

SHORT REPORT

Upregulation of matrix metalloproteinase-9 in the cerebrospinal fluid of patients with acute Lyme neuroborreliosis

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MMP-9, MMP-3, and TIMP-1 concentrations, white blood cell count, and total protein concentrations in the CSF of patients with acute neuroborreliosis and controls with non-inflammatory neurological disorders

CSF parameter	Patients with acute neuroborreliosis (n=31)	Patients with non-inflammatory neurological disorders (n=20)
MMP-9 concentrations (ng/ml)	13.2(29.3)*	1.0(0.0)
MMP-3 concentrations (ng/ml)	7.5(12.2)	3.8(0.0)
TIMP-1 concentrations (ng/ml)	230.7(142.4)*	109.7(112.7)
White blood cell count (cells/ μ l)	576 (613)*	1 (1)
Total protein content (mg/dl)	413 (517)*	42 (17)

Values in parentheses are SD. Concentrations were calculated using a standard curve. The detection limit in the CSF was 1 ng/ml for MMP-9, and 3.75 ng/ml for MMP-3 and TIMP-1. The ELISA system is designed to detect the proform of MMPs in both the free and the complexed form with TIMPs.

* $p < 0.05$, compared with CSF parameters from patients with non-inflammatory neurological disorders.

Abstract

It was investigated (1) whether metalloproteinase-9 (MMP-9), MMP-3, and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1, the natural tissue inhibitor of MMP-9) are increased in the CSF of patients with Lyme neuroborreliosis and (2) whether macrophages can express MMP-9 when stimulated with *Borrelia burgdorferi*. Zymography showed MMP-9 activity in 26 of 31 (84%) CSF samples from patients with acute stage 2 Lyme neuroborreliosis, but not in 20 controls with non-inflammatory neurological disorders. Activity of MMP-2 was detected in all CSF samples in both patients with neuroborreliosis and controls, suggesting a constitutive release of MMP-2. Using enzyme linked immunosorbent assay (ELISA) MMP-3 (which can activate MMP-9) was detected in low concentrations in the CSF of 13 of 29 patients with neuroborreliosis, but not in controls. TIMP-1 was increased twofold in CSF samples from patients with neuroborreliosis in comparison with the controls. MMP-9 activity was induced in vitro in a mouse macrophage cell line (RAW 264.7) when stimulated with two different genotypes of *B burgdorferi* (*B garinii*, *B afzelii*). This MMP-9 activity was reduced in a dose dependent manner when macrophages stimulated with *B burgdorferi* were coincubated with NF- κ B SN50, a cell

permeable peptide which inhibits the translocation of NF- κ B into the nucleus of stimulated cells. The data show that (1) MMP-9 activity is present in the CSF of patients with neuroborreliosis, (2) macrophages stimulated with *B burgdorferi* are a possible source of MMP-9 increase, and (3) activation of NF- κ B may play a part in the upregulation of MMP-9 by *B burgdorferi*. (J Neurol Neurosurg Psychiatry 2000;68:368-371)

Keywords: *Borrelia burgdorferi*; Lyme neuroborreliosis; matrix metalloproteinase; NF- κ B

Lyme borreliosis is a zoonosis transmitted by ticks and caused by *Borrelia burgdorferi*.¹ Typical CSF findings in neuroborreliosis include lymphocytic pleocytosis, intrathecal *B burgdorferi* antibody production, and blood-CSF barrier abnormalities. Matrix metalloproteinases (MMPs), a family of zinc dependent enzymes, can degrade components of the extracellular matrix, thereby inducing blood-CSF/-brain barrier lesions.² Because some of the inflammatory mediators (for example, NO, IL 1 β , TNF- α) inducing MMP expression³⁻⁵ seem to be involved in the pathophysiology of Lyme borreliosis⁶⁻⁹ we investigated (1) whether MMP-9, MMP-3 (which can activate MMP-9), and a tissue inhibitor of matrix metalloproteinase-1 (TIMP-1, the natural tissue inhibitor of MMP-9) are increased in the CSF of patients with neuroborreliosis and (2) whether macrophages can express MMP-9 when stimulated with *B burgdorferi*.

Patients and methods

PATIENTS AND ANALYSIS OF CEREBROSPINAL FLUID SAMPLES

We investigated CSF samples from 31 patients with acute neuroborreliosis (11 females, 20 males; mean age 50.4 (SD 22.1) years), and 20 control patients (10 females, 10 males, mean age 53.5 (16.7) years) with non-inflammatory neurological disorders (for example, tension headache, n=2; epilepsy, n=2; psychogenic movement disorders, n=3). All patients with neuroborreliosis had (1) a CSF lymphocytic pleocytosis and an increased CSF total protein content (table), (2) evidence of an intrathecal *B*

burgdorferi antibody production determined by comparison of enzyme linked immunosorbent assay (ELISA) titres of IgG antibodies to *B burgdorferi* with total IgG in CSF and serum as previously described¹⁰ (the specific CSF/serum index was greater than 2 in all patients (mean 9.4 (SD 7.3)). Patients with neuroborreliosis had the following clinical symptoms and signs: radicular pain (n=15); headache (n=17); facial nerve palsy (n=7); other cranial nerve involvement (n=7); or limb paresis (n=10). Ten patients (32%) had a history of erythema migrans, which appeared within 3 months before the onset of neuroborreliosis. 981235.

