

Matrix Metalloproteinase (MMP)-8 and MMP-9 in Cerebrospinal Fluid during Bacterial Meningitis: Association with Blood-Brain Barrier Damage and Neurological Sequelae

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To evaluate the spectrum and regulation of matrix metalloproteinases (MMPs) in bacterial meningitis (BM), concentrations of MMP-2, MMP-3, MMP-8, and MMP-9 and endogenous inhibitors of metalloproteinases (TIMP-1 and TIMP-2) were measured in the cerebrospinal fluid (CSF) of 27 children with BM. MMP-8 and MMP-9 were detected in 91% and 97%, respectively, of CSF specimens from patients but were not detected in control patients. CSF levels of MMP-9 were higher ($P < .05$) in 5 patients who developed hearing impairment or secondary epilepsy than in those who recovered without neurological deficits. Levels of MMP-9 correlated with concentrations of TIMP-1 ($P < .001$) and tumor necrosis factor- α ($P = .03$). Repeated lumbar punctures showed that levels of MMP-8 and MMP-9 were regulated independently and did not correlate with the CSF cell count. Therefore, MMPs may derive not only from granulocytes infiltrating the CSF space but also from parenchymal cells of the meninges and brain. High concentrations of MMP-9 are a risk factor for the development of postmeningitidal neurological sequelae.

Bacterial meningitis (BM) continues to be an important clinical problem, since mortality and the incidence of neurological sequelae remain high, despite effective antimicrobial therapy [1, 2]. In BM, acute breakdown of the blood-brain barrier (BBB), intrathecal production of TNF- α , and accumulation of blood-derived leukocytes in the CSF lead to brain edema, cerebral vasculitis, and ultimately neuronal injury [3, 4]. An overactive immune response of the host, rather than the bacterial pathogen per se, is thought to be responsible for the neuronal damage, resulting in hearing loss, secondary epilepsy, and cognitive impairment [2, 3].

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that are subdivided according to their substrate affinities for different components of the extracellular matrix [5, 6]. Gelatinases (MMP-2 and MMP-9) have been shown to induce BBB breakdown and to facilitate leu-

kocyte extravasation in experimental BM [7] and other models of neuroinflammation [8–12] of the central and peripheral nervous systems. Collagenases (MMP-1, MMP-8, and MMP-13) and stromelysins (MMP-3, MMP-10, and MMP-11) exert proteolytic activity predominantly against fibrillar collagen types that constitute the interstitial extracellular matrix [5]. Tissue inhibitors of metalloproteinase-1 and -2 (TIMP-1 and TIMP-2) are secreted as heterodimeric complexes with MMP-9 and MMP-2, respectively, and thus modulate their proteolytic activity [5, 6]; MMP-8, in turn, is secreted in free form [13]. TNF- α plays a pre-eminent role as the mediator of inflammation in BM [14] and has been shown to induce the expression of MMP-8 and MMP-9 in different types of leukocytes and resident brain cells [15–19].

The upregulation of MMP-9 in human BM has been described in an anecdotal report (2 patients) [20] and recently in the report of a study of 19 patients [7]. However, the spectrum of MMPs expressed in BM, their kinetic regulation, and their role in the development of long-term neurological sequelae have not been investigated.

In a retrospective study, we quantitated CSF concentrations of MMP-2, MMP-3, MMP-8, and MMP-9 and of TIMP-1 and TIMP-2 in children with BM. We correlated levels of MMPs and TIMPs with parameters of intrathecal inflammation and the occurrence of persistent neurological sequelae.

Patients and Methods

Patients CSF samples from 27 children (median age, 13 months; range, 6.5–45 months) with confirmed BM were evaluated

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The study protocol was reviewed and approved by the committee on medical ethics of the Inselspital, Bern. Oral informed consent was obtained from the parents of all patients before enrollment in the study.

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in a retrospective study. CSF samples from 8 children (median age, 116 months; range, 8.5–142 months) served as controls in cases in which lumbar punctures were performed as routine follow-up after remission of lymphocytic leukemia or when suspected CNS infection was not confirmed. The patients with confirmed BM who were enrolled in this study were admitted 1–5 days after onset of clinical symptoms. The diagnosis of BM was based on detection of the pathogen in the CSF by gram staining, bacterial culture, or antigen testing, and the presence of CSF pleocytosis.

All patients with BM were treated with either ceftriaxone ($n = 12$) or cefuroxime ($n = 15$) as part of an earlier study protocol [21]. Since corticosteroids strongly suppress the CSF expression of MMP-9 [22] and possibly of other MMPs, only patients who did not receive corticosteroid therapy were included. Clinical data were gathered from case report files.

