Our own work (Scarisbrick *et al*., 1997) and that of others (Yamashiro *et al*., 1997; Yousef *et al*., 1999; Petraki *et al*., 2001) has demonstrated at least low to moderate levels of MSP mRNA expression in the normal human spleen, thymus and lymph nodes.

The potential role of MSP in inflammatory cell migration is supported by what has been shown for other neutral proteases, including the matrix metalloproteinases (MMPs), such as MMP-9, and tissue plasminogen activator (tPA). Each, like MSP, has been shown to be present in subsets of inflammatory cells in animal models and in human multiple sclerosis lesions (Gijbels *et al*., 1993; Chandler *et al*., 1995; Cuzner *et al*., 1996; Maeda and Sobel, 1996; Gveric *et al*., 2001; Lindberg *et al*., 2001). MMP-9 (type IV collagenase) has further been shown to affect the transmigration of lymphocytes *in vitro* (Leppert *et al*., 1996; Stuve *et al*., 1996) and *in vivo* (Rosenberg *et al*., 1995). An important consideration in attempting to target proteolysis to abrogate CNS inflammation, as suggested for the MMPs (Kieseier *et al*., 1999), is the distribution of each enzyme outside the brain. Indeed, inhibition of MMP activity can result in widespread deposition of extracellular matrix components. Although MSP is also expressed in human peripheral tissues (Petraki *et al*.,

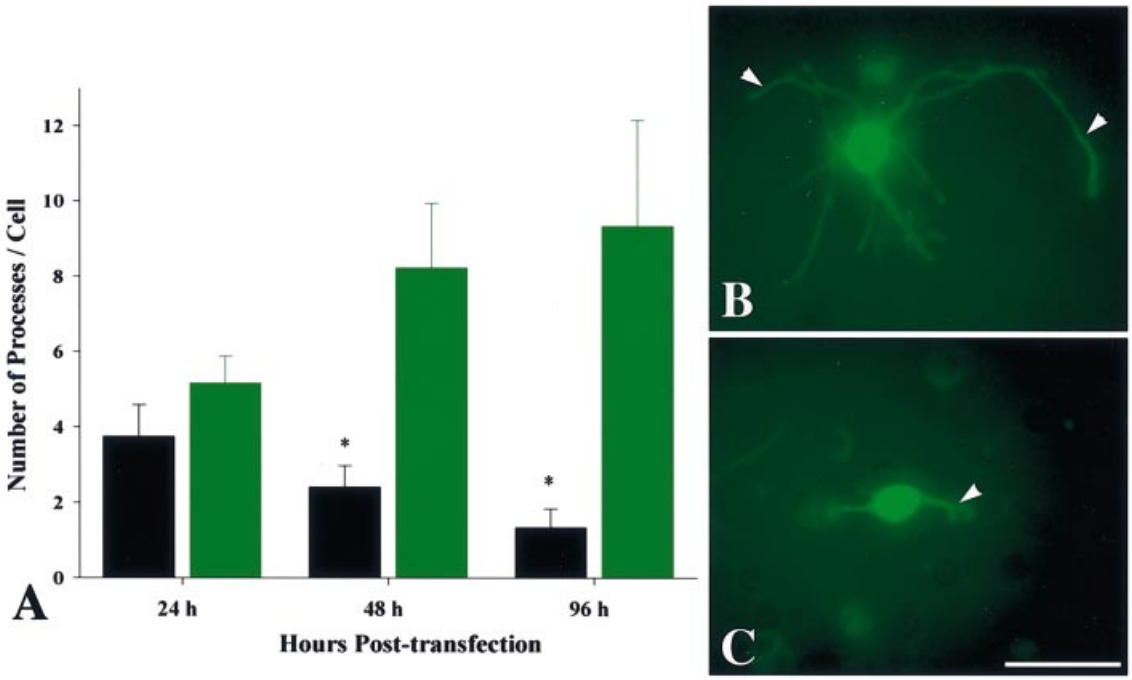

Fig. 7 Effect of MSP overexpression on oligodendrocyte process outgrowth. In **A**, the quantitative assessment of the effects of MSP overexpression in CG4 oligodendrocyte progenitors is shown. CG4 cells transfected with plasmid DNA containing the full-length MSP cDNA fused at the C-terminal to cycle 3 GFP (black bars) had significantly fewer processes by 48 h (*$P < 0.001$), and at 96 h (*$P = 0.005$), post-transfection, compared with CG4 cells transfected with plasmid containing GFP alone (green bars) (Mann–Whitney rank sum test). **B** shows a CG4 oligodendrocyte progenitor 96 h after transfection with plasmid containing GFP alone. **C** shows a CG4 oligodendrocyte progenitor 96 h after transfection with plasmid containing the MSP–GFP construct. Overexpression of MSP in CG4 oligodendrocyte progenitors caused, on average, a four-fold reduction in process elaboration (processes at arrowheads in **B** and **C**). Counts represent data from six independent experiments, with ~40 cells counted per time point. Scale bar is 50 μm.

