Differential expression of matrix metalloproteinases in bacterial meningitis

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Summary
Matrix metalloproteinases (MMPs) are implicated in the pathogenesis of various inflammatory diseases of the central nervous system. Evidence is accumulating that gelatinase B (MMP-9) might be involved in the pathogenesis of meningitis, but the spectrum of different MMPs involved in the inflammatory reaction of this disease has not been determined. We investigated the temporal and spatial mRNA expression pattern of gelatinase B in experimental meningococcal meningitis in rats. In contrast to controls, increased mRNA levels with peak values 6 h after injection with meningococci were found in brain specimens of the animals. Elevated MMP-9 mRNA expression was accompanied by enhanced proteolytic activity, as demonstrated by gelatin zymography, and positive immunoreactivity. The mRNA expression pattern of six other MMPs was investigated.

Keywords: gelatinase B; collagenase-3; stromelysin-1; bacterial meningitis; experimental meningitis

Abbreviations: MMP = matrix metalloproteinase; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; TNF-α = tumour necrosis factor-α; WBC = white blood cell

Introduction
The matrix metalloproteinases (MMPs) belong to a large family of zinc-dependent proteinases which includes the collagenases, gelatinases, stromelysins, matrilysin, membrane-type metalloproteinases and metalloelastase (Woessner, 1994; Birkedal-Hansen, 1995; Yong et al., 1998). Various MMPs have been identified to date which have structural domains in common but differ in cellular sources, substrate specificity and inducibility (Goetzl et al., 1996). However, knowledge about their involvement in physiology as well as pathological conditions remains incomplete. An emerging body of evidence points to MMPs as important factors in the pathogenesis of meningitis. Increased levels of gelatinase B (MMP-9) were detectable in CSF samples from patients suffering from viral (Kolb et al., 1998) or bacterial (Gijbels et al., 1992) meningitis. Furthermore, application of the synthetic broad-spectrum MMP inhibitor batimastat (BB-94) in a rat model of meningococcal meningitis significantly reduced blood–brain barrier disruption and the disease-characteristic increase in intracranial pressure (Paul et al., 1998). Breakdown of the blood–brain barrier is a typical histopathological feature in meningitis, giving rise to the development of vasogenic oedema (Quagliarello and Scheld, 1992). The notion that MMPs play an important role in blood–brain barrier disruption is supported by studies in experimental autoimmune encephalomyelitis. In this animal model of multiple sclerosis, non-specific inhibition of MMPs was shown to protect the blood–brain barrier (Gijbels et al., 1994; Hewson et al., 1995). Among all known MMPs, gelatinase B might be particularly involved in blood–brain barrier disturbance, as raised CSF levels of MMP-9 in MS...
patients are associated with a leaky blood–brain barrier on magnetic resonance imaging (Rosenberg et al., 1996). However, while there is some evidence suggestive of increased gelatinase activity in meningitis, the mRNA expression pattern of gelatinase B and other MMPs in meningitis has not been explored so far. The present study focuses on this important issue in a rat model of meningococcal meningitis, since such information might help in the design of novel MMP-targeted therapies for a disorder that, despite improved antimicrobial therapy, is still associated with high mortality (Quagliarello and Scheld, 1997).

Material and methods

Animals

Male Wistar rats (Charles Rivers, Sulzfeld, Germany) weighing 250–320 g were used. The group challenged with meningococci consisted of eight animals, while four served as controls. In addition, two animals were used to assess the integrity of the blood–brain barrier. All experiments were conducted according to local state regulations for animal experimentation and were approved by the responsible authorities.

Induction of meningococcal meningitis

Bacterial meningitis was induced as described previously (Pfister et al., 1990). Briefly, rats were anaesthetized with thiopental (Trapanal, Byk Gulden, Germany; 100 mg/kg, intraperitoneally), tracheotomized and artificially ventilated. The left femoral artery was cannulated for continuous monitoring of the mean arterial blood pressure and for hourly blood gas analysis. The left femoral vein was cannulated for fluid substitution. Through a burr hole at the occipital bone, a catheter was inserted into the cisterna magna for continuous intracranial pressure monitoring, injection of meningococci and the determination of white blood cell (WBC) counts in the CSF. During the experiment all animals were kept at a constant temperature of 37.5 ± 0.5°C using a rectal thermometer-controlled heating pad. Three, six and nine hours after induction of the disease, given numbers of animals were killed, and each brain was flash-frozen in liquid nitrogen and stored at –70°C.