The development of neurological sequelae was assessed by follow-up examinations, 8–10 weeks after discharge [21]. Hearing capacity was assessed by evaluation of auditory evoked brainstem potentials or determination of behavioral responses to acoustic stimuli. All patients with abnormal results were tested a second time after 4–6 months. Diagnosis of sensorineural hearing loss was based on the results of at least 2 consecutive hearing tests. Additional follow-up examinations, such as electroencephalography and neurocranial CT or MRI, were performed if indicated.

CSF samples. Twenty CSF samples from patients with BM were obtained by lumbar puncture at the time of admission (first lumbar punctures) but before initiation of antibiotic therapy. Sixteen CSF specimens were from lumbar punctures performed 12–72 h after the initiation of antibiotic therapy. Seven patients' CSF from lumbar punctures performed at admission had been used for clinical purposes, and only samples from second lumbar punctures were available for this study. Seventeen CSF samples from 8 patients with BM who underwent serial lumbar punctures were available (1 patient had 3 consecutive spinal taps). Total cell count, differential leukocyte count, and total protein concentration were determined by standard methods. For measurement of MMPs, TIMPs, and cytokines, CSF samples centrifuged at 1500g for 15 min were frozen at -80°C until assayed.

ELISAs for MMPs, TIMPs, and TNF- α . The ELISA for MMP-2 and -3 was performed as described elsewhere [23]. ELISA kits for MMP-8 [13], TIMP-1, and TIMP-2 [23] were obtained from Amersham (Little Chalfont, UK), and kits for MMP-9 from R&D Systems (Abingdon, UK). The ELISAs for MMPs detect proactive, active, and inactivated MMPs. CSF samples were assayed at dilutions of 1:5 to 1:20. The sensitivity limit of assays was defined as 2 SDs above the mean optical density of zero standard replicates. The calculated detection limits with undiluted samples were as follows: 0.51 ng/mL for MMP-2, 0.055 ng/mL for MMP-3, 0.032 ng/mL for MMP-8, 0.03 ng/mL for MMP-9, 1.25 ng/mL for TIMP-1, and 3.0 ng/mL for TIMP-2. TNF- α was determined by a high-sensitivity ELISA (R&D Systems) that detects free and TNF-receptor-bound forms of TNF; the detection limit was 1.3 pg/mL.

Statistical analysis. The first available CSF samples were those obtained by lumbar punctures performed at admission ($n = 20$) or (when these were unavailable) from second lumbar punctures ($n = 7$). Follow-up CSF samples were those from second lumbar punctures ($n = 8$) performed for patients whose CSF samples obtained at the time of admission had been analyzed for a specific

parameter (a sample from a third lumbar puncture is not included). The intraindividual comparison of CSF levels of MMPs and TIMPs at different time points was performed with the Student's paired t test. All other data sets were analyzed with use of non-parametric statistical tests. Spearman rank correlations of levels of MMPs or TIMPs with CSF cell count, TNF- α , and protein concentrations were calculated. The concentrations of MMPs, TIMPs, and TNF- α in BM patients, grouped according to the type of infectious agent, the time point of lumbar puncture, and the occurrence of neurological sequelae, were compared with use of the Mann-Whitney U test and the Kruskal-Wallis test. Calculations of the risk for neurological sequelae were performed with only values for the first available CSF samples from each patient. $P < .05$ was considered significant. Samples less than the detection limit were assigned to the value of the detection limit of the respective assays.

Results

Patients. Twenty-seven patients with BM caused by *Haemophilus influenzae* ($n = 14$), *Neisseria meningitidis* ($n = 11$), or *Streptococcus pneumoniae* ($n = 2$) were included in this study. CSF protein levels ranged from 0.28 to 5.74 g/L (median, 0.92 g/L); the numbers of leukocytes (and of granulocytes, in parentheses) ranged from 464 (313) $\times 10^9/\text{L}$ to 16,200 ($15,400$) $\times 10^9/\text{L}$ (median, 4916 [4241] $\times 10^9/\text{L}$) (values from all 36 samples included).

All patients survived the disease, but 5 (19%) of 27 had permanent neurological sequelae: 3 patients had bilateral and 1 had unilateral hearing impairment, and 1 patient developed focal epilepsy. Among 4 patients with neurological sequelae, the infectious agent was *H. influenzae*, and in 1 patient it was *S. pneumoniae*.

MMP-9, MMP-8, and TIMP-1 values are elevated in the CSF of patients with BM. MMP-9 and MMP-8 were detected in 35 (97%) of 36 and 29 (91%) of 32, respectively, of CSF specimens from patients with BM, whereas all control patients were negative for both MMPs ($P_{\text{MMP-9}} < .001$ and $P_{\text{MMP-8}} < .001$; figure 1A, and 1B).

