GELATINASE ACTIVITIY OF MATRIX METALLOPROTEINASES IN THE CEREBROSPINAL FLUID OF VARIOUS PATIENT POPULATIONS

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ABSTRACT

We have studied the enzymatic gelatinolytic activity of matrix metalloproteinases (MMPs) present in cerebrospinal fluid (CSF) of samples obtained from 67 individuals, twenty-one nonneurological patients (considered controls) and 46 subjects with various neurological disorders e.g., vascular lesions, demyelination, inflammatory, degenerative and prion diseases. Biochemical characterization of MMPs, a family of neutral proteolytic enzymes involved in extracellular matrix modeling, included determination of substrate specificity and Ca^{+2} dependency, as well as the effects of protease inactivators, carboxylic and *His* (histidine) residue modifiers, and antibiotics. Whereas all CSF samples expressed MMP-2 (gelatinase A) activity, it corresponded in most cases (normal and pathological samples) to its latent form (proenzyme; pMMP-2). In general, inflammatory neurological diseases (especially meningitis and neurocisticercosis) were associated with the presence of a second enzyme, MMP-9 (or gelatinase B). Whereas MMP-9 was found in the CSF of every tropical spastic paraparesis patient studied, its presence in samples from individuals with vascular lesions was uncommon. Patients blood-brain barrier damage was ascertained by determining total CSF protein content using both, the conventional polyacrylamide gel electrophoresis procedure under denaturing conditions and capillary zone electrophoresis.

INTRODUCTION

Matrix metalloproteinases (MMPs) have been shown to play a role in numerous physiological and pathological conditions involving remodeling of various tissues' extracellular matrix (ECM) (Matrisian, 1990; Woessner, 1991; Parsons et al., 1997). Enzymes such as MMP-2 (72 kDa gelatinase; type IV collagenase A, or gelatinase A) and MMP-9 (92 kDa gelatinase; type IV collagenase B or gelatinase B) are members of the

gelatinase family whose substrates include types IV and V collagen, fibronectic, elastin and gelatin (Woessner, 1991; Parson et al., 1997). The catalytic domain of these enzymes, which are highly regulated at various levels e.g., genetic expression, proteolytic activity, including activation of the secreted latent enzymes or pro-enzymes, and by various endogenous tissue inhibitors (TIMP) (Parsons et al., 1997), contains conserved *His* and *Asp* residues (Matrisian, 1990; Woessner, 1991).

In the central nervous system (CNS), collagen, fibronectic and laminin are generally restricted to vascular and connective tissue stromal elements (Maeda and Sobel, 1996). In response to injury, axonal degeneration and regeneration leads to remodeling within the nerve, a process associated with the release of proteolytic enzymes and their inhibitors (La Fleur el al., 1996). The recruitment of macrophages to injured nerves play an important role in the removal of axonal and myelin debris, as well as in the production of mitogenic factors for Schwann cells and fibroblasts, thus potentiating the rate of nerve regeneration (La Fleur *et al.*, 1996).

The blood-brain barrier (BBB) is largely responsible for maintaining homeostasis of the CNS environment (De Vries et al., 1997). It has been suggested that perivascular astrocytes regulate endothelial barrier expression and cooperate in eliciting developmental and pathological neovascular responses in brain (Rao et al., 1996). CNS inflammation is accompanied by extensive leukocyte migration into the injured brain area, a process often accompanied by an increased influx of serum proteins into the CSF. Besides cerebral endothelial cells, other cell types e.g., microglial cells and perivascular macrophages may eventually be involved in the neuroimune response (De Vries et al., 1997). Gelatinases A and B have been detected predominantly in astrocytes and microglia throughout white matter obtained from nonneurological patients (controls); additionally, positive mononuclear cells are also present in the perivascular cuffs from white matter samples obtained from multiple sclerosis (MS) patients (Cuzner et al., 1996). In the demyelinating lesion there is prominent, widespread expression of MMP-9 in reactive astrocytes and macrophages.