Intracisternal inoculum

Heat-killed (60°C for 4 h) Neisseria meningitidis (serogroup B, strain B1940) were used for intracisternal application. Before use, the bacteria were subcultured on blood-agar plates and resuspended in phosphate-buffered saline (PBS) (Pfister et al., 1990). Rats were challenged intracisternally with 100 µl of 10⁹ colony-forming units/ml of heat-killed meningococci. Control animals were treated with the same volume of PBS.

Competitive PCR

For quantitation of rat MMP mRNA levels, a polymerase chain reaction (PCR) using a multi-competitor DNA standard was performed (Wells et al., 1996). The synthetic standard DNA contained tandem arrays of 5' and 3' priming sites for cDNAs of different matrix metalloproteinases and β-actin. Primer pairs for collagenase-3, matrilysin, gelatinases A and B, stromelysin-1, -2 and -3 and β-actin were used according to the sequences published elsewhere (Wells et al., 1996). Poly(A⁺) RNA was extracted from frozen brain specimens and used as a template for cDNA synthesis using AMV (avian myeloblastosis virus) reverse transcriptase (Promega, Madison, Wis., USA). Threefold serial dilutions of competitive standard DNA were combined with a fixed amount of sample cDNA, and PCR was performed in 50-µl reactions containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris–HCl (pH 9.0) and 0.1% Triton X-100 in the presence of 200 µM dNTP (deoxyribonucleotide triphosphate; Pharmacia, Freiburg, Germany), 50 pmol of sense and antisense MMP primers, 1 U Taq DNA polymerase (Perkin Elmer, Branchburg, NJ, USA) and 1 µCi [α-³²P]dCTP (Amersham, Braunschweig, Germany). Amplification was carried out using 35 cycles (95°C, 30 s/57°C, 30 s/72°C, 120 s) in a Hybaid Omnigene thermal cycler (MWG Biotech, Ebersberg, Germany). Ten microlitres of the reaction products was electrophoresed on a 6% polyacrylamide gel. Gels were exposed to appropriate screens, and the uptake of [α-³²P]dCTP within each individual PCR product was determined using a phosphor imaging system (Storm 360; Molecular Dynamics, Krefeld, Germany). Levels of MMP mRNA were determined by plotting the ratio of sample cDNA to standard DNA against the standard dilution using a double logarithmic scale.

Gelatin zymography in the animal model

Gelatinase activity was determined by SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) zymography (Brown et al., 1990). In brief, 10 × 10 µm frozen sections of brain were incubated with 25 µl of Tris/glycine SDS sample buffer (Novex, San Diego, Calif., USA), degraded by ultrasound and further homogenized by vortexing for 1 h at 4°C. The samples were applied to a 10% (w/v) polyacrylamide resolving gel containing 0.1% SDS and 0.1% gelatin type A from porcine skin (Sigma, St Louis, Mo., USA). Stacking gels were 3% (w/v) polyacrylamide. After electrophoresis, gels were washed in renaturing buffer (Novex) containing Triton X-100 to remove any SDS and incubated in developing buffer (Novex) for 18 h at 37°C. Gels were stained for 6 h in 30% methanol/10% acetic acid containing 0.5% (w/v) Coomassie brilliant blue G-250 and destained in the same buffer without dye. Gelatinase activity was detected as unstained bands on a blue background representing areas of gelatin digestion.

