# TIMPs and MMPs expression in CSF from patients withTSP/HAM

Ana M. Kettlun[a], Luis Cartier[b], Lorena García[a], Lucía Collados[a], Felipe Vásquez[a], Eugenio Ramírez[c], M. Antonieta Valenzuela[a,*]

[a] *Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Casilla 233 Correo 1, Santiago, Chile*
[b] *Departamento de Ciencias Neurológicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile*
[c] *Departamento de Laboratorios de Salud, Instituto de Salud Pública de Chile, Santiago, Chile*

**Abstract**

The tropical spastic paraparesis or human T-cell lymphotropic virus associated myelopathy (TSP/HAM), has been related with an overexpression of matrix metalloproteinases (MMPs), especially MMP-9. Initial studies of reverse zymography with cerebrospinal fluid (CSF) from TSP/HAM patients, and controls showed the presence of TIMPs, endogenous MMP inhibitors. We determined in CSF the levels of TIMPs by immunoanalysis in 25 patients with TSP/HAM, and compared with two groups: controls and patients with acute and subacute inflammatory neurological diseases. We found that TIMP-2, TIMP-3 and TIMP-4 levels were significantly higher than in controls in both TSP/HAM and inflammatory patients, while TIMP-1 was increased only in the inflammatory group. Levels of MMP-3 and MMP-9 from the two groups of patients showed a significant upregulation in CSF. In the CSF of around the 70% of TSP-HAM and inflammatory patients the presence MMP-9 was detected by zymography, but not in controls. MMP-2 was only overexpressed in the acute inflammatory group. The active form of MMP-2 was observed in both groups of patients with a similar high frequency (60%). MMPs overexpressions are independent of the evolution time of the disease in TSP/HAM. The chronic overexpression of these extracelullar matrix proteins detected in CSF of TSP/HAM should be indirectly produced by secreted viral proteins being responsible for the progression of this disease, accounting for the observed differences with acute inflammatory patients. Our results support the existence of an imbalance between MMPs and their endogenous tissue inhibitors, which could be a pathogenic factor in the chronicity of TSP/HAM.

*Keywords:* TSP/HAM; TIMPs; MMPs; Tropical spastic paraparesis; Neurological inflammatory diseases; Cerebrospinal fluid

* Corresponding author. Tel.: +56-2-678-1648; fax: +56-2-222-7900.
*E-mail address:* mavalenz@uchile.cl (M.A. Valenzuela).

## Introduction

Tropical spastic paraparesis (TSP) due to human T-cell lymphotropic virus associated myelopathy (HAM) is a pathological condition characterized by a progressive weakness and stiffness of the lower limbs expressed by a pyramidal syndrome [1]. The main feature of this slowly progressive neurological disease is a damage that predominantly affects the cortical-spinal tract with demyelinization and axonal degeneration [2].

The mechanisms of axonal degeneration in TSP/HAM remain unknown. In any CNS (central nervous system) lesions, molecular changes in the extracellular environment are accompanied by rapid activation of neuroglial and other nonneural cells, secreting metalloproteinases (MMPs) among several components of the extracellular matrix (ECM) [3–5]. MMPs constitute a family of proteolytic enzymes involved in the remodeling of the ECM and participate in many normal and pathological processes [3,4,6]. MMPs, synthetized initially as zymogens, require processing to expose their catalytic active site, involving a membrane-bound metalloproteinase (MT1-MMP) for proMMP-2 (latent form); plasmin for MMP-3 (stromelysin-1); MMP-3 activates proMMP-9, and MMP-7 (matrilysin) activates proMMP-1, proMMP-2 and proMMP-3 [4–9]. Activity can also be controlled by natural tissue inhibitors of metalloproteinases (TIMPs) [4–6,10,11]. Additionally, the gene expression of MMPs is regulated by several factors, including growth factors, cytokines, etc. [6].

Overproduction of MMPs, could produce an abnormal proteolysis of the ECM resulting in progressive and persistent tissue damage, that has been implied in several neurological diseases [3–5]. The most commonly increased MMPs in CSF of patients with various neurological infectious diseases, including other retroviral infections (like HIV), are MMP-9 and MMP-7 [12,13]. In other infections like viral and bacterial meningitis a parallel upregulation of MMP-9 and TIMP-1 has been observed [14–17]. In inflammatory demyelinating disorders like multiple sclerosis (MS) both in tissue lesions and CSF an increase in MMPs has been described [18–22] and also in Guillain–Barré syndrome [23,24].

In CSF from TSP/HAM patients the presence of MMP-9 has been demonstrated [24–28]. Studies on TSP/HAM patients and asymptomatic carriers of HTLV-I showed that TIMP-1 was detected at a similar frequency in CSF of both cases, being TIMP-3 expression more specifically related to the clinical status [28]. In a preliminary study, our group has detected the presence of the four TIMPs in CSF by immunowestern blot, which pointed out an increase in TIMP-2, -3 and -4 [29]. Tax protein expressed by *tax* gene of HTLV-I infection of T-lymphocytes, activates the expression of MMPs [30] and produces a dysregulation of TIMP genes (TIMP-3) as previously reported [28,30].

In this study we have performed a comparative study of the level of TIMPs and MMPs in CSF from TSP/HAM patients, and compared with several acute and subacute inflammatory patients and controls with two main purposes: a) to find a characteristic pattern in TSP/HAM, and b) to confirm the hypothesis that an imbalance between MMPs and TIMPs sustains the chronicity of TSP/HAM for several years.

