Extracellular matrix protein expression in cerebrospinal fluid from patients with tropical spastic paraparesis associated with HTLV-I and Creutzfeldt-Jakob disease

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Cartier L, García L, Kettlun AM, Castañeda P, Collados L, Vásquez F, Giraudon P, Belin MF, Valenzuela MA. Extracellular matrix protein expression in cerebrospinal fluid from patients with tropical spastic paraparesis associated with HTLV-I and Creutzfeldt-Jakob disease.

The cerebrospinal fluid (CSF) is in direct contact with the extracellular space of the CNS, thus biochemical processes in the CNS could potentially be reflected in the CSF. Changes in extracellular matrix (ECM) proteins can be studied through their analysis in the CSF. ECM plays an essential role in CNS homeostasis and several proteins such as laminin (LN), fibronectin (FN), thrombospondin (TS) and heparan sulphate proteoglycan (HS, perlecan) form part of its structure. Possible changes in the levels of these proteins were investigated in two different pathologies —tropical spastic paraparesis/HTLV-I-associated myelopathy (TSP/HAM) (n = 25) and Creutzfeldt-Jakob disease (CJD) (n = 19)—and compared with those in a control group with or without neurological disease (n = 25). CSF analyses were carried out using monoclonal or monospecific polyclonal antibodies. In comparison with the control group, it was found that TSP/HAM patients presented significantly higher levels of LN, TS and HS, while in CJD patients the levels of FN, TS and HS were increased. In CJD patients the HS level was almost double that of the TSP/HAM patients. These results suggest a distinct pattern of ECM proteins in CSF in relation to the type of neurological disease. TSP/HAM is a chronic motor disease that affects the white matter of the spinal cord, while CJD is a subacute dementia that affects cerebral neurons and their synapsis.

Key words: Cerebrospinal fluid; Creutzfeldt-Jakob disease; extracellular matrix proteins; fibronectin; laminin; perlecan; thrombospondin; TSP/HAM

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INTRODUCTION

In nearly 1% of infected people, HTLV-I is responsible for producing a chronic progressive myelopathy (many years) known as tropical spastic paraparesis/HTLV-I associated myelopathy (TSP/HAM), characterized by progressive axonal degeneration of the cortical spinal tracts [1–3]. The precise mechanisms for this CNS axonal damage and for demyelination processes are not known so far. The HTLV-I inserted in the genome of T lymphocytes encodes the *tax* protein, a gene transactivator known to have pleiotropic functions that may play some role in the TSP-HAM pathogenesis [4]. Creutzfeldt-Jacob disease (CJD) is a subacute, degenerative, fatal process (months), characterized by a rapidly progressive cognitive and neurological impairment [5, 6]. It originates in a prion infection that produces spongiform encephalopathy with significant alteration of neurons and loss of cortical and subcortical structures [7, 8]. Both disorders, TSP/HAM and CJD, from quite different origins and localization could modify the normal functioning of the extracellular matrix (ECM), alterations that could be followed in the cerebrospinal fluid (CSF) of the patients.

In the CSF from TSP/HAM patients, overexpression of metalloproteinases (involved in the normal exchange of the ECM proteins), MMP-3 and MMP-9 and some of their tissular inhibitors, TIMP-2, TIMP-3 and TIMP-4, has been reported [9-12], while in CJD, MMP-9, TIMP-1 and TIMP-2 were up-regulated [13]. In TSP/HAM the imbalance between MMP and TIMP was proposed as one of the pathogenic factors that could result in a more uncontrolled ECM degradation [12].

ECM proteins such as laminin (LN), fibronectin (FN), thrombospondin (TS) and perlecan (HS, a heparan sulphate proteoglycan, HSPG) are involved in axonal growth and plasticity [14]. MMPs and other proteases participate in their physiological degradation [14, 15]. Thus a potential role for some specific alterations of ECM molecules in neuropathological disorders has been proposed [16, 17].

In the present study, we evaluated the relative levels of LN, FN, TS and perlecan in CSF from patients with TSP/HAM and CJD and compared them with the levels in the CSF of the control group.

MATERIALS AND METHODS

CSF samples

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 were routinely measured [18]. Control and patient groups were duly informed of the research protocol approved by the Human Ethics Experimentation Committee at the Hospital del Salvador (Santiago, Chile), and all of them agreed to participate in this study.

