ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN – POLONIA
VOL. LX, N 2, 142 SECTIO D 2005

Chair and Department of Neurology, Chair and Department of Biochemistry and Molecular Biology
Medical University of Lublin

JACEK KURZEPA, HALINA BARTOSIK-PSUJEK, JOANNA WIERZBICKA,
MARTA STRYJECKA-ZIMMER, ZBIGNIEW STELMASIAK

Activity of gelatinases and oligoclonal band presence in cerebrospinal fluid of different multiple sclerosis subtypes

Gelatinases (MMP-2 and MMP-9) belong to matrix metalloproteinases (MMPs), a family of at least 23 endopeptidases targeting extracellular proteins as different types of collagen, elastin, laminin and moreover gelatin and casein. The function of gelatinases is to degrade and remodel the connecting tissue in pathological and physiological conditions. Gelatinases are secreted in a latent form as pro-MMP-2 (75 kDa) and pro-MMP-9 (92 kDa). In the propeptide domain of MMPs, a cysteine residue is present, which blocks a Zn^{2+} ion in the catalytic domain and makes MMPs inactive. The activation involves a disruption of the bond between the active zinc ion and cysteine residue either by chemical and physical agents such as aminophenylmercuric acid (APMA), sodium dodecyl sulfate (SDS), low pH or by removing the catalytic domain by other proteases with simultaneous loss of molecular weight (15). Gelatinases play an important role in numerous neurological diseases as: multiple sclerosis (MS), Alzheimer's disease, amyotrophic lateral sclerosis, Guillain-Bareé syndrome, inflammatory myopathies and stroke (7, 10).

MS is a chronic inflammatory disease of the central nervous system (CNS) white matter characterized by demyelisation (plaques), focal T cell and macrophage infiltrates, axonal injury and loss of neurological functions (3). The typical feature of MS is a relapse remitting course with periods of increased neurological symptoms. MMPs act on several phases of MS development; disrupt the BBB (blood-brain barrier) and in fact increase lymphocyte migration into CNS and also take part in degradation of myelin shield proteins. MMP-9 induces the appearance of the active form of tumor necrosis factor α (TNFα) –a strong proinflammatory cytokine (4). Immunohistochemistry of brain tissue of MS showed that the production of MMP-1, -2, -3, -7, -9 is increased inside and around the plaques (10). Young mice deficient for MMP-9 showed that they are less susceptible for the development of experimental autoimmune encephalitis (EAE) than control mice at the same age (5).

Several investigations suggest an important role of antibodies and B cells in the pathogenesis of MS. Intrathecal synthesis of IgG can be detected as oligoclonal bands (OB) in cerebrospinal fluid (CSF) by isoelectrofocusing (IEF). The OB, a very useful indicator of multiple sclerosis, is detected in over 90 percent of the patients with clinically defined MS (CDMS) and in about 70 percent with possible onset symptoms of MS (POSMS) (14, 16).

The main interest of the study was the evaluation of MMP-2 and MMP-9 activity in CSF of RR-MS and PP-MS patients and comparing these value with OB present and the severity of the disease expressed in EDSS.

MATERIAL AND METHODS

P a t i e n t s. Twenty two MS patients classified according to McDonald's criteria (11) with a relapsing-remitting MS (RR-MS), seven with primary-progressive MS (PP-MS) and nine control patients (NND – non-neurological disorders) with non-inflammatory lower limbs surgical disorders were included in the study. All RR-MS patients had clinical relapse syndromes.

C e r e b r o s p i n a l f l u i d a s s a y s. The samples of CSF of MS (about 3 ml) were obtained by lumbar punctures which were made immediately after admitting patients to hospital and before starting the treatment. The lumbar puncture was a part of the regular diagnostic procedure. Control CSFs were taken during an intrathecal anaestesia, which was performed prior to a lower limbs orthopedic surgery. This group exhibited normal CSF parameters as correct protein's level, absence of pleocytosis and intrathecal production of immunoglobulin. The overview of the patient material is given in the Table 1. After centrifugation of 1 ml of CSF the supernatant was frozen at -30°C. The samples were stored from 1 to 14 days.