Supernatants from astrocyte cell cultures stimulated with tumour necrosis factor-α (TNF-α) served as positive controls.
Matrix metalloproteinases in meningitis

Table 1 Clinical characteristics of patients with bacterial meningitis and non-inflammatory neurological disorders (controls)

<table>
<thead>
<tr>
<th>Patient group</th>
<th>CSF WBC count (cells/µl)</th>
<th>CSF total protein (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial meningitis: 1st LP</td>
<td>2465 (324–8010)</td>
<td>1.87 (1.23–3.37)</td>
</tr>
<tr>
<td>Bacterial meningitis: 2nd LP</td>
<td>315 (132–827)</td>
<td>1.09 (0.6–2.14)</td>
</tr>
<tr>
<td>Control</td>
<td>2 (0–4)</td>
<td>0.38 (0.22–0.49)</td>
</tr>
</tbody>
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Values are given as medians and ranges. WBC = white blood cells; LP = lumbar puncture.

Immunohistochemistry
Frozen sections (10 µm) of rat brain were air-dried and fixed for 10 min in 4% formalin and 2 min in each of 50, 100 and 50% acetone at room temperature. A monoclonal affinity-purified antibody against gelatinase B (R&D Systems, Abingdon, UK) was used at 1.2 µg/ml overnight at 4°C. Thereafter a biotinylated secondary antibody against mouse IgG and an avidin-biotinylated peroxidase complex (Dako, Hamburg, Germany) were used with 3,3'-diaminobenzidine as peroxidase substrate. Endogenous peroxidase activity was suppressed by incubating the sections with 3% H₂O₂ in methanol for 10 min before adding the secondary antibody. Sections were counterstained with haematoxylin, dehydrated, and mounted in Eukitt (Kindler, Freiburg, Germany).

Assessment of blood–brain barrier integrity
To verify disruption of the blood–brain barrier in diseased animals, an additional number of control and meningococcus-injected animals were investigated. Meningococcal meningitis was induced as described above, but 1 h prior to termination of the experiment, 1 ml of 1% (w/v) Evans blue (Sigma Chemicals, Deisenhofen, Germany) was injected intravenously. Six hours after challenge with meningococci, the animals were perfused with 100 ml of ice-cold PBS and the brain was removed and stored at –70°C for further histological evaluation. Frozen sections (10 µm) of the brains were fixed for 20 min with 100% ethanol. Evans blue was observed under green fluorescence microscopy (excitation filter 545 nm, barrier filter 590 nm) and appeared red.

Patients and CSF sampling
Fourteen CSF samples were obtained from 10 patients that suffered from bacterial meningitis (Table 1). Follow-up investigations were performed on four patients. Samples from five patients with non-inflammatory neurological diseases served as controls. After lumbar puncture, CSF samples were immediately centrifuged at 3000 g for 10 min, and supernatants were aliquoted and stored at –70°C. All patients gave informed consent and the study was approved by the local ethics committee.

Gelatin substrate zymography in human CSF samples
Human gelatinase activity was measured as described above using 10 µl of undiluted CSF.

Statistical analysis
One-way analysis of variance and Student–Newman–Keuls multiple comparisons were used as the principal statistical tests. A P value < 0.05 was considered significant. Data are given as mean ± standard error.

Results
Induction of meningococcal meningitis
Intracisternal meningococcal injection in the animals did not result in changes in p(O₂), p(CO₂), pH and mean arterial blood pressure compared with the control group (all results were within normal ranges; data not shown). However, a significant increase in WBC counts in CSF (4229 ± 2959 cells/µl 3 h, 8250 ± 2804 cells/µl 6 h and 7728 ± 5634 cells/µl 9 h after intracisternal injection, compared with 30 ± 11 cells/µl in controls; P < 0.05) and in intracranial pressure (15.8 ± 3.1 mmHg 6 h after intracisternal injection of meningococci compared with 3.2 ± 0.9 mmHg in the control group; P < 0.05) occurred in animals injected with meningococci.

Gelatinase B
Quantitation of mRNA
A constitutive low level of expression of gelatinase B mRNA was found in the PBS-injected animals that served as controls. In contrast, elevated levels were already detected in animals 3 h after injection with meningococci, with peak mRNA levels 6 h after intracisternal injection, compared with 30 ± 11 cells/µl in controls; P < 0.05) and in intracranial pressure (15.8 ± 3.1 mmHg 6 h after intracisternal injection of meningococci compared with 3.2 ± 0.9 mmHg in the control group; P < 0.05) occurred in animals injected with meningococci.