TIMP-1 was constitutively expressed in the CSF of control patients (median [range], 116 [78–1323] ng/mL; figure 1D). Among patients with BM, concentrations of TIMP-1 were significantly greater (750 [19.2–6424] ng/mL; $n = 35$) than in control patients ($P < .03$). In individual patients, levels of TIMP-1 were closely related with those of MMP-9 (ρ , 0.623; $P = .0003$) but not with those of MMP-8 ($P = .89$).

Although median levels of MMP-9, MMP-8, and TIMP-1 were higher in first available than in follow-up CSF specimens (figure 1A, 1B, and 1D), this difference was not significant (not shown). With assignment of the value of the detection limit of the MMP-9 assay (0.15 ng/mL at 1:15 dilution), the MMP-9-to-TIMP-1 ratio of control patients was 1.3×10^{-3} (0.1 – 1.9×10^{-3}). This ratio, a measure of proteolytic capacity [7], was strongly increased in first available (7.7 [0.2 – 393] $\times 10^{-3}$; $n = 26$) and follow-up (2.9 [2.0 – 19.2] $\times 10^{-3}$; $n = 8$) lumbar

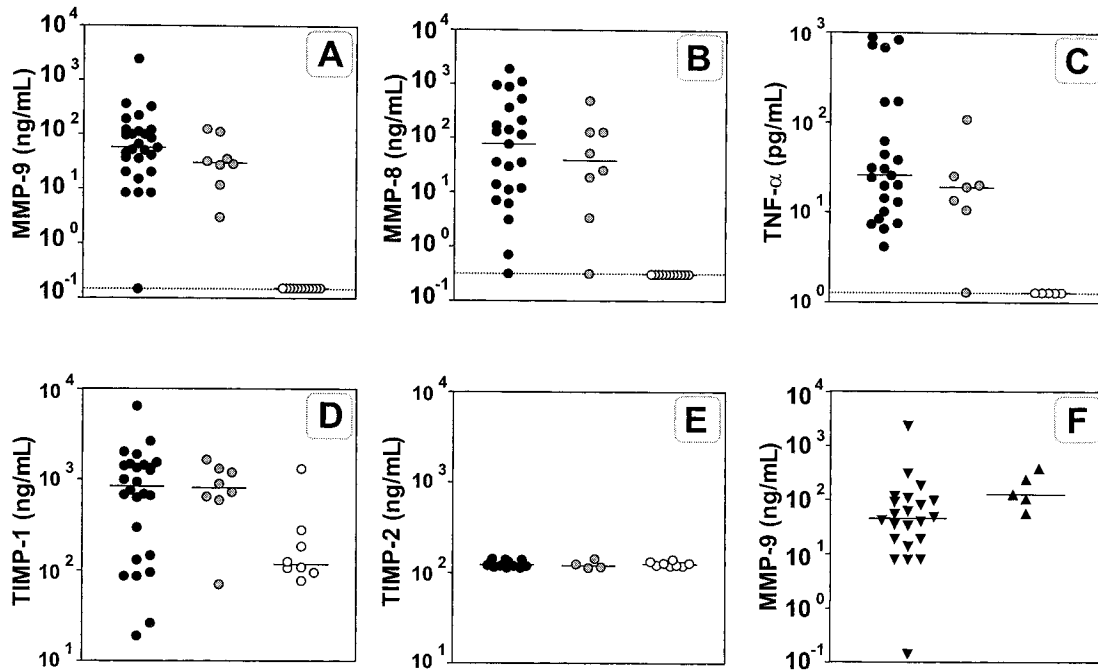


Figure 1. Concentrations of matrix metalloproteinase (MMP)-9 (A), MMP-8 (B), TNF- α (C), tissue inhibitors of metalloproteinase (TIMP)-1 (D), and TIMP-2 (E) in the CSF (first available [●] and follow-up specimens [grey dots]) of patients with bacterial meningitis and of controls (○). Detection limit for MMPs at the CSF dilutions used (MMP-9, 0.15 ng/mL; MMP-8, 0.32 ng/mL) are indicated by the long dotted horizontal lines. Values from a third lumbar puncture for 1 patient are not depicted; those levels were 25 ng/mL for MMP-9, not detectable for MMP-8, 10.6 pg/mL for TNF- α , and 460 ng/mL for TIMP-1. F, Comparison of CSF levels of MMP-9 in patients without (▼) and with (▲) postmeningitidal neurological sequelae. Levels of MMP-9 were significantly ($P < .05$) higher in patients with ($n = 5$) vs. those without ($n = 22$) neurological sequelae. Short horizontal bars indicate medians.

punctures ($P < .001$; the difference of the MMP-9-to-TIMP-1 ratio from first available vs. follow-up lumbar punctures missed statistical significance: $P = .068$).