Table 1. The overview of patients. RR-MS – relapse-remitting MS, PP-MS – primary-progressive MS, NND – non-neurological diseases

	RR-MS	PP-MS	NND
Number of patients	22	7	9
Sex	female 19 (86%)	female 4 (57%)	Female 6 (66%)
Age (years)	30.7 (SD 7,5)	40.2 (SD 6,9)	38.6 (SD 5,9)
Duration of disease (years)	4.5 (range 0-16)	6.2 (3-14)	-
Presence of oligoclonal bands	15 (68%)	4 (57%)	0 %
EDSS (mean)	2.5 (range 1.5-4)	4 (range 2.5-6)	-
Presence of MMP-2 (75 kDa) in CSF (%)	100	100	100
Presence of MMP-9 (92 kDa) in CSF (%)	9 (41%)	4 (57%)	0

The IEF method and silver staining according to Mehta et al. (12) was carried out to show OBs in cerebrospinal fluid and serum. The detection of OB on agarose plates (IsoGel agarose IEF plates pH 3-10 BMA products) was made once 8 samples of CSF from different patients were collected. Briefly, the samples of fourteen-µl of unconcentrated CSF were ran with 1500 V, 15 mA/plate for 45 min. at 10°C (IsoLab Ing. Akron, Ohio). After isoelectrofocusing, the agarose plates were consolidated by 30% trichloroacetic acid (TCA). Washing was carried out for two 1-hour periods with 5% glycerol. The plates were stained for 3 min. with 0.133% $AgNO_3$. The staining was stopped by washing in 10% acetic acid for 5 min. To avoid false-positive results the IEF of serum samples was performed together with CSF. Only the absence of OB in serum and the presence in CSF showed intrathecal synthesis of oligoclonal proteins and allowed to recognize the positive OB results. Before the IEF serum samples were diluted 300-fold with PBS to make protein concentration similar to CSF.

Zymography was carried out according to Azeh et al. (2) with minor modifications. Briefly, the sample which consisted of 15 ul of CSF + 5 ul of sample buffer with 10% sodium dodecyl sulfate (SDS) was applied on 10% polyacrylamide gel to which 0.06% gelatin was added. Stacking gel was 4% polyacrylamide gel and did not contain gelatin. The gels were run for 1.5 h with 30 mA/gel in

room temperature. Washing was carried out for two 30 min periods with buffer (50 mM Tris-HCl pH 5, 7, containing 10 mM $CaCl_2$, 0.02% NaN_3 and 2.5% Triton X-100) to remove the SDS. Incubation was performed for 18 h at 37°C in the above buffer, but containing 1% Triton X-100 to develop of the enzyme activity. The gels were stained with 0.1% Coomassie Blue R-250 in 30% ethanol and 10% acetic acid and destained in 30% ethanol and 10% acetic acid for two 15 min periods. Gelatinases activity was detected as unstained bands on a blue background. Enzymes were identified by co-localization with human pro-MMP-2 (Roche, No. 1782916) and pro-MMP-9 (Roche, No. 1758896) and with molecular mass standard (Fermentas, SM0441). Quantification of zymograms was done using a computer scanner (600x1200 dpi) and ZERO-Dscan Image Analysis System v 1.0. Gelatinase activity was expressed in arbitrary scanning units (a.s.u.) representing the optical density of the substrate lysis zone.

For statistical quantification we used the computer assisted statistical program GraphPad InStat v. 3.06. The two-tailed Mann-Whitney test was used to compare variables in RR-MS and PP-MS patients groups. To correlate all studied variables the two-tailed Spearman's rank test was used. Chi-square test was used to compare OB present in two groups.

RESULTS

PP-MS patients were on average 10 years older than RR-MS patients (mean age was 40.2 and 30.7 years, respectively) and the severity of the disease measured with EDSS was serious in the first group (see Table 1).