Proteolytic activity in the animal model
To demonstrate that increased expression of gelatinase B mRNA was associated with enhanced enzymatic activity, zymography of brain tissue taken from animals injected with meningococci was performed. A pronounced rise in gelatinase B activity was observed in meningococcus-injected animals compared with control animals, in line with the mRNA data obtained by competitive PCR. Thus, higher expression of mRNA was accompanied by augmented gelatin digestion.
In contrast, no increase in the activity of gelatinase A, which is detected in the same experiment, could be found using this assay (Fig. 2).
Fig. 1 MMP mRNA expression in brain specimens of Wistar rats injected with heat-killed meningococci (HKM) and PBS (control). Increased mRNA expression of (A) gelatinase B can be detected 3 h after intracisternal injection of meningococci, with maximum levels after 6 h. The mRNAs for (B) stromelysin-1 and (C) collagenase-3 were upregulated at all time points studied. Results are given as mean ± SD. Values for 3 h before intracisternal injection and controls (6 h after injection of PBS) represent data from four animals.

Proteolytic activity in human CSF samples

In CSF samples taken from patients with bacterial meningitis, augmented activity of gelatinase B was recorded in comparison with non-inflammatory controls. In cases where repeated puncture was carried out, a pronounced decrease in proteolytic activity was detected at the second time point compared with the first. In contrast, the activity of gelatinase A appeared to be unchanged between meningitis and control cases, the size of the unstained bands remaining constant (Fig. 3).

Immunohistochemistry

In control animals no immunoreactivity for gelatinase B could be detected. In contrast, positive signals were noted in diseased animals, primarily around the blood vessels and localized to cells with polymorphic or round nuclei, most likely representing invading neutrophils and mononuclear cells (Fig. 4). Control sections after omission of the primary antibody showed only background staining.

Assessment of blood–brain barrier integrity

Weak Evans blue fluorescence was demonstrable in the subarachnoid space of control rats. No extravasation of the dye could be observed in the underlying brain parenchyma. In contrast, enhanced staining within the leptomeninges and a diffuse extravasation of Evans blue dye in the underlying brain parenchyma, indicating disruption of the blood–brain barrier, was detected in meningococcus-injected rats 6 h after challenge (Fig. 5).

Other matrix metalloproteinases

To investigate whether MMPs other than MMP-9 are regulated in bacterial meningitis, we studied the mRNA expression of collagenase-3, stromelysin-1, -2 and -3, gelatinase A and matrilysin. The last four MMPs were constitutively expressed at low levels, and did not exhibit any difference in mRNA levels between the experimental and the control group. The constitutive low mRNA expression pattern of gelatinase A underlines the finding that no change in protease activity measured by gelatin zymography could be detected for this MMP within the brain specimens.
In contrast, mRNAs for collagenase-3 and stromelysin-1 were upregulated 3, 6 and 9 h after injection of meningococci (Fig. 1).

Discussion
In the present study the expression pattern of gelatinase B and six other MMPs in experimental meningitis was investigated. In meningococcus-injected animals, upregulation of MMP-9 mRNA was found, associated with increased enzymatic activity and protein immunoreactivity. On immunohistochemistry, positive signals for MMP-9 could be localized along the meninges and to cellular structures suggestive of infiltrating polymorphonuclear and mononuclear cells. Given that monocytes (Welgus et al., 1990), T cells (Leppert et al., 1995), neutrophils (Masure et al., 1991) and macrophages (Nielsen et al., 1996) are known to be capable of producing MMP-9, we assume that these cells, and especially neutrophils, which are important effector cells in mediating the pathogenesis of bacterial meningitis, have to be considered as the primary sources of MMP-9 in the present study. Degranulation of neutrophil gelatinase B appears to be a fast process, since increased gelatinase B mRNA as well as proteolytic activity could already be detected 3 h after intracisternal challenge with meningococci. Since heat-killed meningococci were used in the present experiment, the decrease in MMP expression noticeable 9 h after intracisternal injection reflects a decrease in immunoreactivity when no bacteria are present to perpetuate the infection any further.