At the CSF dilutions used (1:15), small amounts of MMP-2 and MMP-3 were detected in both BM and control CSF. However, the values ranged consistently below the lowest value of the standard curve of the ELISA (7.6 ng/mL for MMP-2 and 0.82 ng/mL for MMP-3), which precluded an accurate comparison (not shown). Compatible with the stable expression of MMP-2, levels of TIMP-2 were similar in BM patients (122 [114–143] ng/mL; $n = 19$) and control patients (125 [120–140] ng/mL; figure 1E).

Correlation of TNF- α with MMP-8 and -9, TIMP-1, and the CSF cell count. Sufficient material was available from 30 CSF samples from patients to test for the presence of TNF- α . TNF- α was not detectable (detection limit, 1.3 pg/mL) in control patients ($n = 5$) but was upregulated in 29 (97%) of 30 of CSF specimens from patients; the 1 sample scoring negative was from a follow-up lumbar puncture (figure 1C). However, the concentrations of TNF- α were not different in first available and follow-up lumbar punctures (not shown). Levels of TNF- α were closely correlated with those of MMP-9 ($\rho, 0.409$; $P = .031$) but not with those of MMP-8 or TIMP-1. Both

TNF- α ($\rho, 0.529$; $P_{n=17} = .034$) and MMP-9 levels ($\rho, 0.488$; $P_{n=17} = .034$) correlated with the total protein content in CSF specimens taken at admission but not in those from subsequent lumbar punctures. MMP-8 and TIMP-1 values did not correlate with protein levels at any time point (not shown). The CSF cell count (total cells, mononuclear cells, or neutrophils only) and the disease duration before admission did not relate to any of those parameters (TNF- α , MMP-9, MMP-8, or TIMP-1) at any time point (not shown). Furthermore, levels of MMP-8 and -9, TIMP-1, and TNF- α were not dependent on the type of infectious agent (in the first lumbar puncture specimen) or on antibiotic therapy (not shown).

Time course of MMP and TIMP-1 expression in patients with repeated lumbar punctures. The heterogeneity of the study population regarding acuity of disease at hospital admission rendered the collective comparison of kinetic regulation of MMPs, TIMP-1, and other parameters inconclusive. We therefore compared concentrations of CSF samples from individual patients who had serial lumbar punctures. MMP-9 and MMP-8 underwent rapid kinetic changes, but the absolute concentrations or relative changes did not relate to each other or to the CSF cell count (not shown). At admission, levels of MMP-9 were higher than those of MMP-8 in 3 patients and lower in

3 others (1 patient had equal amounts of MMP-9 and MMP-8 in the first lumbar puncture specimen). The relative kinetic changes of TNF- α and MMP-9 in individual patients who underwent repeated lumbar punctures were significantly correlated (ρ , 0.943; $P_{n=6} = .035$); this was not the case for TNF- α and MMP-8 (not shown).

Correlation of CSF levels of MMPs with long-term clinical outcome. To evaluate whether upregulation of MMPs represents a risk factor for the development of persistent neurological complications after BM, we compared levels of MMP-9 and MMP-8 in first available CSF samples from patients with ($n = 5$) and without ($n = 22$) neurological sequelae. Figure 1 *F* shows that median CSF concentrations of MMP-9 were 2.5 times higher ($P < .05$) in patients who developed long-term complications (120 ng/mL; range, 53–363 ng/mL) than in those who recovered without deficits (47.5 ng/mL; range, not detectable to 2461 ng/mL). In contrast, CSF levels of TIMP-1, MMP-8, and TNF- α , as well as the cell count and total protein content in the CSF, were not discriminative for the occurrence of neurological deficits in patients with BM (not shown).

Discussion

This study demonstrates the upregulation of MMP-9 and MMP-8 in CSF specimens from children with BM, whereas noninflammatory CSF samples from control patients scored negative for both proteases. The endogenous MMP inhibitor TIMP-1 is constitutively expressed in control CSF. In patients with BM, CSF concentrations of TIMP-1 increased in parallel with those of MMP-9, with a 10- to 20-fold molar excess. TIMP-1 forms heterodimers with MMP-9 and MMP-8; this formation decreases their detectability in the ELISAs used (information from manufacturers and [13]). This might explain why the levels of MMP-8 fell below the detection threshold in 3 CSF samples from patients with BM. In contrast, CSF concentrations of MMP-2, its inhibitor TIMP-2, and MMP-3 were not upregulated in cases of BM.