## Methods

### *CSF samples*

The control group (25 cases) comprised both people without neurological disorders from gynecology (spinal anesthesia) and with neurological diseases, without inflammatory or degenerative

pathologies that required the study of the CSF (cephalalgia, neuropathy), and were aged 21–80 years. Among the inflammatory patients we studied: 14 patients with HIV (including 6 cases with additional infections), 4 patients with meningitis, 4 patients with neurocisticercosis, 4 with MS, and 2 cases of Guillain–Barré. TSP/HAM patients (25 cases) were identified both clinically and following the determination of HTLV-I antibodies in serum [31], or the presence of the *tax* gene in lymphocytes by PCR [32]. Controls were seronegative. Control and patient groups were duly informed of the research protocol and all of them freely agreed to participate in this study.

CSF samples were obtained by atraumatic lumbar puncture as part of the normal diagnostic procedures and were frozen at −20 °C. Cell count and protein [33] were routinely measured.

*Detection of TIMPs by reverse zymography*

Reverse zymography of gelatinase inhibitors was done on SDS-PAGE with 12% polyacrylamide, containing 1 mg/ml gelatin according to Ref. [34]. Samples containing 15 μl CSF plus 5 μl sampling buffer 4× (under non-reducing conditions) were used per well. After the electrophoretic run, the gel was incubated at room temperature on an orbital shaker for 30 min in 100 ml of 2.5% Triton X-100 to remove the SDS. This solution was then replaced by 50 ml of 0.2 μg/ml of a mixture of MMP-2 and MMP-9 (Chemicon, Cat. CC073) in 50 mM Tris–HCl, pH 7.5, 10 mM $CaCl_2$, 1 mM APMA (p-aminophenylmercuric acetate, metalloproteinase activator), 0.025% $NaN_3$, and incubated for 18 h at 37 °C. Following the Coomassie staining (0.1% Coomassie Brilliant Blue R-250 in methanol:water:glacial acetic acid with a proportion 5:5:2 v/v) and destaining with 10% acetic acid. Areas of inhibition of gelatinase activity were visualized as blue stained regions on a clear background in the zone of low *Mr* (around 20–30 kDa) [35].

*CSF levels of TIMP-1 and MMP-9 in TSP/HAM by enzyme-linked immunoabsorbent assay*

ELISA assays were performed using Biotrak assays (Amersham Biosciences, Piscataway, NJ, USA) for MMP-9 (code RPN 2614) and TIMP-1 (code RPN 2611), in accordance with the manufacturer's instructions, using 100 μl of CSF sample at a dilution of 1:10.

*Detection of the relative amount of TIMPs and MMPs by immunodot blot*

Relative amounts of these proteins were determined separately applying portions of 2.5–10 μl of CSF to a nitrocellulose membrane (BioRad, Hercules, CA. USA). Samples from controls (18–25 cases), TSP/HAM patients (16–25 cases), and with inflammatory diseases (28 cases) were tested simultaneously in the same nitrocellullose membrane. The membrane was blocked for 4 h with 6% nonfat milk in TBS-T buffer (20 mM Tris–HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20). The following monoclonal antibodies have been used: antiTIMP-1 (Chemicon International, Temecula, CA, USA, Cat. MAB3301), antiTIMP-2 (Chemicon, Cat. MAB3310), and antiTIMP-3 (Chemicon, Cat. MAB3318), diluted 1:250 in the same blocking buffer; and antiMMP-9 (Chemicon Cat. MAB1347) diluted 1:500. The following monospecific polyclonal antibodies have been used: antiTIMP-4 (Chemicon, Cat. AB816) diluted 1:250, antiMMP-2 (Chemicon Cat AB809) diluted 1:3,500, and antiMMP-3 (Santa Cruz Biotecnology, Santa Cruz, CA, USA, Cat. CS-6839), diluted

1:500. After rinsing with TBS-T buffer, the membrane was treated with the adequated secondary antibody conjugated with peroxidase for 1 h, using an anti-mouse antibody (Pierce, IL, USA, Cat. 31452) diluted 1:20,000 in TBS-T for the development of the monoclonal antibodies, or anti-rabbit antibody, diluted 1:5,000 in TBS-T (Pierce, Cat. 31462) or anti-goat antibody, immunoabsorbed against human proteins diluted 1:2,000 (Pierce, Cat. 314022). After rinsing with TBS-T, a positive reaction was identified using enhanced chemiluminescence (Super Signal West Pico Chemiluminescent Substrate, Pierce). Dots were scanned and quantified using the "Uni-Scan-it Gel Automated Digitizing System" software. Values of integrated density are reported in units of intensity (pixels) per μl of CSF. Control experiments in the absence of the primary antibody and using any one of the three secondary antibodies did not yield chemiluminescence signal. Immunodot blot determinations were done at least in duplicate using different nitrocellulose membranes, and further averaging the integration data. The analysis of the CSF from TSP/HAM and inflammatory patients was done using the same control samples.