It should be noted also that astrocytes (Wells et al., 1996), microglia (Gottschall and Yu, 1995) and endothelial cells (Herron et al., 1986) have been reported to synthesize gelatinase B after stimulation with various cytokines, such as interleukin (IL)-1 and TNF-α in vitro; however, there is no histological evidence that these cells were sources of this metalloproteinase in the current study.

Gelatin zymography revealed that the upregulation of the message accompanied enhanced proteolytic activity of MMP-9, underscoring the pathogenic relevance of this finding. In the CSF of animals with bacterial meningitis, increased proteolytic activity of MMP-9 and MMP-2 has been observed (Paul et al., 1998). Upregulation of MMP-9 mRNA, augmented proteolytic activity and positive immunoreactivity were accompanied by both a significant increase in CSF WBC counts and raised intracranial pressure. These findings point to a potential role of MMP-9 in the pathogenesis of meningitis. Breakdown of the blood–brain barrier is a pathological hallmark in bacterial meningitis that (i) leads to vasogenic brain oedema with a consecutive increase in the intracranial pressure, and (ii) facilitates leucocyte recruitment across the endothelium into the CSF (Gray and Nordmann, 1997). Studies in experimental meningitis support the concept that endogenous inflammatory mediators play a crucial role in promoting impairment of the blood–brain barrier. Waage and coworkers demonstrated release of TNF-α, IL-1 and IL-6 into the CSF after intracisternal inoculation of meningoococcal lipopolysaccharide into rabbits (Waage et al., 1989). Intracisternal challenge of rats by IL-1 precipitated blood–brain barrier disturbance in a dose-dependent manner, an effect which was synergistically enhanced by the additional administration of TNF-α (Quagliarello et al., 1991). The mediators thought to be involved in cytokine modulation of capillary permeability include MMPs, and it has been suggested that these proteases play a central role in the opening of the blood–brain barrier. Intracerebral injection or induction of gelatinases A and B resulted in the breakdown of the extracellular matrix and the opening of the blood–brain barrier in rats (Rosenberg et al., 1992, 1994). Our data, showing upregulated gelatinase B mRNA and augmented proteolytic activity in association with increased intracranial pressure and Evans blue extravasation in experimental meningitis, support the concept that MMP-9 is a key mediator of blood–brain barrier breakdown.

Knowledge about the mechanisms involved in the passage of leucocytes across the endothelium, leading to increased WBC counts in the CSF, is still incomplete. It is commonly accepted that the process of homing and transmigration of circulating leucocytes through the blood–brain barrier encompasses the complex interaction of a variety of adhesion molecules and different cytokines (Hartung, 1995; Archelos and Hartung, 1997). Some of these adhesion molecules and cytokines are also known to regulate MMP expression (Romanic and Madri, 1994). Various studies point to the involvement of gelatinase B in the migration of leucocytes.

![Fig. 3 Detection of proteolytic activity in CSF samples from patients with bacterial meningitis (lanes 1–10) and controls (lanes 11–15). Follow-up investigations were performed on the first four patients (a = first lumbar puncture; b = second lumbar puncture). Molecular mass is indicated by arrows at 72 kDa for gelatinase A and 92 kDa for gelatinase B.](image-url)
Fig. 4 Immunohistochemistry for gelatinase B in frozen sections of brain specimens from Wistar rats. Positive signals occur primarily along the meninges 3 h after intracisternal injection of meningococci (A), with an increase in density of immunoreactivity 6 h after injection (B), followed by decreasing positive signal intensity 9 h after intracisternal injection of meningococci (C). Control animals do not reveal any positive immunoreactivity (D). Higher magnification of the meninges in animals injected with meningococci clearly links positive immunoreactivity to infiltrating polymorphonuclear and mononuclear cells, marked with arrows; arrowheads point to cells without immunoreactivity (E). Magnification: A and C, ×200; B and D, ×400; E, ×600.

through artificial basement membrane systems in vitro (Huber and Weiss, 1989; Leppert et al., 1995). Since migration of inflammatory cells from the blood to the CNS is believed to be of paramount importance in the genesis of an inflammatory reaction along the meninges, such an action would assign MMPs a strategic role in this process. The upregulation of gelatinase B associated with a significantly increased WBC count in the CSF of diseased animals, as demonstrated in the present study, supports this notion.