2001), it exhibits a more limited distribution relative to both MMPs and tPA (Scarisbrick *et al*., 1997), and therefore may represent an important alternative enzymatic target to modulate CNS inflammation.

Effect of MSP on oligodendroglia

In addition to inflammatory cell transmigration, MSP may participate in more indirect 'bystander' activities at sites of demyelination. Our data suggest that a potential consequence of elevated MSP at sites of inflammation may be a 'dying back' of oligodendroglial processes (Rodriguez, 1989). It has previously been established that infiltrating immune cells can cause both oligodendrocyte and myelin damage in multiple sclerosis, and in animal models of this disease (Huitinga *et al*., 1990; Tran *et al*., 1998). For example, immune cells and CNS resident cells can release toxic molecules such as tumour necrosis factor-α (Selmaj and Raine, 1988; Renno *et al*., 1995), interleukin-1 (Bauer *et al*., 1993), reactive oxygen species (Ruuls *et al*., 1995), nitric oxide (Okuda *et al*., 1995) and MMPs (Chandler *et al*., 1995), each of which has been implicated as a cytopathic mediator of demyelination. We demonstrate that application of exogenous r-MSP, causes a retraction of oligodendroglial process from mature cells, and inhibits process extension from immature cells *in vitro*. Moreover, the effect of excess MSP on process extension was shown to be mediated either by overexpression internally, using an MSP–GFP construct, by exogenous application over the period of culture or by pre-incubation with oligodendrocytes prior to culture, but not by the pre-treatment of the substratum alone. These studies thereby demonstrate that MSP may exert its activity, at least in part, at the cellular surface, perhaps by modifying cell surface integrin receptors, thereby affecting the ability of oligodendrocytes to maintain or extend processes.

While the presence of excess, unregulated levels of MSP appears to compromise the integrity of oligodendroglial processes, it should be noted that MSP is densely produced by oligodendrocytes of the normal adult rodent and human CNS, and is normally localized to the growth tips of oligodendrocytes in cell culture (Scarisbrick *et al*., 2000, 2001). In this regard, it is also important to emphasize that while excess MSP had a profound effect on oligodendrocyte process number, it did not affect oligodendrocyte survival or differentiation towards the sulphatide-positive lineage. These data support the hypothesis that regulated expression of MSP promotes normal oligodendrocyte function, but that an excess, as is present in inflammatory demyelinating lesions,

may have a detrimental effect, both on the processes of existing oligodendroglia and any precursors known to be in the area poised to effect repair (Chang *et al*., 2000).

MSP-mediated myelin breakdown

Evidence is also presented that myelin degradation may be an additional consequence of excess MSP at sites of CNS inflammation. We demonstrate that MBP, and to a lesser extent αMOG, both potentially important auto-antigens in multiple sclerosis (Linington *et al*., 1993; Genain *et al*., 1994), are cleaved by MSP *in vitro*. Taken with the demonstration of MSP within macrophages and T cell subsets, secreted MSP is clearly in a position to participate in myelin breakdown at sites of CNS inflammation. In addition, since both MSP protein and mRNA are present within macrophages, it is likely that MSP participates in the breakdown of myelin debris taken up by these cells. It remains for future work to determine whether cleavage of myelin proteins by MSP results in the generation of encephalitogenic epitopes, known to be presented to TH1-positive T cells by macrophages, and thought to drive epitope spreading in demyelinating disease (Miller *et al*., 1997; Katz-Levy *et al*., 2000). It will be important to identify and characterize the activators and endogenous inhibitor(s) of MSP, as well as regulators of this enzyme at the transcriptional level, in order to better understand the full range of MSP activity in the orchestration of the immuno-inflammatory response and the pathogenesis of CNS demyelination.

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