Immunowestern blots of CSF employing primary antibodies against TIMP-1, TIMP-3, TIMP-4, MMP-2, MMP-3 and MMP-9, at the same dilution as in immunodot blot analysis, showed the same immunoreactive pattern as the corresponding positive controls. Instead, TIMP-2 immunorective band observed in CSF exhibited a *Mr* close to the double of that of the positive control; no changes were observed using stronger denaturing conditions.

### *MMP activity determination by zymography using gelatin or laminin as substrates*

MMP-2 and MMP-9 detection in CSF samples was performed by zymography using 1 mg/ml gelatin copolymerized with 7.5% acrylamide [16], and for MMP-3 detection 0.1 mg/ml laminin as substrate was used. Samples containing 7.5 μl CSF plus 2.5 μl sampling buffer 4× (under non reducing conditions) were used per well. MMP-3 (Chemicon, Cat. CC1035), and a mixture of MMP-2 and MMP-9 (Chemicon, Cat. CC073) were utilized as positive controls.

### *Statistical analysis*

Differences between the means in TIMP and MMP levels of controls, TSP/HAM and inflammatory samples were evaluated using the nonparametric test of Mann-Whitney U for two-independent-samples. Data were expressed as mean ± SEM. Spearman rank correlation of levels of MMPs with TIMPs was used.

## Results

### *Initial detection of the presence of TIMPs in CSF from TSP/HAM patients*

The novel system called reverse zymography is an electrophoretic technique designed to detect TIMP inhibitory activity of gelatinase within acrylamide gels copolymerized with gelatin [35]. In an initial study testing CSF samples from 4 TSP/HAM patients and 3 controls we observed inhibitory bands of gelatinase activity with *Mr* around 30 kDa similar to TIMP-1 and TIMP-2 standards (Fig. 1).

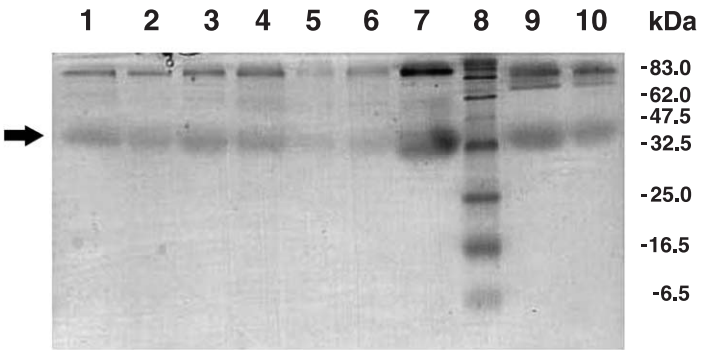


Fig. 1. Reverse zymography in SDS/PAGE under non-reducing conditions of CSF from some patients and controls using gelatin as substrate. After electrophoresis the gel was digested with a mixture of MMP-2 and MMP-9 (standards for zymography: 0.2 μg/ml), and gelatinase inhibitory activity was detected as Coomassie Brilliant Blue stained bands in the zone of low molecular weight. Lanes 1, 2 and 3 correspond to CSF from controls, lanes 4–7 to CSF from TSP/HAM, lane 8 to molecular weight standards, and lanes 9 and 10 to the positive controls TIMP-2 and TIMP-1, respectively. The arrow indicates the presence of inhibitory gelatinase activity.

*CSF level of TIMP-1 in TSP/HAM by ELISA*

In a preliminary study with some samples (13 CSF from TSP/HAM, and 11 controls) we used an ELISA kit for quantifying TIMP-1. This method allows the determination of both free and MMP-bound TIMP-1. The mean value ± SEM of TIMP-1 in CSF samples from TSP/HAM was 383.6 ± 44.1 ng/ml, which was not significantly different from that of controls, 403.1 ± 20.8 ng/ml.

*Relative levels of TIMPs in CSF followed by immunodot blot*

We were interested in screening the levels of the different TIMPs in CSF from TSP/HAM and compare them with inflammatory cases and controls. Because the lack of available ELISA kits for TIMP-3 and TIMP-4, we proposed to perform a comparative study by immunodot blot applying in the same nitrocellulose membrane a similar portion of CSF from patients and controls. We standarized this study using the same volume of CSF, considering that this would be a better representation of what is occuring in vivo. This comparative determination of TIMP relative levels in CSF of both patients and controls was validated determining TIMP-1 values by immunodot blot of 21 TSP-HAM samples together with 25 controls, which included the samples where the quantitative determination by ELISA had been done. Data of TIMP-1 levels, determined by immunodot blot in TSP/HAM samples, normalized according to the mean value of controls, is presented in Table 1, together with some patients' clinical data, including age, sex, length of the disease and CSF parameters. A comparison of mean values ± SEM (expressed as the scanner data in pixels) in controls and TSP/HAM is shown in Table 2. In accordance with ELISA analysis, the mean value of TIMP-1 in TSP/HAM (determined in a larger number of cases) was not significantly different from controls.

