Matrix metalloproteinases and tissue inhibitors of metalloproteinases in cerebrospinal fluid differ in multiple sclerosis and Devic’s neuromyelitis optica

Raul N. Mandler,1,2,* John D. Dencoff,1 Fatma Midani,1,† Corey C. Ford,1 Waseem Ahmed1,‡ and Gary A. Rosenberg1,2,3

Departments of 1Neurology, 2Neuroscience and 3Cell Biology and Physiology, University of New Mexico Health Sciences Center, Albuquerque, New Mexico, USA

Summary

Matrix metalloproteinases (MMPs) are increased in the CSF of patients with multiple sclerosis. Devic’s neuromyelitis optica (DNO) is a demyelinating syndrome that involves the optic nerve and cervical cord but differs pathologically from multiple sclerosis. Therefore, we hypothesized that the type of inflammatory reaction that causes MMPs to be elevated in multiple sclerosis would be absent in patients with DNO. CSF was collected from 23 patients with relapsing–remitting or secondary progressive multiple sclerosis, all of whom were experiencing acute symptoms, from seven patients with DNO, and from seven normal volunteers. Diagnoses were made according to current criteria on the basis of clinical manifestations, imaging results and CSF studies. IgG synthesis was increased in the CSF of multiple sclerosis patients but not in that of DNO patients. Zymography, reverse zymography and ELISA (enzyme-linked immunosorbent assay) were used to measure gelatinase A (MMP-2), gelatinase B (MMP-9) and tissue inhibitors of metalloproteinases (TIMPs). Zymograms showed that multiple sclerosis patients had elevated MMP-9 compared with DNO patients and controls (P < 0.05). TIMP-1 and TIMP-2 levels were similar in all three groups. We conclude that multiple sclerosis patients have higher MMP-9 levels in the CSF than patients with DNO, which supports the different pathological mechanisms of these diseases.

Keywords: gelatinase B; Devic’s neuromyelitis optica; matrix metalloproteinases; multiple sclerosis; tissue inhibitors of metalloproteinases

Abbreviations: BBB = blood–brain barrier; DNO = Devic’s neuromyelitis optica; ELISA = enzyme-linked immunosorbent assay; MMP = matrix metalloproteinase; TIMP = tissue inhibitor of metalloproteinase

Introduction

Multiple sclerosis and Devic’s neuromyelitis optica (DNO) are neuroinflammatory syndromes that have been separated on the basis of clinical manifestations, imaging, CSF chemistry and pathology (Piccolo et al., 1990; Mandler et al., 1993; Wingerchuk et al., 1999). Separation of the two remains controversial, because the mechanisms producing demyelination and axonal transection in multiple sclerosis and necrosis and cavitation in DNO are poorly understood. Elucidation of these mechanisms is vital for the development of rational, science-based therapies, which are required because of the severe, often fulminate disease course suffered by patients with DNO.

Proteolytic enzymes have been detected in the CSF and brain of patients with multiple sclerosis (Rinne and Riekkinen,
Neutral proteases are increased during acute exacerbation of multiple sclerosis. Matrix metalloproteinases (MMPs) are neutral proteases that attack the extracellular matrix. Patients with multiple sclerosis have elevated levels of the MMP, gelatinase B (MMP-9), in the CSF (Gijbels et al., 1992; Paemen et al., 1994), and blood levels of MMP-9 are increased before an acute attack of multiple sclerosis (Waubant et al., 1999). Intracerebral injection of gelatinase A (MMP-2) opens the blood–brain barrier (BBB) (Rosenberg et al., 1992). Tumour necrosis factor-α injected into brain induces MMP-9 synthesis, causing a delayed opening of the BBB (Rosenberg et al., 1995). T lymphocytes use MMP-9 to attack the capillary basal lamina, allowing them to cross the BBB (Leppert et al., 1995). Experimental allergic encephalomyelitis causes an increase in the levels of MMP-9 in the rodent brain, and inhibitors of MMPs block the manifestations of the disease in animals (Gijbels et al., 1994; Hewson et al., 1995). Thus, considerable evidence in experimental animals and man implicates the MMPs in the acute pathophysiology of multiple sclerosis.

MMPs are a gene family with 20 members that comprise four major groups differing in protein structure and substrate specificity (Birkedal-Hansen et al., 1993). The major MMPs identified in the brain include gelatinase A (MMP-2), stromelysin 1 (MMP-3), matrilysin (MMP-7), gelatinase B (MMP-9) and membrane-type metalloproteinases (Yong et al., 1993). Gelatinases (MMP-2 and -9) attack the basal lamina surrounding the blood vessels, altering permeability. MMP-2 is a constitutive enzyme that is normally found in the CSF. MMP-9, which is induced during the neuroinflammatory response, is increased in the CSF of patients with gadolinium-enhancing lesions on MRI; treatment with high-dose intravenous methylprednisolone returned the MMP-9 levels to normal (Rosenberg et al., 1996). Because of the toxicity of MMPs to tissues, their proteolytic activity is controlled at several levels, including transcription, activation and inhibition. Tissue inhibitors of metalloproteinases (TIMPs) are the main inhibitors. MMPs and TIMPs can be measured in CSF by zymography and reverse zymography, respectively. Enzyme-linked immunoassay (ELISA) methods have been developed recently.

