Title: Expression pattern of matrix metalloproteinases-2 and-9 and their tissue inhibitors in patients with chronic inflammatory demyelinating polyneuropathy

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Abstract

Background. Matrix metalloproteinases (MMPs) are a heterogeneous family of endopeptidases that play a role in many physiological functions, including the immune response. An imbalance between activity of MMPs and their physiological tissue inhibitors (TIMPs) has been proposed in the pathophysiology of different autoimmune disorders. We aimed to assess the plasmatic levels of MMP-2, MMP-9 and their inhibitors TIMP-1 and -2 in patients with chronic inflammatory demyelinating polyneuropathy (CIDP).

Subjects and methods. Twenty patients with CIDP and 20 age- and sex-matched healthy controls were enrolled. Plasma concentrations of MMP-2, MMP-9, TIMP-1 and TIMP-2 were determined by the enzyme-linked immunosorbent assay.

Results. CIDP subjects had higher MMP-9 concentrations along with TIMP-1 downregulation when compared to controls, with the consequent increase in the MMP-9/TIMP-1 ratio ($p<0.000002$ for all measures). Conversely, the concentration of MMP-2 was lower in the CIDP group ($p<0.01$) without changes in the TIMP-2 concentration. The MMP-2/TIMP-2 ratio was decreased in the patients' group ($p<0.02$).

Discussion. A dysregulation of the plasmatic pattern of MMPs and TIMPs, with increase of MMP-9 and decrease of TIMP-1 and MMP-2 levels, may represent a useful biomarker of CIDP. Future studies are needed to clarify the link between altered expression pattern of MMPs and TIMPs and pathogenesis of the peripheral nerve damage in CIDP.

Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that regulate extracellular matrix (ECM) turnover and cell-cell and cell-matrix interactions in many organs and tissues[12]. MMPs are synthesized in many cell types as inactive precursors (proenzymes) and stored in intracellular vesicles. Proenzymes are secreted by exocytosis and activated in the extracellular space through complex mechanisms. Besides acting on ECM macromolecules, MMPs have numerous substrates: cellular receptors, factors regulating cell motility and cytokines[1]. This explains their involvement in several physiological and pathophysiological processes, including cell migration during organs development and tissue repair following damage [2]. MMPs are also involved in inflammatory processes as they allow immune cells migration from the blood into various organs[3–5].

MMP-9 and MMP-2 are two gelatinases that act on collagen type IV, the main collagen component of the basement membrane [6]. MMP-9 is synthetized as pro-MMP-9. The cascade of proteolytic steps needed to transform this proenzyme into the active form involves enzymes of the plasminogen-plasmin system, transmembrane peptidases (e.g., furin and membrane type 1-matrix metalloproteinase, MT1-MMP) and the same MMP-2 [7]. Once activated, MMP-9 is rapidly inactivated by the tissue inhibitor of metalloproteinases-1 (TIMP-1). MMP-2 is also synthetized as a proenzyme (pro-MMP-2) and activated in the extracellular space. The activation process requires an interaction of the proenzyme with two identical MT1-MMP proteins, only one of which must bind a third protein, the tissue inhibitor of metalloprotease-2 (TIMP-2). Of note, high concentrations of TIMP-2 inhibit this activation step by saturating the transmembrane peptidases MT1-MMP. Thus, though low TIMP-2 levels are necessary for activation of pro-MMP-2, at higher concentrations TIMP-2 represents the main physiological inhibitor of MMP-2 [8].

Both MMP-9 and MMP-2 allow migration of immune cells through the blood-nerve barrier (BNB) [5,8,9]. MMP-9 also plays a role in myelination processes in the central and peripheral nervous system [10]. On these bases, a pathogenetic role of these proteases has been hypothesized in inflammatory neuromuscular diseases, such as myositis, myasthenia gravis, and chronic inflammatory demyelinating polyneuropathy (CIDP) [3,4,8].

To date, only three studies have investigated the potential role of MMPs and their inhibitors in the pathogenesis of CIDP in sural nerve biopsies [11–13]. Upregulation of MMP-2 and MMP-9 has been found in

nerve biopsy specimens of subjects with CIDP and nonsystemic vasculitic neuropathies, but not in patients with noninflammatory neuropathies, thus suggesting their role in inflammatory tissue damage [11,12]. Moreover, an increased MMP-9 immunoreactivity in sural nerve biopsies has proved useful in discriminating diabetic patients with CIDP from patients with diabetic neuropathy [13].

To the best of our knowledge, no systematic studies have instead been conducted on plasma of subjects with CIDP.