Apart from the role of MMPs in BBB breakdown [7, 22] and extravasation of inflammatory cells into the CNS, recent animal studies suggested a role of MMPs in glial and neuronal cell death [11], a feature of BM with serious clinical implications [24]. In patients with persistent hearing impairment and secondary epilepsy, the median CSF level of MMP-9 was >2.5 times higher than in patients who recovered without long-term neurological sequelae. Conversely, routine CSF parameters and levels of TNF- α were, as found by others [25], not indicative of persistent neurological deficits. Results of the present study suggest that excessive increase of MMP-9 is a risk factor for the development of postmeningitidal neurological sequelae.

CSF levels of MMP-9 in cases of BM were 10- to 1000-fold higher than in cases of viral meningitis [26] and multiple sclerosis [23]. Furthermore, BM is the first neuroinflammatory disease in which the upregulation of a second metalloproteinase, MMP-8,

in the CSF can be demonstrated. MMPs have been shown to mediate BBB damage in experimental BM [7] and in other animal models of neuroinflammation [8, 9, 11, 16]. That MMP-9 plays a role in BBB breakdown in human BM can be postulated by the correlation with CSF protein content in the present and earlier studies [7]. In light of the rapid kinetic changes of MMPs and the sustained BBB disruption in fully established BM, this correlation is restricted to early phases of disease.

MMP-9 and MMP-8 reach similar concentrations and may have a synergistic effect on BBB breakdown. MMP-8 targets primarily collagen types of interstitial extracellular matrix, and its increase may also mediate parenchymal damage. MMP-8 has been believed to be an exclusive product of granulocytes and was therefore initially named neutrophil collagenase. In acute BM, the cellular immune response is dominated by granulocytes, and upregulation of MMP-8 in the CSF is therefore not surprising. However, the lack of correlation between the number of CSF granulocytes and the level of MMP-8 in the collective analysis, as well as the comparison of kinetic changes in serial lumbar puncture specimens from individual patients, suggests that cells other than granulocytes could be additional sources of MMP-8. This is supported by the upregulation of MMP-8 in a model of autoimmune encephalitis in which granulocytes are not involved [11] and in various cell types in vitro [18].

TNF- α is a strong inducer of MMP-9 in vitro [15, 17, 19] and in experimental neuroinflammation [12, 16]. As in recent findings concerning animal BM [27], the 2 molecules are simultaneously upregulated in human disease. In rabbit models of BM, maximal levels of TNF- α occurred *before* invasion of leukocytes into the CSF was detectable [14, 28]. Accordingly, we have found that upregulation of MMP-9 in CSF occurs as early as 15 min after initiation of experimental BM [29]. Congruent with this, the findings from repeated lumbar punctures demonstrate that MMP-9 is upregulated independently of MMP-8.

Adjunctive therapy with corticosteroids is used in some centers with the aim of limiting the deleterious effects of an inflammatory host response in infant BM. According to some study findings, this therapeutic regimen correlates with reductions in mortality and long-term neurological morbidity [30]. However, corticosteroids may also exert detrimental effects, since they were shown to increase hippocampal neuronal loss in an animal model of BM [31]. Moreover, among patients whose therapy was begun in an advanced phase of BM, the incidence of deaths and neurological sequelae was higher in the group that received adjunctive corticosteroid therapy than for patients treated only with antibiotics [32].

Corticosteroids have been shown to suppress the expression of TNF- α [33] and MMP-9 [22] in the CSF during acute CNS inflammation. It is therefore conceivable that the beneficial effect of corticosteroids on long-term neurological sequelae is the result of their downregulatory effect on MMP expression, among other factors.

In experimental BM, synthetic inhibitors of MMPs effectively

reduced leukocyte transmigration of basal lamina layers [34]. In early phases of experimental BM, they decreased CSF pleocytosis and the development of intracranial pressure [7]. In rats with fully established BM, systemic pretreatment with the MMP inhibitor GM6001 lowered the CSF concentrations of MMP-9 and TNF- α , and, most important, diminished cortical damage [27]. Other than corticosteroids, which act predominantly on transcriptional regulation, MMP inhibitors have the conceptual advantage of directly antagonizing proteolytic activity of MMPs.

Present findings suggest a critical role for MMP-9 and MMP-8 as effectors of BBB damage and of neuronal injury in human BM. The evidence from this study and the results obtained in a number of models of BM provide a compelling molecular basis for future trials in which MMP inhibitors are used as adjunctive therapy for BM.

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