Due to the agreement between ELISA and immunodot blot, we proceeded to the determination of the relative amounts of the other TIMPs by immunodot blot analysis. Other alternative method for this

Table 1
Patient's clinical data, CSF parameters, presence of MMP-2 (in its active form of 68 kDa) and MMP-9 (in its latent form of 92 kDa, proMMP-9) followed by zymographic analysis, and the relative amount of TIMPs and MMP-3 determined by immunodot blot analysis

| Patient no. | Age, sex | Years of disease | Cells per μl | Protein mg per ml | TIMP-1 relative level | TIMP-2 relative level | TIMP-3 relative level | TIMP-4 relative level | MMP-3 relative level | Presence of proMMP-9 zymography | Presence of active MMP-2 zymography |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 01 | 45, F | 4 | 12 | 0.60 | 1.07 | 1.88 | 1.81 | 1.41 | 2.40 | + | + |
| 02 | 67, F | 5 | 3 | 0.23 | 0.89 | 1.63 | 1.30 | 1.69 | 2.21 | + | + |
| 03 | 49, F | 4 | 13 | 0.24 | 1.90 | 2.16 | 0.88 | 1.28 | 3.49 | + | + |
| 04 | 61, F | 2 | 14 | 0.29 | 0.74 | 0.68 | 0.54 | 1.60 | 2.11 | + | + |
| 05 | 65, M | 11 | 1 | 0.22 | 0.96 | 1.50 | 1.12 | 0.96 | 1.79 | + | + |
| 06 | 63, M | 19 | 0 | 0.17 | 0.99 | 1.19 | 1.21 | 1.10 | 1.93 | + | + |
| 07 | 47, F | 3 | 0 | 0.57 | 0.96 | 1.47 | 1.30 | 1.67 | 2.20 | + | + |
| 08 | 42, M | 2 | 11 | 0.57 | 0.43 | 1.09 | 1.35 | 1.31 | 1.92 | + | – |
| 09 | 40, F | 4 | 10 | 0.40 | 1.02 | 0.95 | 1.14 | 1.28 | 3.77 | + | – |
| 10 | 64, F | 8 | 10 | 0.50 | 0.96 | 0.88 | 1.09 | 0.90 | 3.55 | – | + |
| 11 | 66, F | 4 | 4 | 0.30 | 1.20 | 1.02 | 1.68 | 1.31 | 2.64 | – | – |
| 12 | 59, F | 12 | 2 | 0.30 | 1.14 | 1.11 | 1.91 | 0.77 | 1.89 | + | + |
| 13 | 66, M | 8 | 16 | 1.00 | 0.92 | 1.28 | 2.19 | 2.09 | 2.11 | + | + |
| 14 | 68, M | 20 | 2 | 0.20 | 0.93 | 0.82 | 1.04 | 1.12 | 2.35 | – | – |
| 15 | 75, F | 30 | 2 | 0.20 | 1.30 | 20.5 | 0.86 | 1.31 | 2.77 | – | – |
| 16 | 48, F | 2 | 38 | 0.30 | 1.15 | 1.66 | 1.25 | 1.37 | 1.97 | + | – |
| 17 | 68, F | 11 | 0 | 0.10 | 1.31 | 1.29 | 0.66 | 1.51 | 1.38 | + | + |
| 18 | 57, F | 6 | 27 | 0.40 | 0.93 | 1.36 | 2.64 | 1.88 | 1.67 | – | – |
| 19 | 68, F | 8 | 5 | 0.41 | 0.90 | 1.28 | 1.70 | 0.95 | 1.62 | – | – |
| 20 | 58, F | 10 | 7 | 0.38 | 1.02 | 0.92 | 0.83 | 1.30 | 3.64 | + | + |
| 21 | 39, M | 7 | 6 | 0.40 | 1.05 | 1.47 | 1.32 | 0.81 | 3.98 | + | – |
| 22 | 47, M | 4 | 3 | 0.20 | nd | nd | nd | nd | 2.58 | – | – |
| 23 | 64, M | 6 | 5 | 0.46 | nd | nd | nd | nd | 1.46 | + | + |
| 24 | 71, F | 8 | 80 | 0.29 | nd | nd | nd | nd | 2.14 | + | + |
| 25 | 59, F | 5 | 20 | 0.22 | nd | nd | nd | nd | 3.17 | + | + |

Data from immunoanalysis correspond to an average of at least two independent experiments, and have been normalized according to the corresponding mean value of the control group; nd: not determined; (+): presence; (–): not observed.

Table 2
Mean values of TIMPs and MMPs levels in controls, TSP/HAM and inflammatory patients

| | Controls ($\times 10^{-2}$ pixels)/μl | TSP/HAM ($\times 10^{-2}$ pixels)/μl | Inflammatory diseases ($\times 10^{-2}$ pixels)/μl |
|---|---|---|---|
| TIMP-1 | 162 ± 5 (n = 25) | 168[#] ± 10 (n = 21), $p > 0.50$ | 342* ± 28 (n = 28), $p < 0.000$ |
| TIMP-2 | 381 ± 25 (n = 25) | 489[*,#] ± 32 (n = 21), $p < 0.02$ | 1372* ± 235 (n = 28), $p < 0.000$ |
| TIMP-3 | 161 ± 12 (n = 25) | 213[*,#] ± 18 (n = 21), $p < 0.03$ | 340* ± 37 (n = 28), $p < 0.000$ |
| TIMP-4 | 407 ± 16 (n = 25) | 535[*,#] ± 31 (n = 21), $p < 0.001$ | 885* ± 77 (n = 28), $p < 0.000$ |
| MMP-3 | 229 ± 30 (n = 25) | 592[*,#] ± 34 (n = 25), $p < 0.000$ | 1840* ± 339 (n = 28), $p < 0.000$ |
| MMP-2 | 292 ± 29 (n = 18) | 413[#] ± 58 (n = 16), $p < 0.101$ | 1803* ± 274 (n = 28), $p < 0.000$ |
| MMP-9 | 152 ± 17 (n = 18) | 245[*,#] ± 25 (n = 16), $p < 0.004$ | 1182* ± 230 (n = 28), $p < 0.000$ |

Data are expressed as pixels ± SEM per μl of CSF; n = number of samples, significance was calculated using Student's paired t test.