Because DNO has different histopathological features and lacks the typical neuroinflammatory markers in the CSF that are generally seen in multiple sclerosis, we hypothesized that elevated levels of MMPs that are uncompensated by a rise in TIMPs would be seen in the CSF of patients with multiple sclerosis but not in that of patients with DNO.

Patients and methods

Patients were selected if they had symptoms suggestive of DNO or clinically active multiple sclerosis; patients with relapsing–remitting and secondary progressive courses of multiple sclerosis were included. CSF was collected from patients during a diagnostic lumbar puncture, and analyses were done after informed consent had been obtained. All patients had an active disease process that required lumbar puncture and treatment. Subsequently, the multiple sclerosis patients were separated into those with relapsing–remitting and secondary progressive disease on the basis of a chart review. Diagnoses of multiple sclerosis and DNO were made by clinicians experienced in the diagnosis and treatment of multiple sclerosis and of DNO. Multiple sclerosis was diagnosed by the Poser criteria (Poser et al., 1983), and DNO was diagnosed as described in an earlier publication (Mandler et al., 1993). The study was approved by the Human Research Review Committee at the University of New Mexico, and complied with NIH Guidelines. All patients gave informed consent for the use of CSF in these assays.

Zymography

The methods we used for the measurement of the MMPs in the CSF by quantitative zymography have been reported (Kleiner and Stetler-Stevenson, 1994; Rosenberg et al., 1996). Briefly, 5 µl CSF was mixed with 3 µl of 1.5 mM Tris (pH 8.8) and 2 µl of ×4 concentrated zymography buffer [0.0625 M Tris–HCl (pH 6.8), 10% glycerol, 2% SDS (sodium dodecyl sulphate) and 0.00125% bromophenol blue]. CSF was analysed with gelatin gel polyacrylamide gels (SDS–PAGE). Ten per cent polyacrylamide gels were prepared [1.5 ml distilled water, 1.18 ml of 1.5 M Tris–HCl (pH 8.8), 45 µl of 10% SDS; 1.5 ml acrylamide-bis (30% T, 2.67% C), 2.2 µl TEMED (l N,N,N′,N′-tetramethyl-ethylenediamine) and 22 µl ammonium persulphate solution, 100 mg/ml], with gelatin (300 µl of 15 mg/ml solution; Sigma G-2500, St Louis, Mo., USA) copolymerized into the gel matrix. Prestained protein standards and HT1080 fibrosarcoma media, which contains MMP-2 and MMP-9, were run in every gel to determine molecular weights. After electrophoresis, gels were agitated in 2.5% Triton X-100 to remove the SDS and restore enzyme activity during incubation. Gels were then incubated for 24 h at 37°C in 50 ml of 50 mM Tris–HCl (pH 7.6, containing 0.2 M NaCl, 5 mM CaCl₂, 0.02% Brij-35 and 0.02% sodium azide). Finally, gels were stained for 1 h in 50% methanol/1% acetic acid with 0.125% Coomassie G blue dye, then destained in 10% acetic acid. Zones of proteolytic activity were evident as clear bands against a dark blue background. Dried gels were scanned (Agfa Duo scanner), and analysed with computer image analysis software (NIH Image, v. 1.56) running on a Macintosh Power PC computer. Individual lanes were analysed with the electrophoretic gel lane calculation option in the program. Standardization was done with an optical density step tablet.