After lumbar puncture the CSF samples were centrifuged at 150 g and the supernatants were stored at -30°C until analysis. Gelatinase activity of all CSF samples and cell culture supernatants was determined by zymography as previously described.¹¹ The CSF concentrations of MMP-9, MMP-3, and TIMP-1 were determined by a commercially available ELISA (Amersham Life Science, UK).

CELL CULTURE EXPERIMENTS

We investigated whether a mouse macrophage cell line RAW 264.7 could express MMPs when stimulated with *B afzelii* (strain PKo, skin isolate, OspA serotype 2) and *B garinii* (strain PBi, CSF isolate, OspA serotype 4).¹² *Borrelia* strains at early passage were cultured, pelleted by centrifugation, lysed by ultrasonication, and recentrifuged; the protein concentrations were then determined.

Mouse macrophages were cultured in RPMI (Sigma Chemicals, Deisenhofen, Germany), supplemented with 10 % fetal calf serum, penicillin (100 U/ml), and 100 µg/ml streptomycin. Twenty four hours before stimulation cells were resuspended in serum free medium (Life Technologies, Eggenstein, Germany) containing penicillin and streptomycin in the same concentrations. Adherent cells were stimulated for different time intervals (6 h, 9 h, 12 h, 18 h) with the following concentrations of

ultrasonicated *B burgdorferi* : 0.1 µg/ml, 1 µg/ml, 10 µg/ml. After stimulation cell culture supernatants were collected, centrifuged at 150 g, and assayed for MMP-9 production. Supernatants of unstimulated macrophages served as controls.

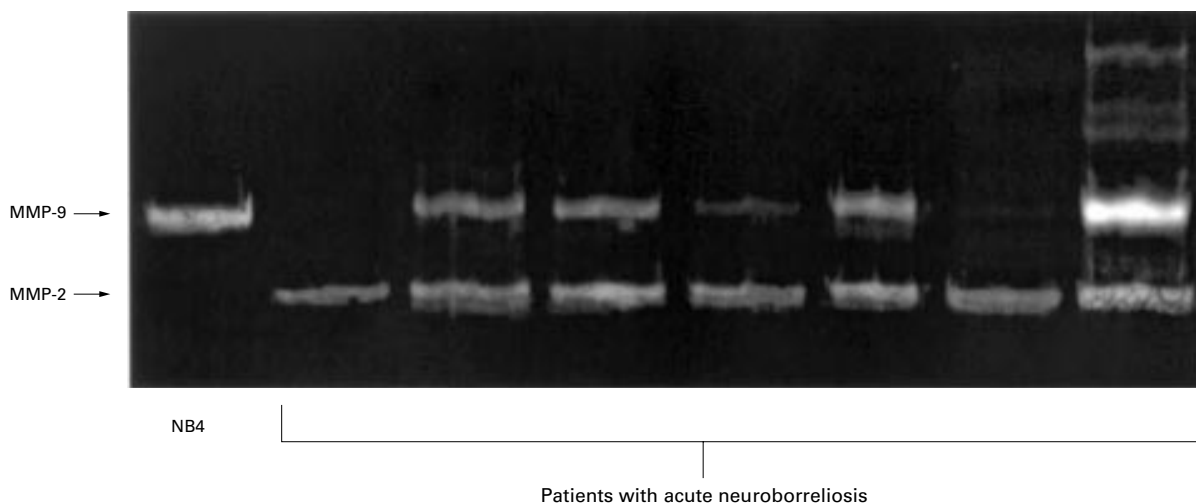
To investigate the role of NF-κB in MMP-9 induction by *B burgdorferi*, macrophages stimulated with *B burgdorferi* ultrasonicate were coincubated with NF-κB SN50 (BIOMOL, Hamburg, Germany), a cell permeable peptide which inhibits the translocation of NF-κB into the nucleus of stimulated cells by specific binding to the nuclear localisation signal of NF-κB.¹³

STATISTICAL ANALYSIS

The significance of differences in the concentration of MMPs and TIMPs was calculated by Mann-Whitney *U* test. A *p* value < 0.05 was considered significant. Values below the detection limit were arbitrarily assigned to the value for the detection limit in the assays. Non-parametric analysis was done by Spearman rank correlation test.

Results

Zymography showed MMP-9 activity in 26 of 31 (84%) CSF samples from patients with neuroborreliosis but in none of 20 controls (figure). ELISA indicated increased MMP-9 concentrations in 17 of 31 (55%) CSF samples of patients with neuroborreliosis, but not in controls. The discrepancy between the MMP-9 ELISA and zymography data may be due to the fact that the detection limit of the ELISA assay was much higher than that of zymography. MMP-2 activity was detected in all CSF samples in both patients with neuroborreliosis and controls, suggesting a constitutive release. MMP-3 was detected in low concentrations in the CSF of 13 of 29 patients with neuroborreliosis, but not in controls. TIMP-1 was increased twofold in CSF samples from patients with neuroborreliosis compared with controls.