A number of CNS diseases appear to be accompanied by significant changes in the pattern of CSF's MMPs e.g., CSF of Lyme neuroborreliosis patients contained MMP-2, MMP-9 and gelatinolytic activity at 130 and 250 kDa (Perides et al., 1998), a specific CSF MMPs profile has been reported to correlate with the presence of malignant astrocytomas, brain metastases, and carcinomatous meningitis (Friedberg et al., 1998), patients with positive CSF cytologies show activated MMP-2 (Friedberg et al., 1998), and upregulation of MMP-9 and TIMP-1 in CSF was observed in viral meningitis (Kolb et al., 1998). The additional presence of MMP-9 activity in the CSF of HIV-infected patients, which is associated with an increased white blood cell count and elevated amounts of human serum albumin (HSA), suggest that increased MMP activity may play a role in BBB leakage in HIV- positive individuals (Sporer et al., 1998). Preliminary studies on patients with tropical spastic paraparesis (TSP) suggests, depending on the patient's clinical status, a better correlation of the existence of this condition with the CSF's presence of MMP-9 than with MS (Giraudon et al., 1998). Occurrence of this slowly progressive neurologic disease has been suggested to be associated with the presence of human T cell leukemia virus type 1 (HAM/TSP) (Miura et al., 1997).

In this study we have biochemically characterized various MMPs present in the CSF, and identified the presence of MMP-9 in patients with vascular, demyelination, neuroinflammatory (meningitis, encephalitis, neuritis, neurocisticercosis), and TSP diseases.

MATERIALS AND METHODS

CSF samples were obtained from neurological patients as part of normal diagnostic procedures (n=46; atraumatic lumbar puncture) or from nonneurological patients (n=21; epidural anesthesia, used as controls), and immediately frozen (-20 °C). Patients were duly informed of this research protocol and each and everyone freely agreed to participate in the study. Cytosis and total protein (Lowry et al., 1951) were routinely measured. In addition, the integrity of the BBB was determined by gel electrophoresis [sodium dodecyl sulfate-polyacrylamide (SDS/PAGE)] (Laemmli, 1970) and by HSA quantification using capillary zone electrophoresis (CZE) (Wehr et al., 1997). In brief, CZE was carried out using a fused silica uncoated capillary (75 um in diameter and 68 cm long), an electrolyte solution containing 150 mM phosphoric acid, 5% acetonitrile, 4.58 mM n-octylamine and 10% methanol, and voltage of 18 kV. HSA peak was assigned by comparison with that of added internal standard (analysis was done using Millennium software from Waters and Associates). Proteins with gelatinolytic or neutral non-specific proteolytic activity were identified using SDS polyacrylamide gels (7.5% polyacrylamide) containing gelatin, collagen I, collagen V or other proteins (1 mg/ml) like casein, bovine serum albumin (BSA), and ovoalbumin (Galis et al., 1994). After electrophoresis, gels were washed to remove the SDS (30 min incubation with 2.5% Triton X-100), incubated overnight to develop the enzymatic activity (17 h at 37 °C in a 50 mM Tris-HCl, 5 mM $CaCl_2$, 0.02% NaN_3, pH 7.5 solution), stained (Coomassie brilliant Blue R-250), and destained (7 % acetic acid). Proteolytic activity was detected as unstained bands on a blue background. The effect of various protease inactivators, chemical modifiers of amino acid residues and metal ion requirements, was assessed after adding these agents to the overnight incubation medium. Blockage of hydroxyl groups and sulfhydryl residues was performed with 0.2 mM p-methylsulphonyl fluoride (PMSF) and 1.0 mM *bis*-dithionitrobenzoic acid (DTNB), respectively (Means and Feeney, 1964). Modification of acid amino acids and *His* residues was accomplished using 50 mM water soluble carbodiimide (WSC) in the presence of either 1 M methylglycine ester or 50 mM diethylpirocarbonate (DEP), respectively (Means and Feeney, 1964). The inhibitory effect of 2 mM tetracycline and 4 mM doxycycline was also tested by their addition to the assay medium. The effect of mercaptoethanol or dithiotreitol in the electrophoresis process was examined by their addition to the sample buffer, which contained 60% sucrose, 0.1% SDS and bromophenol blue. To detect the active form of MMPs in CSF, samples were incubated (1 to 48h at 37 °C) with 1 mM aminophenylmercuricacetate (APMA) previous to the electrophoresis asay (Rucklidge et al., 1990).