[a]p values correspond to the statistic significancy of both patient gropus with the control group.

* Significant differences of patients (TSP/HAM and inflammatory) with controls with $p < 0.05$.

[#]Significant differences of TSP/HAM with the inflammatory group with $p < 0.05$.

comparative study could be immunowestern blot, however this method presents difficulties for a quantitative analysis in the transference process to the nitrocellulose membrane, and also in the simultaneous analysis of a large number of samples.

Normalized values of TIMP-2, -3 and -4 in TSP/HAM patients determined by immunodot blot are shown in Table 1, and the corresponding mean values ± SEM in Table 2. Unlike TIMP-1, relative mean values of these TIMPs were significantly higher than those of controls. In CSF of the 71% of the patients the relative mean value of TIMP-2 was over the mean value found in controls, and in the 76% of the cases TIMP-3 and TIMP-4 showed higher values than those in controls.

We have also measured TIMPs levels in CSF of a group of 28 patients with inflammatory neurological diseases for comparing with TSP/HAM patients. Normalized levels of TIMPs are shown in Table 3, together with some patients' clinical data, including age, sex, protein and cell count in CSF. The mean values shown in Table 2 indicate that, in the inflammatory group, the four TIMPs are significantly increased compared to both controls and TSP/HAM patients ($p < 0.05$).

*Detection of MMPs activities in CSF*

The possible implication of a dysregulated MMP/TIMP balance in TSP/HAM has been suggested by earlier studies demonstrating an overexpression of MMP-9 in CSF from TSP/HAM [25–28]. To test this proposal we determined MMP activity in the same patients and controls where we had measured TIMPs. MMP-2 and MMP-9 were followed by zymography in SDS-polyacrylamide gels copolymerized with gelatin (MMP-2 and -9 substrate). Following the electrophoresis, the SDS removal produced both renaturation of the active form of gelatinase and activation of the latent form [35]. As an example, the zymography of two controls and four TSP/HAM CSF samples is shown in Fig. 2.

We have observed in CSF the presence of MMP-9, in its 92 kDa proenzyme form in the 72% of TSP/HAM samples (Table 1), and in the 71% of the inflammatory group (Table 3). No evidence of MMP-9 either in the latent form (proMMP-9) or active form was found in the CSF from control subjects. The CSF of the two group of patients (TSP/HAM and inflammatory) and control subjects exhibited the 72 kDa proenzyme form of MMP-2. In around a 60% of TSP/HAM and inflammatory patients, the active form of MMP-2 was also detected, since a band of 68 kDa is observed. In contrast, only the 11% of

Table 3
Patient's clinical data, CSF parameters, presence of proMMP-9 followed by zymographic analysis, and relative levels of TIMPs and MMPs determined by immunodot blot