Detection of MMP-9 activity in five of seven CSF samples (examples) of patients with acute neuroborreliosis using zymography. Altogether, MMP-9 activity was found in 26 of 31 CSF samples of patients with neuroborreliosis but not in controls. NB4: cell culture media from NB4 leukaemia cells were used as MMP-9 standard.

There was a significant correlation between MMP-9 and TIMP-1 concentrations in the neuroborreliosis group. No significant correlation was found between MMP-9, or MMP-9/TIMP-1 ratio and white blood cell count or total protein content of CSF, respectively, in the neuroborreliosis group.

Supernatants of unstimulated RAW 264.7 showed no gelatinolytic activity. When cells were stimulated with *B burgdorferi*, zymography of the conditioned media displayed dose dependent gelatinolytic bands at 92 kDa, which corresponded to MMP-9. When supernatants collected at different times of stimulation were investigated, there was only a very small MMP-9 signal at 6 hours and an increasing signal at 9 and 12 hours, respectively. RAW 264.7 could be induced to express MMP-9 by both *Borrelia* genospecies. When PKo and PBi were dissolved in serum free medium in the absence of macrophages no gelatinolytic activity was detected by zymography. When macrophages stimulated with 10 µg/ml of *B burgdorferi* ultrasonicate for 18 hours were coincubated with NF-κB SN50 at 50 µg/ml and 100 µg/ml concentration, a dose dependent reduction of MMP-9 activity was found by zymography. This effect was shown for both *B burgdorferi* strains investigated.

Discussion

The major findings of this study were three-fold: (1) zymography detected MMP-9 activity in 84% of CSF samples from patients with neuroborreliosis, (2) macrophages can release MMP-9 when stimulated with *B burgdorferi*, and (3) NF-κB might be involved in *B burgdorferi*-induced MMP-9 release from macrophages.

Increased CSF protein content indicates a blood-CSF barrier disruption and is often found in patients with neuroborreliosis. This raises the question of whether the MMP-9 detected in the CSF is involved in *B burgdorferi* associated blood-CSF barrier lesion. In this study we did not find an association between CSF-MMP-9 activity and CSF protein content in patients with neuroborreliosis. However, this missing association does not definitively exclude involvement of MMP-9 in *B burgdorferi* associated blood-CSF barrier lesions. To further clarify this issue, a larger number of patients must be examined. Furthermore, use of MMP inhibitors (for example, in an in vitro blood-brain barrier model) may help to obtain further information on the potential role of MMPs in *B burgdorferi* associated blood-CSF barrier lesions.

Apart from their capacity to degrade the extracellular matrix, other effector mechanisms of MMPs have been described which may have a role in *B burgdorferi* infection. For example, activated MMPs expressed in immune cells may contribute to the release of active cytokines such as TNFα and cytokine receptors from the cell membrane.^{14, 15} Furthermore, MMP-2 and MMP-9 may have substrates other than extracellular matrix such as myelin basic protein.¹⁶ Myelin basic protein concen-

trations were increased in the CSF in anecdotal cases of neuroborreliosis.¹⁷

Various cell types can express MMPs—for example, macrophages, microglia, lymphocytes, endothelial cells, neurons, and astrocytes.¹⁸ The missing correlation between CSF-MMP-9 activity and CSF total protein content as well as CSF white blood cells in our study implies that the production of MMP-9 is predominantly intrathecal. It has recently been reported that microglia are morphologically, immunophenotypically, and functionally related to cells of the monocyte/macrophage lineage.¹⁹ Both *B burgdorferi* genospecies investigated were capable of inducing MMP-9 expression in mouse macrophages. Activity of MMP-9 in *B burgdorferi* stimulated macrophages was reduced by coincubation with a NF-κB inhibiting peptide. This suggests that the activation of NF-κB may have a role in the induction of MMP-9 by *B burgdorferi*. Recent in vitro studies have shown that *B burgdorferi* (in particular OspA) can activate NFκB in different cell types (for example, endothelial cells, fibroblasts²⁰).

Recently, Perides *et al* reported that CSF MMP-9 activity was detectable in only one of 14 patients with Lyme neuroborreliosis.²¹ Possible explanations for the discrepancies between these findings and our results are differences in activity and duration of the disease. For example, in the study of Perides *et al*, CSF pleocytosis was present in only three of 14 patients and only six of 14 patients had an increased CSF total protein content. By contrast, pleocytosis was present and concentrations of CSF protein content were increased in all patients in our study. Furthermore, most of the patients in the study of Perides *et al* had chronic neuroborreliosis; by contrast, all of our patients had acute disease that lasted less than 3 months.

In conclusion, our data show that CSF MMP-9 activity was detected in a large number of patients with active neuroborreliosis. MMP-9 might be involved in the pathophysiology of *B burgdorferi* infection by acting as an immunomodulating or cytotoxic agent or by degrading the extracellular matrix and thereby causing blood-CSF barrier alterations.

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