RESULTS

Biochemical Characterization of Gelatinolytic Activity

1. Substrate Specificity

Fig. 1 was obtained using an assay which detects both the active and zymogen (latent, pMMP) forms of MMPs. It shows a similar proteolytic activity on gelatin and collagen V for CFS samples obtained from a control individual and from a patient with an inflammatory disease presenting both MMP-2 and MMP-9 activity. Both samples, however showed a fairly poor activity on collagen I, and failed to hydrolyze casein, bovine serum albumin (BSA) or ovoalbumin (results not shown); these results agree with previously reported MMP-2 and MMP-9 specificity.

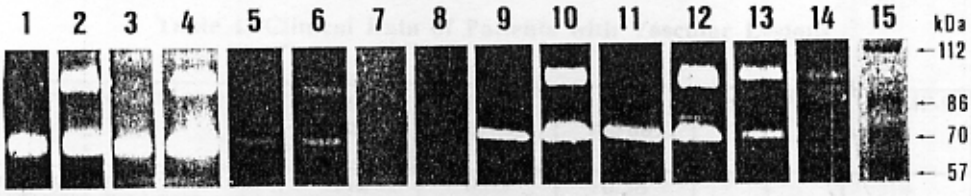

Figure 1. Zymograms of CSF from a control individual (odd numbers, single proteolytic band except # 13 and 15), and of a TSP patient (pair numbers, two proteolytic bands). A) Gels (lanes 1 through 6) were prepared in the 1% presence of either gelatin, collagen type V or collagen type I. Activity was developed as described in Materials and Methods. B) The same samples run in gels prepared with 1% gelatin and developed in the presence of either 10 mM EDTA (lanes 7 and 8), or of two irreversible proteinase inhibitors [(0.2 mM PMSF (lanes 9 and 10) or 1 mM DTNB (lanes 11 and 12)]. C) Samples (see above) previously incubated with 1 mM APMA (48 h at 37 °C; lanes 13 and 14). Lane 15 show prestained molecular weight standards.

2. Metal Ion Dependence and Effect of Chemical Modifiers and Antibiotics

Gel incubation in the presence of 10 mM ethylenediaminetetraacetic acid (EDTA) completely abolished the activity of both MMPs. Whereas CSF proteolytic activity was essentially unchanged by irreversible seryl and sulfhydryl protease inhibitors (PMSF and DTNB, respectively) (Figure 1), it almost completely disappeared in samples pretreated with the reducing agents mercaptoethanol or dithiotreitol (data not shown). Chemical modification of acid residues and *His* (with WSC and DEP, respectively) results in a complete blockage of MMP-2 and MMP-9 hydrolytic activity. Incubation with 2 mM tetracycline reduced significantly MMP activity, however a larger concentration of doxycycline was required (4 mM) to produce some measurable inhibitory effects.

3. Activation of Latent MMPs with APMA

APMA CSF incubation (from 1 to 48h; 37 °C) prior to electrophoretic analysis, resulted in partial activation of both pMMP-2 and pMMP-9; a significant increased MMP-2 to MMP-9 proenzyme form ratio was seen

after 24 h pretreatment. In these conditions, extrapolated pMMP-2 (70 kDa) and pMMP-9 (100 kDa) Mr (molecular weight) were decreased to 67 kDa and 90 kDa, respectively. Fig. 1 shows the partial activation after 48h preincubation.

Zimographies of CSF samples obtained from Control and Patients

Controls samples [CSF HSA, average ± SD of 0.13 mg/mL ± 0.05] showed only MMP-2 activity, mostly in its latent form, with some lower Mr activity visualized in just 3 of the 22 specimens tested. Validation of results obtained using CZE was accomplished by analyzing the same CSF samples by a conventional electrophoretic procedure under denaturing conditions (SDS/PAGE) and measuring the protein band corresponding to HSA.