| Patient no. | Diagnosis | Age, sex | Cells per μl | Protein mg per ml | TIMP-1 relative level | TIMP-2 relative level | TIMP-3 relative level | TIMP-4 relative level | MMP-3 relative level | MMP-2 relative level | MMP-9 relative level | Presence of proMMP-9 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 01 | HIV cognitive-motor S | 36, F | 18 | 1.50 | 2.48 | 8.02 | 3.60 | 2.23 | 8.10 | 7.43 | 7.96 | + |
| 02 | HIV cognitive-motor S | 29, M | 7 | 0.38 | 1.78 | 4.42 | 1.44 | 1.49 | 6.61 | 4.10 | 4.39 | + |
| 03 | HIV cognitive-motor S | 27, M | 30 | 0.29 | 1.23 | 1.29 | 1.00 | 3.90 | 1.76 | 1.69 | 1.81 | – |
| 04 | HIV cognitive-motor S | 34, M | 6 | 0.45 | 1.90 | 2.30 | 1.76 | 1.70 | 4.53 | 3.35 | 3.59 | + |
| 05 | HIV cognitive-motor S | 29, M | 3 | 0.32 | 2.27 | 2.83 | 1.71 | 1.95 | 5.91 | 3.88 | 4.16 | + |
| 06 | HIV cognitive-motor S | 37, M | 2 | 0.47 | 1.46 | 1.21 | 0.31 | 1.43 | 4.18 | 3.68 | 3.94 | + |
| 07 | HIV cognitive-motor S | 28, M | 5 | 0.40 | 1.83 | 1.47 | 1.90 | 3.35 | 3.44 | 6.39 | 6.85 | + |
| 08 | HIV | 33, M | 7 | 0.54 | 2.19 | 3.34 | 2.02 | 2.07 | 8.16 | 4.74 | 5.07 | + |
| 09 | HIV, syphilis | 42, M | 7 | 0.25 | 1.57 | 1.41 | 0.32 | 0.86 | 3.93 | 2.44 | 2.62 | – |
| 10 | HIV, syphilis | 30, M | 5 | 0.63 | 1.99 | 2.36 | 1.90 | 1.57 | 0.85 | 2.82 | 3.03 | + |
| 11 | HIV, cytomegalovirus | 32, M | 6 | 0.56 | 1.74 | 2.90 | 3.15 | 1.48 | 8.14 | 4.97 | 5.33 | + |
| 12 | HIV, cytomegalovirus | 26, M | 6 | 0.65 | 1.54 | 3.32 | 2.12 | 1.93 | 6.73 | 4.33 | 4.65 | + |
| 13 | HIV, toxoplasmosis | 39, M | 3 | 0.80 | 2.89 | 8.20 | 2.33 | 2.62 | 17.38 | 12.29 | 13.18 | + |
| 14 | HIV, cryptococcal M | 35, M | 47 | 0.86 | 2.50 | 4.98 | 2.43 | 2.18 | 12.01 | 12.20 | 13.01 | + |
| 15 | Viral meningitis | 27, M | 40 | 0.70 | 3.04 | 11.30 | 3.42 | 5.92 | 15.31 | 11.28 | 12.09 | + |
| 16 | Viral meningitis | 30, M | 60 | 0.60 | 1.48 | 2.56 | 1.57 | 2.01 | 2.90 | 5.03 | 5.40 | + |
| 17 | Viral meningitis | 28, M | 90 | 0.10 | 2.07 | 7.38 | 0.91 | 1.46 | 4.65 | 4.80 | 5.15 | + |
| 18 | Chronic meningitis | 27, M | 10 | 0.70 | 1.16 | 0.52 | 1.03 | 1.61 | 1.31 | 1.11 | 1.41 | – |
| 19 | Neurocisticercosis | 37, M | 59 | 0.35 | 3.68 | 10.68 | 3.23 | 2.78 | 33.5 | 18.11 | 19.42 | + |
| 20 | Neurocisticercosis | 32, M | 53 | 4.10 | 5.13 | 6.09 | 5.54 | 2.77 | 22.48 | 17.91 | 19.20 | + |
| 21 | Neurocisticercosis | 56, F | 10 | 0.60 | 2.54 | 3.71 | 1.65 | 1.69 | 20.48 | 14.31 | 15.34 | + |
| 22 | Neurocisticercosis | 20, M | 3 | 0.60 | 3.87 | 12.58 | 2.75 | 1.13 | 2.75 | 2.75 | 2.94 | – |
| 23 | Multiple Sclerosis | 23, F | 7 | 0.41 | 1.65 | 1.43 | 1.46 | 2.15 | 1.57 | 1.95 | 2.09 | – |
| 24 | Multiple Sclerosis | 57, F | 10 | 0.22 | 0.87 | 0.92 | 0.60 | 1.58 | 2.65 | 2.63 | 2.82 | – |
| 25 | Multiple Sclerosis | 30, F | 5 | 0.40 | 1.75 | 0.32 | 1.43 | 1.72 | 3.66 | 2.65 | 2.84 | + |
| 26 | Multiple Sclerosis | 51, F | 4 | 0.26 | 1.36 | 1.44 | 2.52 | 1.63 | 2.09 | 3.66 | 2.24 | – |
| 27 | Guillain–Barré S | 45, M | 4 | 0.90 | 3.32 | 11.80 | 3.71 | 2.89 | 11.07 | 13.36 | 11.87 | + |
| 28 | Guillain–Barré S | 25, M | 3 | 0.85 | 1.20 | 0.76 | 4.51 | 2.75 | 2.27 | 2.33 | 2.44 | – |

Immunoanalysis data, normalized according to mean values of the control group, correspond to an average of at least two independent experiments. (+) gelatinolytic band observed; (–) not observed. S: syndrome; M: meningitis.

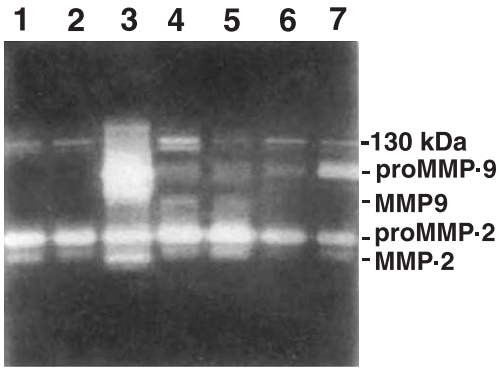


Fig. 2. Zymogram in SDS/PAGE under non-reducing conditions using gelatin as substrate. Portions of 10 μl of sample (7.5 μl CSF or positive control plus 2.5 μl of sample buffer) were placed in each lane. Lanes 1 and 2 correspond to CSF from controls, lane 3: positive control for zymography of MMP2-/MMP-9 previously dituted 1:500 in 50 mM Tris–HCl, pH 7.5, 10 mM $CaCl_2$; lanes 4–7 represent CSF from TSP/HAM.

controls had this active form of MMP-2. In a previous report we have shown in CSF from TSP/HAM patients, the partial activation of latent MMPs (proMMP-9 with 92 kDa, and proMMP-2 with 72 kDa), after incubation with APMA (p-aminophenylmercuric acetate), to their active forms with lower *Mr* [24], similar to the additional bands pointed at Fig. 2.

The gelatinolytic activity with a *Mr* of 130-kDa found in CSF samples has been suggested that correspond to lipocalin-associated MMP-9, stable to SDS treatment [28]. The active form of MMP-9 has been detected in 4 of 25 samples from TSP/HAM, and in 5 of 28 samples from the inflammatory patients.