Reverse zymography

TIMP-1 and TIMP-2 were measured in CSF by quantitative reverse zymography (Oliver et al., 1997). Briefly, samples of CSF (5 µl) were mixed with 3 µl of 1.5 mM Tris...
Table 1: Results of CSF demyelinating profile [oligoclonal bands (OCB), myelin basic protein (MBP) and IgG index], disease duration and steroid (prednisone) immunosuppressant medication in the Devic’s neuromyelitis optica patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>CSF protein (mg%)</th>
<th>CSF cells (per mm³)</th>
<th>OCB</th>
<th>MBP</th>
<th>IgG index</th>
<th>Disease duration (months)</th>
<th>Medication</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.T.</td>
<td>47</td>
<td>3</td>
<td>Negative</td>
<td>1.5</td>
<td>0.55</td>
<td>4</td>
<td>None</td>
</tr>
<tr>
<td>A.M.</td>
<td>22</td>
<td>1</td>
<td>Negative</td>
<td>0.4</td>
<td>–</td>
<td>3</td>
<td>Prednisone 60 mg/day</td>
</tr>
<tr>
<td>Y.M.</td>
<td>59</td>
<td>–</td>
<td>Negative</td>
<td>0</td>
<td>0</td>
<td>48</td>
<td>None</td>
</tr>
<tr>
<td>J.R.</td>
<td>57</td>
<td>2</td>
<td>Negative</td>
<td>0</td>
<td>&lt;0.6</td>
<td>36</td>
<td>None</td>
</tr>
<tr>
<td>N.C.</td>
<td>38</td>
<td>1</td>
<td>Negative</td>
<td>0.6</td>
<td>0.71</td>
<td>6</td>
<td>Prednisone 60 mg/day</td>
</tr>
<tr>
<td>M.K.</td>
<td>39</td>
<td>0</td>
<td>Negative</td>
<td>0.5</td>
<td>0.50</td>
<td>9</td>
<td>None</td>
</tr>
<tr>
<td>D.O.</td>
<td>56</td>
<td>10</td>
<td>Negative</td>
<td>0</td>
<td>0.57</td>
<td>8</td>
<td>None</td>
</tr>
</tbody>
</table>

Results

All of the 23 multiple sclerosis patients were experiencing acute symptoms at the time of the lumbar puncture. The seven patients with DNO all had optic neuritis, the presence of an acute lesion in the upper cervical spinal cord and the absence of supratentorial lesions on MRI. The CSF results for the DNO patients are given in Table 1. Multiple sclerosis patients had a mean CSF protein concentration of 53.2 ± 9.6 mg% and a cell count of 6.4 ± 1.6 mm (mean ± SEM). CSF showed IgG synthesis in the multiple sclerosis patients with a mean value of 15.2 ± 3.8. Mean CSF protein concentration for the DNO patients was 45.3 ± 13.5 mg% and the mean cell count was 2.8 ± 3.7 mm³, which was statistically similar to multiple sclerosis patients. Elevated IgG synthesis and oligoclonal bands were absent in the CSF of DNO patients. Some of the multiple sclerosis patients were included in an earlier report (Rosenberg et al., 1997), and several of the DNO patients were also reported earlier (Mandler et al., 1993, 1998). The control group comprised seven patients with non-neurological illnesses undergoing spinal anaesthesia. Because of the small amount of CSF obtained from controls, only MMPs and TIMPs were measured.

MMP-2 values for the 72-kDa inactive species were measured by zymography only, and were similar in the three groups (multiple sclerosis, 1162 ± 57 relative lysis units; DNO, 961 ± 88; controls, 1226 ± 97). There were no bands seen with a molecular weight <72 kDa, indicating the absence of activated forms of MMP-2 (Fig. 1A). The highest levels of MMP-9 were found in the CSF of patients with multiple sclerosis (339 ± 52 relative lysis units/mg protein) (Fig. 1B). Values of MMP-9 in patients with multiple sclerosis were significantly higher than those found in DNO patients (114 ± 16) (P < 0.05) and controls (151 ± 20). MMP-9 measured by ELISA was found in only three of the 37 samples, whereas zymography showed the presence of MMP-9 in all but one of the samples (data not shown). In addition, an 84-kDa band, representing the active form of the 92-kDa species, was seen in four multiple sclerosis patients and two controls, but in none of the patients with DNO.
TIMPs were detected in the CSF of all patients. Reverse zymography and ELISA gave similar values for TIMP-1, and the values from the ELISA were used. TIMP-2 was measured only by reverse zymography. TIMP-1 was statistically similar in the three groups (multiple sclerosis, 12.0 ± 2.3 ng/ml; DNO, 13.9 ± 4.3 ng/ml; controls, 15.9 ± 1.5 ng/ml) (Fig. 2A). TIMP-2 values were also similar in all three groups (Fig. 2B).

Separation of the patients with multiple sclerosis into groups with relapsing–remitting and secondary progressive disease revealed that the MMP-9 values were significantly higher in the relapsing–remitting than in the DNO group (Fig. 3A). In addition, TIMP-1 values were significantly lower in the group with multiple sclerosis with a relapsing–remitting course than in the control group (Fig. 3B).

**Discussion**

We found elevated levels of MMP-9 in the CSF of patients with an acute attack of multiple sclerosis, but not in those with DNO, suggesting different pathological mechanisms. These results suggest an excess of MMP-related proteolytic activity in the brains of multiple sclerosis patients. These results are consistent with earlier reports showing elevated levels of MMP-9 in the CSF of multiple sclerosis patients (Gijbels et al., 1992; Paemen et al., 1994; Rosenberg et al., 1996). They are also compatible with the finding that elevated levels of MMP-9 in the serum of multiple sclerosis patients are related to acute attacks (Trojano et al., 1999; Waubant et al., 1999).