Table 1. Clinical Data of Patients with Vascular Lesions

Diagnosis (sex and age)	Cytosis cells/ul	Protein mg/mL	HSA mg/mL	pMMP-2	pMMP-9
Subarach.hemorrh.(F, 82)	112	0.33	0.38	+	+
Trunk Infaction (F, 20)	18	1.60	2.00	+	+
Trunk Infarction (M, 84)	1	0.29	0.22	+	-
Trunk Infarction (M, 50)	0	0.15	0.18	+	Weak
Infarction,VasculitisF,19)	3	0.41	0.36	+	Weak
Cerebral Infarction(M, 44)	3	0.44	0.35	+	-
Cerebral Infarction (F, 54)	0.8	0.18	0.12	+	-
Cerebral Infarction(M, 76)	0	0.33	0.29	+	+
Cerebral Infarction (F, 82)	0	0.32	0.37	+	Weak
Cerebral Infarction (F, 75)	0	0.35	0.33	+	-
Cerebral Infarction (F, 38)	0	0.39	0.28	+	-
Cerebellum Infarc. (M, 57)	2	0.10	0.11	+	Weak
Cerebellum C Infarc. (F, 53)	0	0.14	0.12	+	-
Multiple Infarction (F, 47)	2	0.18	0.14	+	-
Migraine, Infarction (F, 24)	0.4	0.36	0.24	+	-
Parietal Hemorrhage (F, 55)	0	0.23	0.17	+	-

Data from 16 patients with vascular lesions (Table 1) indicates that only two cases with abnormal cytosis values (cells/ul of 112 and 18) showed similar, positive pMMP-2 and pMMP-9 activity. A weak pMMP-9 band was observed in four samples; in two of them, this finding can be attributed to the lower number of white cells present. A pMMP-9 band, as strong as the normal pMMP-2, was visualized in a single patient without abnormal cytosis, and corresponds to a sample from the only patient within this group with a damaged BBB.

Table 2. Clinical Data of Patients with Inflammatory Alterations Produced by Infectious [1] Diseases.

Diagnosis (sex and age)	Cytosis cells/ul	Protein mg/mL	HSA mg/mL	pMMP-2	pMMP-9
Viral Meningitis (F, 27)	142	0.35	0.34	+	+
Viral Meningitis (F, 44)	74	0.36	0.31	+	+
Viral Encephalitis (F, 17)	0	0.23	0.23	+	-
HIV(+), Meningitis Cryptococ. (M, 36)	46	0.72	0.60	+	+
HIV(+), Motor Complex (M, 33)	0	0.16	0.21	+	-
HIV(+), Motor Complex (M, 30)	0	0.38	0.29	+	+
Chronic Meningitis (F, 57)	60	1.50	1.53	+	+
Chronic Meningitis (F, 67)	32	0.40	0.45	+	-
Chronic Meningitis (F, 53)	19	0.27	0.30	+	+
Neurolues (F, 53)	96	0.22	0.18	+	+
Neurolues (M, 54)	3.2	0.54	0.43	+	+
Neurocisticercosis (M, 26)	174	1.10	1.20	+	+
Neurocisticercosis (M, 57)	59	0.50	0.41	+	+[2]
Neurocisticercosis (F, 57)	14	1.10	1.00	+	+[2]
Neurocisticercosis (F, 29)	3.2	0.32	0.34	+	-
Creutzfeldt-Jacob (M, 61)	9.6	0.52	0.49	+	+
Creutzfeldt-Jacob (F, 57)	2.4	0.78	0.63	+	+[2]

[1]Caused by virus, bacteria, fungi, parasites or prions. [2]In addition to pMMP-9 activity, weaker gelatinolytic effects corresponding to activated MMP-9 were also observed.

CSF samples (patients in Table 2) showed a significant increase in the incidence of pMMP-9 expression which, with the exception of a patient with chronic meningitis, was associated with the presence of white cells. Also, conditions described above are more commonly associated with alterations in the BBB [(average ± SD and range protein (CSF values for patients and controls) of 0.33 ± 0.40 and 0.16 to 1.10 mg/mL, and 0.13 ± 0.05 and 0.20 to 0.45 mg/mL, respectively)] than the other types of pMMP-9 associated pathologies included in this study (Tables 1, 3 and 4).