In this study, we evaluated the plasma levels of MMP-9, MMP-2, TIMP-1 and TIMP-2 in a cohort of subjects with CIDP in comparison with age- and sex-matched healthy controls, aiming to explore the potential usefulness of these parameters as biomarkers of the disease.

Subjects and methods

We collected the plasma from 20 patients with CIDP (typical n=16, atypical n=4) according to the European Federation of Neurological Societies/Peripheral Nerve Society (EFNS/PNS) criteria [14]. In CIDP subjects treated by intravenous or subcutaneous immunoglobulins (IVIg and SCIg) (Table 1), all biological measures were assessed just before starting a new treatment cycle. Samples from 20 age- and sex-matched healthy volunteers were tested as controls.

The clinical evaluation at enrollment included assessment of muscle strength with the Medical Research Council (MRC) scale (score ranging from 1 to 60) [15], sensory function with the Inflammatory Neuropathy Cause and Treatment (INCAT) sensory sum score (ISS) (score ranging from 1 to 20) [16], and disability level with INCAT scale (score ranging from 0 to 10) [17]. Main demographic and clinical findings of the subjects enrolled are shown in Table 1. All subjects with CIDP were receiving immunomodulators and/or immunosuppressants at the time of the investigation (Table 1).

In both patients and controls, plasma concentrations of gelatinases MMP-9 and MMP-2, and inhibitors TIMP-1 and TIMP-2 were determined using the Human MMP-2 and MMP-9 and the Human TIMP-1 and TIMP-2 ELISA kits (Boster Biological Technology, Ltd.).

Statistical analyses. This is a pilot study where we investigated plasma variables with unknow values in this type of subjects. Thus we could not calculate a formal sample size calculation. Rather, we estimated that we would recruit 20 participants and 20 control subjects over a reasonable time-frame of six months. Mean values of each measurement were compared by using independent-samples t-test analysis, as data showed a normal distribution at the Shapiro-Wilk Test. Along with comparison between subjects with CIDP and controls, we also compared data from patients with and without steroid treatment.

Correlation between plasmatic values of MMP-9, MMP-2, TIMP-1, TIMP-2, MMP-9/TIMP-1 ratio, MMP-2/TIMP-2 ratio, and demographic and clinical data were tested by Pearson's correlation test.

All tests were performed by using SPSS (v26) with level of significance set at 0.05.

The study was approved by local institutional ethical committees. Written informed consent was obtained from all subjects participating in the study.

Results

Plasmatic MMPs and TIMPs levels along with MMPs/TIMPs ratios in patients and controls are shown in Fig. 1. The MMP-9 concentration was significantly higher in the CIDP group compared to controls ($p<0.000001$). Conversely, MMP-2 and TIMP-1 levels were lower in the CIDP group compared to controls ($p<0.01$ and $p<0.000002$, respectively). No significant difference was found for TIMP-2 concentration between CIDP subjects and controls ($p=0.95$). As regards the MMP/TIMP ratios, a more than twofold higher MMP-9/TIMP-1 ratio was observed in the CIDP subjects ($p<0.000001$), whilst their MMP-2/TIMP-2 ratio was significantly lower ($p<0.02$) than controls. To test the possible role of steroid treatment on the experimental measures, we compared the levels of MMPs and TIMPs of CIDP subjects with (n=11) or without (n=9) steroid treatment. The analysis did not show significant differences for all parameters (Table 2).

No significant correlations were found between values of MMPs, TIMPs and MMP/TIMP ratios and different clinical measures in the CIDP subjects. We detected only a trend towards significance TIMP-2 and MRC sum score ($p=0.08$).

Discussion

This is the first study exploring the potential role of plasma concentration of MMP-9 and -2 and respective inhibitors TIMP-1 and -2 as biomarkers for CIDP. We observed a relevant increase of MMP-9 level in CIDP, which, along with the decrease concentration of its physiological inhibitor TIMP-1, led to a notable alteration of the physiological MMP-9/TIMP-1 ratio. Albeit to a lesser extent, also the expression pattern of MMP-2 and TIMP-2 was altered, with low levels of MMP-2 in the presence of normal TIMP-2 concentration, with the consequence reduction in the MMP-2/TIMP-2 ratio.

Our results are in line with previous evidence from 3 single subjects with CIDP that belonged to a series of patients with different neuromuscular disorders [3]. In that study, higher levels of MMP-9 and slightly lower MMP-2 concentrations were shown in the subjects with CIDP, with imbalanced ratios between MMPs and TIMPs resembling those we observed. The authors also assessed the effects of the IVIg treatment in these 3 individual patients, showing a transient and partial reduction in the MMP-9 together with increase in the MMP-2 concentration, though normal MMPs levels could not be restored immediately after treatment.