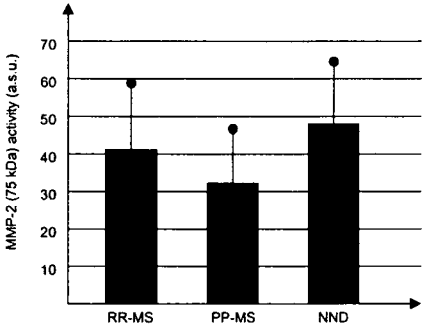

Fig. 1. Activity of MMP-2 (75 kDa) in CSF of RR-MS, PP-MS and NND patients expressed in arbitrary scanning units. There are not significant differences between these values (p>0.05)

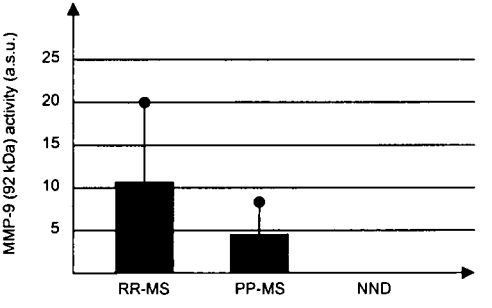

Fig. 2. Activity of MMP-9 (92 kDa) in CSF of RR-MS and PP-MS patients expressed in arbitrary scanning units (p>0,05). In NND patients MMP-9 was not detectable

Comparing zymography results with human pro-MMP-2 (75 kDa), pro-MMP-9 (92 kDa) and molecular mass standard in all cases we observed pro-forms of both enzymes which were activated during zymography process. MMP-2 (75 kDa) activity was detected in CSF of all patients. MMP-9 (92 kDa) activity was detected in 41% of RR-MS and 57% of PP-MS patients. We noticed higher both MMP-2 (22% more) and MMP-9 (58% more) activities in CSF of patients with RR-MS comparing to PP-MS patients but statistical difference was not significant (p>0.05). In control patients MMP-9 activity was not detectable but MMP-2 activity was higher than in both subtypes of MS (Fig. 1, 2). Oligoclonal bands were present in 68 percent of RR-MS patients and in 57 percent of PP-MS patients in CSF but statistical difference between these values was not noticed (p>0.05). There was not any

statistical correlation between activity of both gelatinases, OB present in CSF, severity of the disease, duration of MS and the age of patients.

DISCUSSION

Gelatin zymography is a semiquantitive method used to detect MMPs activity. It is extremely sensitive because levels of 10 pg of MMP-2 can already be detected (15). Geltinase activity *in vivo* is regulated by tissue inhibitors of MMPs (TIMPs). During the zymographical analysis, the gelatinase-TIMP complexes are dissociated by the SDS used for electrophoresis therefore results of zymography show gelatinase activity without inhibition which is present *in vivo* (13). The activation of pro-form of both enzymes during zymography is caused by the dissociation of cysteine from zinc molecule due to SDS without loss of molecular weight (15).

Our investigations showed higher MMP-9 (92 kDa) activity in CSF of RR-MS patients than PP-MS patients, but results differ not significantly ($p>0.05$). MMP-9 is not presented in the CSF of control patients and we are consistent with previous studies (6) suggesting that presence of MMP-9 in CSF is involved in a pathological process of CNS.

Avolio et al. indicate that serum MMP-9 level is associated with active RR-MS disease. On the other hand, serum MMP-2 level is characteristic of long duration progressive stages of MS (1). We noticed higher MMP-2 (75 kDa) activity in CSF of RR-MS patients comparing to PP-MS patients, but the results differ insignificantly. The high activity of both types of gelatinases of RR-MS patients in CSF obtained during a relapse of the disease suggested that this enzymes play an important role in the active stage of this MS subtype.

An unexpected result was a higher MMP-2 activity observed in the control group than in MS patients. The control patients were subjects suffering form an non-inflammatory lower limbs orthopedic disorders such as the rupture of ligamentum cruciatum. Although in all NND controls patients inflammatory symptoms (fever, high c-reactive protein level) were not observed, there is a probability that in these cases MMP-2 activity was higher than in healthy donors. Using IEF and silver staining we found oligoclonal bands only in 57 percent of PP-MS patients and 68 percent of RR-MS patients in CSF. In previous studies with sixty-nine RR-MS patients we found OB in over 70 percent of cases using this method (8). This result is lower than in studies in which polyacrylamid plates and concentrated CSF were used instead of agarose plates and unconcenrtated CSF (16). We noticed more OB in CSF of patients with relapse of RR-MS. This result could suggest an important role of humoral response in this subtype of MS, but it requires further investigations.