Table 3. Clinical Data of Patients with Tropical Spastic Paraparesis

Sex; Diagnosis	Age	Cytosis cells/ml	Protein mg/mL	HSA mg/mL	pMMP-2	pMMP-9
F; HTVL-I+	38	38	0.31	0.30	+	+
F; HTVL- I+	45	12	0.70	0349	+	+
F; HTVL- I+	69	11	0.45	0.42	+	+
F; HTVL- I+	58	7	0.38	0.35	+	+
M; HTVL-I+	64	5	0.46	0.42	+	+
M; *tax*-	48	3	0.30	0.27	+	+
M; *tax*-	55	0.8	0.28	0.26	+	+

Table 3 illustrates results obtained from seven TSP patients [five positive human T cell leukemia virus Type 1, and two seronegative for both this virus and the *tax gene* from HTVL-I provirus (Ramírez et al., 1998)]. We failed to show an association between the presence of the pMMP-9 enzyme and white cells in the CSF; except for one patient, no alteration in the BBB was observed.

Table 4. Clinical Data of Patients with Demyelinated or Degenerative Diseases

Diagnosis (sex and age)	Cytosis cells/ml	Protein mg/mL	HSA mg/mL	pMMP-2	pMMP-9
Amyotrophic Lat Sclerosis (F, 57)	4.8	0.14	0.09	+	+
Multiple Sclerosis (F, 36)	36	0.47	0.43	+	-
Multiple Sclerosis (F, 33)	33	0.35	0.25	+	-
Craneal Mononeuritis (F, 24)	1.6	0.13	0.11	+	-
Guillen-Barré (M, 50)	16	0.83	0.83	+	-
Multiple Mononeuritis (F, 47)	0.4	0.15	0.12	+	+

Fig. 4 shows CSF MMP-9 presence in one out of three multiple sclerosis patients. Also, we failed to detect MMP of higher molecular mass in samples from the three patients with abnormally high white cell count.

DISCUSSION

We have attempted the biochemical characterization of CSF proteolytic enzymes with similar requirements and properties as MMPs e.g., absolute need for Ca^{2+}, significant hydrolytic activity on gelatin and collagen V, low activity on collagen I, and lacking such activity on BSA and ovoalbumin. Type IV collagenases/gelatinases are known to degrade basement membrane collagen, and interstitial and neutrophil collagenases hydrolyze collagen type I (Matrisian, 1990; Woessner, 1991; Parsons et al., 1997). The lack of effect of specific inhibitors led us to discard the presence of significant CSF proteolytic activity due to seryl and sufhydryl proteases, however the inactivating effect of specific acid and *His* residue modifiers agree with the essentiality of these two groups in CSF proteolytic activity, where they have been suggested to participate as zinc ligands (Woesner, 1991). Tetracycline (more potent) and doxycycline have been suggested to be used as therapeutic agents for their inhibitory effect on MMP-2 and MMP-9 activity (Boyle et al., 1998).

CSF MMP-9 analysis in a group of 67 subjects, including individuals diagnosed with various neurological diseases and healthy controls, showed that this enzyme is not generally found in the CSF (Tables 1-4). Its appearance appears to reflect a pathological condition generally associated with the presence of lymphocytes in the CSF; 20 out of the 46 patients studied showing MMP-9 activity had lymphocyte count (per uL CSF) of at least five. Simultaneous, intense MMP-9 activity and low cell count was observed in various patient populations e.g., three individuals with TSP, three with infectious diseases, one with vascular stroke, one

with multiple mononeuritis, and in one subject diagnosed with amyothrophic lateral sclerosis. In nine patients we failed to find an association between cytosis and expression of MMP-9 activity. Recent studies in $CD8^+$ T cells from patients with TSP suggest that cytotoxic lymphocytes are an important source of proinflammatory soluble mediators contributing to the pathogenesis of this disease (Biddison et al., 1998). Our findings indicate that MMP-9 activity in some pathologies cannot be attributed only to T-lymphocytes, therefore implying that other cells e.g., glial and vessel adventitia could also be responsible for at least part of this activity (Rao, et al., 1996; Perides et al., 1998).