Zymography was more sensitive than ELISA for the detection of MMP-9 (Kleiner and Stetler-Stevenson, 1994). Major differences exist in the two assay methods. ELISA detects inactive forms of the enzyme. Zymography separates molecules by molecular weight, and shows an inactive, higher molecular weight form and a lower active form. The level of sensitivity of the zymograms is in the picogram range, while ELISA is sensitive to nanogram amounts. Reverse zymography measures active forms of the TIMPs that have blocked the activity of the MMPs, while the ELISA measures inactive forms of MMP-9 (Oliver et al., 1997). Reverse zymograms and ELISA gave similar results in the measurement of the TIMPs.

All cell types in the brain produce the matrix-degrading metalloproteinases by the invading leucocytes and macrophages. The enzymes attack all components of the extracellular matrix, and participate in the opening of the BBB by disrupting the basal lamina around the blood vessels.
DNO (Mandler et al.) findings showed that the lower doses affected the CSF, and recent reports showed that oral high-dose methylprednisolone remitting and secondary progressive multiple sclerosis (MS-SP) subgroups. A, MMP-9 was significantly elevated compared with the DNO group (P < 0.05, ANOVA). (B) TIMP-1 measured by ELISA showed a significant difference between the relapsing–remitting multiple sclerosis subgroup and the control group (P < 0.05).

In addition to the absence of MMP-9, the CSF of patients with DNO lacks other markers of acute inflammation, such as elevated IgG synthesis, fragments of myelin basic protein and oligoclonal bands. Leucocytes have been suggested as a possible source for the MMPs (Gijbels et al., 1992), and levels of the MMPs in the CSF tend to be higher with leucocytosis. MMP-9 has been shown immunohistochemically to be present in infiltrating neutrophils and in endothelial cells (Anthony et al., 1997). Gelatinases are secreted by neutrophils and monocytes, suggesting that they may contribute to the MMP-9 detected in the CSF of patients with multiple sclerosis (Hibbs et al., 1985). However, considerable experimental evidence suggests that endogenous sources also contribute to the production of the MMPs. Isolated cerebral capillaries produce MMP-9 during an inflammatory stimulus (Herron et al., 1986; Harkness et al., 2000). MMP production takes place when astrocytes and microglia in culture receive an inflammatory stimulus (Gottschall and Deb, 1996). Activated microglia secrete MMP-9 (Colton et al., 1993). Thus, brain cells as well as infiltrating leucocytes could be involved in MMP production during neuroinflammation, and further studies will be needed to clarify this. Several of the DNO patients were taking low doses of oral prednisone at the time of the lumbar puncture. A recent report showed that oral high-dose methylprednisolone failed to lower the CSF levels of MMP-9, so it is unlikely that the lower doses affected the CSF findings in our patients (Sellebjerg et al., 2000).

The relationship of DNO to multiple sclerosis is controversial, complicating the diagnosis of patients with DNO (Mandler et al., 1993; Wingerchuk et al., 1999). We used the criteria described in our earlier report because the modified criteria appeared after the completion of the study. When the optic nerve and the cervical cord are the only sites of involvement in a patient with severe illness, DNO is generally accepted as the diagnosis. Although patients are found in whom the two conditions overlap, there are differences in the clinical course and the pathological findings that support the concept that separate pathological mechanisms are present. Our finding of reduced levels of the proinflammatory marker MMP-9 in the CSF of patients with DNO is consistent with different disease processes. However, patients with multiple sclerosis showed variability in the levels of MMP-9 in the CSF, which prevented the separation of individual patients on the basis of the MMPs alone. TIMPs block the action of the MMPs. Neither TIMP-1 nor TIMP-2 was significantly different in the full group analysis. However, when the multiple sclerosis patients were separated into subgroups we found a significant reduction of TIMP-1 in the relapsing form of multiple sclerosis compared with DNO.

Pathological changes differ in patients with multiple sclerosis and DNO. Necrosis and macrophages are seen in the cervical spinal cord in DNO, which might suggest involvement of soluble immune mediators, or a vascular mechanism. Few inflammatory cells were detected in the CSF of multiple sclerosis patients, suggesting that the MMP-9 was produced endogenously. There may also be regional differences in the types of cells producing MMPs.

Since DNO is a rare syndrome with diagnostic criteria that vary between clinical centres, a collaborative study with a larger group of patients will be needed to verify these findings. Ideally, a prospective study is needed with patients selected according to similar diagnostic criteria. There may also be differences between MMP production in relapsing–remitting and secondary progressive multiple sclerosis patients that will further complicate the analysis. We propose that measurement of MMPs in the CSF may provide another parameter to aid in the separation of patients with various types of demyelinating process, and may help define the pathophysiological basis for treatment.

References
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