Finally, MMP-9 might be involved in the cleavage or regulation of proteins including type IV collagen of the basement membrane, myelin proteins, and cytokines (Opdenakker and Van Damme, 1994; Ries and Petrides, 1995). Release of the proinflammatory cytokine TNF-α appears to depend on an MMP-like mechanism of action (Gearing et al., 1994; Mohler et al., 1994), emphasizing the complexity of interaction between cytokines and MMPs in neuroinflammation. However, the recently described TNF-α-converting enzyme (TACE) seems more likely to be a novel metalloproteinase, distinct from the classical MMPs (Black et al., 1997; Moss et al., 1997). Thus, it is difficult at present to determine the contributory effect of known MMPs to the
release of surface cytokines and receptors, which play an important role in the modulation of the inflammatory response. Further studies are needed to shed light on the involvement of MMPs in this process.

To demonstrate that our finding of upregulated gelatinase B is not restricted to the experimental model but also has clinical relevance, we performed gelatinase zymography in CSF samples from patients with bacterial meningitis. The markedly increased proteolytic activity of MMP-9 detectable in the acutely ill patients, with an obvious decrease following treatment, and our experimental data drawn from the rat model, reveal, at least in part, similarities between this model and the human disease. This finding is consistent with previous observations in patients suffering from viral meningitis: biology, pathogenesis and therapeutic implications. Mol Med Today 1997; 3: 310–21.

Fig. 5 Assessment of blood–brain barrier integrity. Only weak Evans blue fluorescence is observed in the arachnoid space of control animals (A), whereas in meningococcus-injected rats there is enhanced staining within the leptomeninges and diffuse extravasation of Evans blue dye in the underlying brain parenchyma, indicating disruption of the blood–brain barrier (B). Magnification: A and B, ×200.

in the present study deviates from the pattern of MMP mRNA expression described in experimental autoimmune encephalomyelitis (Kieseier et al., 1998), an inflammatory demyelinating disease associated with blood–brain barrier breakdown.

The role of selectively upregulated collagenase-3 and stromelysin-1 in the inflammatory reaction remains conjectural. Both MMPs could be implicated in the disruption of the blood–brain barrier, since collagenase-3 is known to degrade various collagens and gelatins such as stromelysin-1, which has been reported to degrade fibronectin, laminin, elastin, gelatin and different collagens (Cawston, 1998). In addition, increased mRNA expression of collagenase-3 and stromelysin-1, as seen in our study, may be involved in controlling the inflammatory reaction, as both metalloproteinases are capable of activating progelatinase B, the latent proform of MMP-9, by sequential cleavage of the propeptide (Okada et al., 1992; Knauper et al., 1997). Furthermore, it has been demonstrated that the pro-inflammatory cytokines IL-1 and IL-6, which have been implicated as playing a disease-promoting role in bacterial meningitis, can induce the expression of stromelysin-1, collagenase-3 and gelatinase B (Kusano et al., 1998).

Along with the study by Paul and colleagues (Paul et al., 1998), demonstrating that MMP inhibition effectively reduces blood–brain barrier damage, the present study provides strong circumstantial evidence that MMPs play a critical role in experimental meningitis. Disruption of the blood–brain barrier is a crucial step in the pathogenesis of this disease, and experimental studies provide evidence that various mediators, such as nitric oxide (Koedel et al., 1995; Paul et al., 1997), reactive oxygen species (Pfister et al., 1992; Koedel and Pfister, 1997) and prostaglandin E2 (Pfister et al., 1990), are involved in this process. The present findings emphasize the role of MMPs in the disruption of the blood–brain barrier. Further research is needed to elucidate the precise mechanisms of action and the interplay between MMPs and other factors involved in damage to the blood–brain barrier. It is hoped that increased knowledge of MMP-mediated actions in the pathogenesis of meningitis will aid in the design of inhibitors targeted to specific MMPs that could be administered at critical points in the evolution of this inflammatory disease, and that such compounds may enlarge our therapeutic arsenal to improve the clinical outcome, frequently still dubious, in bacterial meningitis.

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