Our results corroborate recent findings of an increased CSF MMP-9 activity associated with TSP (Giraudon et al., 1998; Lezin et al., 1999), and show a similar effect present in patients with bacterial, fungal or parasitic infections (infective-inflammatory). We failed, however to find significant MMP-9 changes in samples obtained from subjects with vascular lesions e.g., cerebral infarction. Analysis, for the first recorded time, of MMP-9 pattern in CSF obtained from Creutzfeldt-Jacob patients (only two subjects) suggests the presence of MMP-9 activity in this prion-produced disease; clearly more patients should be studied to confirm the validity and understand the possible biological significance of this preliminary finding.

A variety of neurological diseases have been shown to significantly alter the BBB e.g., MS (Gijbels et al., 1992), bacterial meningitis, ischemia during and after stroke, and cerebral injury (De Vries et al., 1997; Rosenberg and Navratil, 1997), brain edema, Alzheimer's disease and AIDS (De Vries et al., 1997). MMPs have the potential to degrade basement membrane and other ECM components, leading to tissue infiltration by circulating inflammatory cells. Thus, activation of these enzymes in CNS tissues may constitute an important, albeit nonspecific, pathogenic mechanism for disruption of the BBB and the myelin sheath (Cuzner et al., 1996). Furthermore, MMPs release from cells present at the site of damaged tissue, notably monocytes and/or macrophages is stimulated by various cytokines, including interleukin-1 and tumor necrosis factor-α (TNFα); activation of these cytokines has, in turn, shown to be catalyzed by MMPs (Cuzner et al., 1996). Recent analysis of CSF obtained from HIV-infected patients indicates an association between the presence of MMP-9 activity and white cell count, together with an elevated amount of serum albumin. These results suggest that at least one MMP (MMP-9) may play a role in the BBB/CSF barrier leakage observed in HIV infected patients (Sporer et al., 1998). The present study documents significant BBB alterations only in three patients with infectious or parasitic diseases (one diagnosed with chronic meningitis and two with neurocisticercosis). The latter condition represents one of the steps in the development of *Tenia salinum*, which frequently progresses into chronic meningitis which may, after extense fibroblastic proliferation, result in pachimeningitis.

Myelin proteolysis is central to the demyelination process which, in association with perivenular inflammation, is a major pathological feature of MS, a condition reported to be frequently associated with positive CSF MMP-9 activity (Gijbels et al., 1992). Although our preliminary results did not show the presence of this enzyme in the only such patients studied, one could speculate that the appearance of significant MMP-9 activity depends on the stage or level of the demyelinization process. Certainly, our work

shows the need for more research aimed at elucidating the possible role of MMP-9 in the etiology and/or pathophysiology of MS and other conditions described in this paper.

In this work we have demonstrated the presence of, and characterized the enzymes gelatinases A and B in CSF and shown that they have a similar kinetic behavior than that of gelatinases present in other human tissues and fluids. We have also shown that a significant number of patients with neurocisticercosis or Creutzfeldt-Jacob disease express CSF MMP-9 activity, that no such association is present in patients suffering from various vascular lesions, and that CSF MMP-9 expression in TSP patients is not only reduced to white cells (inflammatory processes). Furthermore, occurrence of certain disease conditions (see above) appears to be associated with the activation of one or more biological structures e.g., subarachnoid and glial tissues which, in turn, would result in the release of natural enhancers of MMP-2 activity (Giraudon et al., 1999). Our recent work (Cartier et al., 1997) and present results from TSP patients, clinically identical to the HTLV-1 seropositive subjects, would suggest that degenerative CNS lesions may result in activation of MMP-9 mediated proteolysis.

When discussing and interpreting the possible biological significance of our results one should take in consideration, among other factors, the diverse etiology and complex pathophysiology of the medical condition of the subjects partipating in this study. One also should be aware of the relatively small number of controls and patients included in the different groups studied and of the shortcomings of the various analytical techniques used in the laboratory. Accepting these important limitations, one could suggest that the presence of MMP-9 in the CSF could be considered as one of a number of useful markers to follow the progress as well as to formulate differential diagnoses for TSP and various other diseases of infectious origin.

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