The lack of relevant correlations probably come from the low number of patients included in the study.

CONCLUSION

Because MMP-9 (92 kDa) activity in CSF was observed in both multiple sclerosis subtypes (RR-MS and PP-MS) and it was not detectable in NND patients we suggest that this enzyme plays an important role in the pathogenesis of the disease. Oligoclonal bands also appear only in MS CSFs. In RR-MS they appear more often than in PP-MS patients, but in our study the results differ not significantly.

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SUMMARY

Gelatinases act on several phases of multiple sclerosis (MS) development; they disrupt the BBB (blood-brain barrier), take part in degradation of myelin shield proteins and induce appearance of active form of tumor necrosis factor α (TNFα). Oligoclonal antibodies which are represented as oligoclonal bands (OB) in CSF also play an important role in the pathogenesis of MS. The main interest of the study was an evaluation of MMP-2 and MMP-9 activity in CSF of RR-MS (n=22), PP-MS (n=7) and NND (non-neurological diseases) (n=9) patients using zymography and comparing these values with OB presence and severity of disease expressed in EDSS. In all cases we observed pro-form of both enzymes. MMP-2 (75 kDa) activity was detected in CSF in all patients. MMP-9 (92 kDa) activity was detected in 41% of RR-MS, 57% of PP-MS and not detected in any NND patients.

We noticed higher MMP-2 (22% more) and MMP-9 (58% more) activity in CSF of patients with RR-MS in comparison to PP-MS patients but statistical difference was not significant ($p>0.05$). Oligoclonal bands were present in 68 percent of RR-MS patients and in 57 percent of PP-MS patients. There was not statistical correlation between activity of both gelatinases, OB presence in CSF, severity of the disease, duration of MS and the age of patients. As a conclusion both humoral response and MMP-9 (92 kDa) are involved in pathogenesis of MS.

Aktywność żelatynaz oraz obecność prążków oligoklonalnych w płynie mózgowo-rdzeniowym w różnych postaciach stwardnienia rozsianego

Żelatynazy działają na kilka etapów rozwoju stwardnienia rozsianego (SR): uszkadzają barierę krew-mózg, biorą udział w degradowaniu białek osłonki mielinowej oraz przyczyniają się do powstania aktywnej formy czynnika martwicy nowotworów (TNFα). Wcześniejsze badania podkreślają istotną rolę przeciwciał oligoklonalnych w patogenezie (SR) oznaczanych w płynie mózgowo-rdzeniowym (PMR) jako prążki oligoklonalne (PO). Celem pracy była ocena aktywności MMP-2 oraz MMP-9 w PMR pacjentów z nawracająco-remitującą (NR) (n=22) oraz przewlekle-postępującą (PP) (n=7) postacią SR w porównaniu w z grupą kontrolną (n=9). Metodą wykorzystaną do oznaczenia aktywności żelatynaz była zymografia. Wyniki zestawiono z obecnością PO oraz stopniem zaawansowania choroby, wyrażonym w skali EDSS. We wszystkich przypadkach obserwowano obecność proenzymów żelatynaz. Aktywność MMP-2 (75 kDa) stwierdzono w PMR u wszystkich badanych pacjentów. MMP-9 (92 kDa) oznaczono u 41% pacjentów z NR postacią SR oraz u 57% pacjentów z PP postacią SR, a nie stwierdzono jej aktywności u pacjentów z grupy kontrolnej. Wyższą aktywność MMP-2 (o 22%) oraz MMP-9 (o 58%) zauważono u pacjentów z NR-SR w porównaniu z PP-SR, jednakże różnica okazała się nieistotna statystycznie. PO oznaczono u 68% pacjentów z NR-SR oraz 57% pacjentów z PP-SR. Nie stwierdzono statystycznej korelacji między aktywnością żelatynaz, obecnością PO, stopniem zaawansowania choroby oraz długością jej trwania. Zarówno odpowiedź humoralna, jak i MMP-9 (92 kDa) są zaangażowane w patogenezę SR.