Elevated plasmatic MMP-9 concentration has been reported in several autoimmune diseases, ranging from lupus erythematosus to multiple sclerosis, and linked to inflammatory cell invasion [18,19]. MMP-9 mainly derives from blood-derived immune cells, especially T cells, and it represents an important effector of extravasation and early interstitial infiltration, which is a common pathogenetic mechanism of inflammatory neuropathies [12]. Therefore, we can hypothesize that upregulation of MMP-9, along with reduced concentration of TIMP-1, might favor the breakdown of the blood-nerve barrier (BNB), that represents a key step in CIDP process.

As regards MMP-2, our findings showed lower plasmatic levels, which is apparently in contrast with the investigations on nerve biopsy specimens of patients with CIDP, where MMP-2 was upregulated [11,12]. This discrepancy may be explained by the fact that MMP-2 is mainly expressed within the nerve parenchyma by stromal cells of the endo-, peri- and epineurium [12]. To explain the finding of reduced plasmatic MMP-2 levels we could hypothesize a compensatory mechanism towards increased MMP-9 levels, when considering that presence of MMP-2 is required for activation of pro-MMP-9 [7,8].

In the present study, we evaluated plasmatic levels of MMPs and TIMPs in CIDP as a possible expression of disease activity. However, we failed to find significant correlations between clinical and biological parameters. It is therefore possible that current clinical picture likely correlated more with sequelae of previous damage than with the levels of ongoing inflammation. Therefore, further studies on patients with homogeneous clinical features (e.g. with similar clinical course and duration of the disease) and follow-up evaluations in the same patients' population will be needed to clarify the role of MMPs and TIMPs patterns as biomarkers of disease activity.

Some limitations and considerations of the present study need to be addressed. First of all, we did not test naïve patients and we cannot exclude that ongoing treatments may have, at least partially, affected the biological measurements. Some considerations, however, make the role of immunosuppressive and/or immunomodulatory treatment regimens unlikely to determine the observed alterations. Indeed, a regards steroid treatment, we recorded very similar values in treated and untreated CIDP subjects for all biological measures (Table 2). As regards Ig treatment, we collected patients' samples just before the next treatment cycle. Moreover, we have already mentioned preliminary evidence of only transient and slight modifications of the MMPs and TIMPs expression pattern after IVIg treatment[3], that were in the opposite direction to what we observed in CIDP patients. On these bases, the Ig treatment could not explain the alterations recorded in CIDP subjects, though we cannot exclude that different administration routes (i.e., SC vs. IV) could differently affect the MMPs and TIMPs system.

Another consideration is that we did not discriminate between active and non-active forms of MMPs, as our ELISA kit measured only total enzyme concentrations, and quantitative zymography was not employed to specifically detect MMP active forms. However, it has been shown that results of zymography for detection of plasma MMPs are closely related to those obtained by ELISA, thus the two techniques could be used interchangeably for MMPs detection [20].

At this time it is elusive whether the observed alterations of MMPs and TIMPs profile have a specific pathogenetic role in peripheral demyelination or are non-specific expression of the inflammatory processes affecting the peripheral nerve. Findings in patients with different autoimmune diseases and vasculitic neuropathies seem to mainly support the second hypothesis [11,12]. Instead, evidence that immunoreactivity for MMP-9 on sural biopsies is increased in patients with CIDP, but not in patients with diabetic

polyneuropathy, could rule out a significant influence of the tissue repair processes on the MMPs and TIMPs profile [13]. Notwithstanding, targeted studies are needed to clarify the exact role of the MMPs and TIMPs system in the pathogenesis of CIDP. This kind of information is also valuable, considering the recently renewed interest in innovative treatment strategies for autoimmune disorders that aim to interact with function of MMPs and TIMPs [21].

In conclusion, in the present study we describe a dysregulation of the plasmatic pattern of MMPs and TIMPs, in CIDP. Future investigations are hoped to assess the potential usefulness of these biological parameters in the clinical setting, including the differential diagnosis of the peripheral neuropathies, and the prognostic stratification of subjects with CIDP. They may also provide useful candidate drug targets or antitargets.

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Figure legend

Fig.1 Mean, standard deviation and minimal and maximal values of MMP-2, MMP-9, TIMP-1 and TIMP-2 plasmatic concentrations, and MMP-9/TIMP-1 and MMP-2/TIMP-2 ratios, presented for CIDP patients (pts) and controls. Mean values are indicated by the small squares. The box-blot represents standard deviations, and vertical lines mark minimal and maximal values.

***CIDP**, chronic inflammatory demyelinating polyneuropathy; **MMP**: Matrix metalloprotease; **TIMP**: tissue inhibitor of matrix metalloprotease*. * $p<0.05$; ** $p<0.000002$.