Zymographic analysis using either gelatin (1 mg/ml) or laminin (0.1 mg/ml) in the gel was not sensitive enough for the detection of MMP-3 in CSF from controls or patients. However, MMP-3 activity of a positive control was observed in a laminin gel (data not shown). Thus the low sensitivity of this analysis precluded us from determining MMP-3 activity in CSF samples using zymography.

*Level of MMPs in CSF*

Attemps for the quantification of MMP-9 level in CSF using ELISA failed because we could measure MMP-9 in only 3 of the 13 TSP/HAM samples tested (ranging from 11.9 to 62 ng/ml), nevertheless, all these samples exhibited intense 92 kDa gelatinolytic band in the zymogram. Therefore, to confirm the possible upregulation of MMP-9 deduced from zymography, we compared MMP-9 levels in the inflammatory patients with controls by immunodot blot analysis. Table 3 shows the normalized data found in patients together with those of MMP-2. Both enzymes showed to be upregulated in the inflammatory patients (Table 2). We could make this analysis with only some of TSP/HAM available samples (including 12 of the previously CSF studied samples), finding that only MMP-9 was statistically significantly increased but to a lesser extent than the inflammatory group (Table 2).

These results indicate that the ELISA kit was not sensitive enough for detecting MMP-9 in CSF samples with lower MMP-9 gelatinolytic activity. Instead, zymographic and immunodot blot analyses

Table 4
Spearman rank correlations of MMPs with TIMPs in CSF from TSP/HAM and inflammatory patients

| | MMP-2 | MMP-9 | MMP-3 |
|---|---|---|---|
| *TSP/HAM* | | | |
| TIMP-1 | $r = -0.680$*, $p < 0.011$ | $r = 0.018$, $p > 0.957$ | $r = 0.165$, $p > 0.475$ |
| TIMP-2 | $r = 0.231$, $p > 0.448$ | $r = -0.014$, $p > 0.966$ | $r = -0.047$, $p > 0.841$ |
| TIMP-3 | $r = 0.473$, $p > 0.103$ | $r = 0.273$, $p > 0.391$ | $r = -0.074$, $p > 0.750$ |
| TIMP-4 | $r = 0.572$*, $p < 0.041$ | $r = 0.336$, $p > 0.286$ | $r = -.0.205$, $p > 0.372$ |
| *Inflammatory* | | | |
| TIMP-1 | $r = 0.700$**, $p < 0.000$ | $r = 0.736$**, $p < 0.000$ | $r = 0.652$**, $p < 0.000$ |
| TIMP-2 | $r = 0.784$**, $p < 0.000$ | $r = 0.778$**, $p < 0.000$ | $r = 0.772$**, $p < 0.000$ |
| TIMP-3 | $r = 0.500$**, $p < 0.007$ | $r = 0.576$**, $p < 0.001$ | $r = 0.479$*, $p < 0.010$ |
| TIMP-4 | $r = 0.393$*. $p < 0.038$ | $r = 0.235$, $p > 0.229$ | $r = 0.302$, $p > 0.118$ |

In TSP/HAM correlations: n = 13 for MMP-2; n = 12 for MMP-9 and n = 21 for MMP-3. In all inflammatory cases n = 28.
*Significant differences $p < 0.05$.
**Significant differences $p < 0.01$.

were sensitive assays for the measurement of this MMP. The two last methods produce concordant results (see Table 3).

Normalized levels of MMP-3 in CSF from TSP/HAM, followed by immunodot blot analysis, are shown in Table 1. The mean value ± SEM of MMP-3 in TSP/HAM was significantly higher than that of control subjects. The results in the inflammatory group indicated higher levels of MMP-3 in this group compared to controls and TSP/HAM (Table 2).

### *Correlation between levels of MMPs and TIMPs*

In general no significant correlation was found between MMP and TIMP levels in TSP/HAM, except for MMP-2, and TIMP 1 and -4, where according to the nonparametric Spearman's rho correlation coefficient, a negative and a positive significant correlation was found, respectively (Table 4). On the other hand, several correlations were found in inflammatory diseases, summarized in Table 4. The best correlations in the inflammatory group corresponded to TIMP-1 and -2 with the three MMPs, while the lower were obtained between TIMP-3 and -4, and MMPs, with no correlation between TIMP-4, and MMP-9 and MMP-3.

## Discussion

This is the first report of a parallel study done on CSF levels of the four TIMPs and several MMPs in TSP/HAM and their comparison with acute inflammatory patients and controls. The major finding of this study is the lack of increase in TIMP-1 in CSF of TSP/HAM patients compared to inflammatory patients. The overexpression of these extracelullar matrix proteins detected in CSF of TSP/HAM should be indirectly produced by the chronic secretion of viral proteins like Tax, being responsible for the progressive character of this disease, different to the acute or subacute inflammatory neurological diseases. Although in TSP/HAM the CSF levels of TIMP-2, -3 and 4 are significantly increased

compared to control, however these increases are smaller compared to the acute inflammatory cases. In CSF of all TSP/HAM patients we have found increased at least one of the TIMPs compared to the average value from controls. The three TIMPs were elevated in the 38% of the cases, two or three of them in the 81% of the patients, and at least one in the remaining 19%. Except for some preliminary reports in TSP/HAM, levels of TIMP-3 and -4 in CSF of inflammatory disorders or other neurological alterations have not been described so far. As was previously mentioned, a qualitative study on TSP/HAM patients shows a similar frequency of TIMP-1 compared to asymptomatic carriers, while TIMP-3 was detected in the 77.4% of the patients [28]. In the present quantitative study, the 71% of the cases showed higher levels of TIMP-3 in CSF compared to controls.

Overexpression of TIMP-1 together with MMP-9 in CSF has been previously reported in some acute infectious diseases like viral and bacterial meningitis, and fungal or tuberculous meningoencephalitis [14–17,36]. In those cases the elevation of MMPs and TIMPs is attributed to the presence of polymorphonuclear cells in CSF, while in TSP/HAM and HIV patients, except one case complicated with cryptococcal meningitis (Table 3 patient number 14) no correlation between cell count and increase in MMPs in the CSF was observed (compare Tables 1 and 3). Therefore, in these two viral diseases characterized by the release of viral proteins with gene transactivation activity, MMPs and TIMPs overproduction should arise from activation of CNS cells [37], because in the 70% of CSF from TSP/HAM and 79% of HIV, the white cell number was quite normal (Tables 1 and 3). In addition of the upregulation of MMP-9, in meningitis and human immunodeficiency virus dementia increased levels of MMP-2 in CSF have been reported [12,17]. As far as regards the MMP-3 level in meningitis, probably due to the low sensitivity of the ELISA kit used, no upregulation has been described [15,16].

The two reports on TIMP-1 or TIMP-2 levels in CSF from MS patients did not describe upregulation of these inhibitors [20–22]. Accordingly, the four MS samples show the smaller changes in TIMP-1 and TIMP-2. CSF levels of TIMPs in the other inflammatory disorders (HIV, Guillain–Barré, neurocisticercosis) have not been described. CSF levels of the four TIMPs showed larger upregulation in HIV (14 patients), Guillain–Barré (2 patients), and neurocisticercosis (4 patients). Interestingly, several correlations between MMPs with TIMP-1, -2 and 3 have been found in these inflammatory samples, pointing out to another difference with TSP/HAM where no correlation between MMPs and TIMPs was observed, except for MMP-2, and TIMP-1 and TIMP-4.

MMPs are regulated by formation of tight, 1:1 non-covalent complexes with TIMPs [3]. The four TIMPs share many properties but also have distinct activities. TIMPs form complexes with both the proenzyme and active forms of MMPs. Complexes like pro-MMP-9/TIMP-1, pro-MMP-2/TIMP-2 and pro-MMP-2/TIMP-3 control the rate at which physiological factors activate MMPs. While in some cases MMPs and their inhibitors (TIMPs) can be coordinately induced, in anothers, opposite patterns of regulation of MMPs and TIMPs have been found [3]. Under physiological conditions, MMP activity is precisely controlled, an excess of MMP production and activation could be a key feature of the pathology of many inflammatory and malignant diseases [3,4].

CSF samples from TSP/HAM patients revealed an increment in MMPs, involving a significant increase in MMP-3 in all samples, and the presence of a gelatinolytic band of 92 kDa (proMMP-9) in 72% of CSF from TSP/HAM. Therefore, the chronic overexpression of MMP-9, MMP-3 and the higher frequency of the active form of MMP-2, independent of the years of evolution of TSP/HAM, could alter the ECM components of basal lamina.

This overexpression could be caused by the action of the viral protein Tax, which modulates (transactivation/repression) the expression and function of many cellular genes through binding to

groups of transcriptional factors and coactivator proteins, being metalloproteinase-9 gene one of the targets [38–40]. This is supported by an in vitro model mimicking the HTLV-I infected T-cell/astrocyte interaction, where an enhanced secretion of MMP-3 and active MMP-9 from astrocytes was demonstrated [30]. Thus, protein Tax or other secreted viral proteins could be responsible of MMPs (MMP-9 and -3) upregulation accounting for the chronicity and progression of TSP/HAM. The upregulation of some TIMPs in CNS could be in response to the increasing proteolytic activity, however, part of the damage associated to HTLV-I infection could be due to an abnormal stimulus in the proteolysis of ECM components. There is no explanation for the lack of changes of TIMP-1 level, while a selective upregulation of TIMP-2, -3 and -4 is produced.

In summary, we could establish differences in the pattern of overexpression of MMPs and TIMPs in CSF between three types of neurological diseases: a) the chronic progressive TSP/HAM presenting an increase in MMP-9 and MMP-3, together with a parallel but lower augmentation of TIMP-2, TIMP-3 and TIMP-4; b) the subacute HIV, MS and Guillain–Barré syndrome also with low cells count, where the four TIMPs and MMPs are upregulated, being less notorious in MS; and c) acute diseases like meningitis and neurocisticercosis with higher cell count than the normal values reported in CSF, with the most significant increase of both TIMPs and MMPs. In addition, our results support the stated hypothesis of an imbalance between MMPs and TIMPs, because increases in 30% of each TIMP (TIMP-2, -3 and -4) are not able to counterbalance the larger augmentation of MMPs, with an increase around a 1.5 times of MMP-2 and MMP-9, together with an elevation of 2.5 times of MMP-3.

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