TSP/HAM patients (21 cases, mean age 59 years) were identified both clinically and after determination of HTLV-I antibodies in serum [19], or by the presence of the *tax* gene in the PBMC fraction (peripheral blood mononuclear cells) by polymerase chain reaction (PCR) [20]. The CJD group comprised 19 patients (mean age 57 years) who were clinically and electroencephalographically diagnosed with CJD, including determination of the 14-3-3 proteins in the CSF [21, 22]. TSP/HAM and CJD patients' clinical data are presented in Tables I and II. The control group comprised 25 individuals including 10 patients from gynaecology (spinal anaesthesia) aged 23–54 years, 2 patients with headache aged 29 and 79 years, respectively, 6 patients with cognitive compromise (no Alzheimer's disease) aged 57–80 years, 4 patients with lacunar infarction aged 52–80 years, one patient with pontine myelinolysis aged 49 years and 2 patients with chronic polyneuropathy aged 57 and 62 years, respectively This heterogeneous control group could be divided in two subgroups: one group including 10 subjects with no neurological pathology (those that received spinal anaesthesia) and the other subgroup including 15 patients with some clinical neurological findings.

Detection of LN, FN, TS, HS and 14-3-3 proteins by immunodot blot

Concentrations of the ECM and the 14-3-3 proteins were determined separately by applying CSF (3.0–7.5 μL) to a nitrocellulose membrane (BioRad, Hercules, CA, USA). The samples from each group (TSP/HAM, CJD and control) were tested simultaneously on the same nitrocellulose membrane. The membrane was blocked for 4 h in TBS-T buffer (Tris Buffer Saline with Tween)

TABLE I. TSP/HAM patients' clinical data and the relative amount of LN, FN, TS and HS (expressed in pixels/μL of CSF) determined by dot-blotting analysis. Data correspond to an average of at least two independent experiments where samples from patients and controls were tested at the same time.

Patient no.	Age, sex	Evolution time (years)	Functional level	LN	FN	TS	HS
1	47, F	2	Gait without support	1328	507	1230	334
2	60, F	3	Gait without support	1323	503	1231	316
3	66, M	3	Gait without support	1446	574	1114	384
4	57, F	3	Gait without support	1050	586	372	299
5	66, F	4	Gait without support	958	472	726	193
6	49, F	5	Gait without support	1168	926	545	419
7	60, F	6	Gait without support	1485	519	608	318
8	60, F	13	Gait without support	1192	393	198	147
9	41, M	2	Gait with support	1457	537	922	356
10	40, F	3	Gait with support	1525	529	913	276
11	58, F	6	Gait with support	1490	482	1172	291
12	70, F	7	Gait with support	1537	1018	1286	479
13	66, M	10	Gait with support	1063	671	1161	430
14	68, M	10	Gait with support	1227	506	782	310
15	67, F	10	Gait with support	1054	306	764	269
16	62, M	11	Gait with support	1136	484	836	270
17	63, M	20	Gait with support	1288	574	755	359
18	52, F	4	Wheelchair	1204	640	790	307
19	57, F	5	Wheelchair	1201	313	180	228
20	51, F	5	Wheelchair	1230	474	894	269
21	75, F	30	Bedridden	1733	607	811	228

TSP/HAM = tropical spastic paraparesis/HTLV-I-associated pyelopathy; LN = laminin; FN = fibronectin; TS = thrombospondin; HS = heparan sulphate; CSF = cerebrospinal fluid.

TABLE II. CJD patients' clinical data and the relative amount of LN, FN, TS and HS (expressed in pixels/μL of CSF) determined by dot-blotting analysis. Data correspond to an average of at least two independent experiments where samples from patients and controls were tested at the same time.

Patient no.	Age, sex	Evolution time (months)	Functional level	LN	FN	TS	HS
1	70, F	2	Motor cognitive S.	1019	928	3105	525
2	58, M	2	Motor cognitive S.	860	736	2010	840
3	52, F	3	Motor cognitive S.	1418	645	1285	519
4	55, F	3	Motor cognitive S.	1124	711	1042	730
5	53, M	6	Motor cognitive S.	951	670	213	793
6	47, M	3	Myoclonic dementia	1038	553	175	693
7	57, F	3	Myoclonic dementia	1007	629	234	686
8	57, M	3	Myoclonic dementia	1188	880	1332	433
9	54, F	3	Myoclonic dementia	1089	630	1461	496
10	60, M	4	Myoclonic dementia	1372	336	742	576
11	62, F	4	Myoclonic dementia	1129	648	286	615
12	63, F	4	Myoclonic dementia	1064	782	423	266
13	63, F	4	Myoclonic dementia	1661	416	1130	915
14	50, F	6	Myoclonic dementia	1159	594	887	342
15	62, F	5	Myoclonic dementia	1266	707	967	311
16	47, F	5	Myoclonic dementia	903	697	1007	327
17	61, M	6	Akinetics mutism	1042	465	2427	313
18	59, F	6	Akinetics mutism	1041	745	411	559
19	60, M	4	Akinetics mutism	1249	539	1655	698

CJD = Creutzfeldt-Jacob disease; LN = laminin; FN = fibronectin; TS = thrombospondin; HS = heparan sulphate; CSF = cerebrospinal fluid; S = syndrome.

composed of 20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween 20, 6% non-fat milk, pH 7.6. Primary antibodies were purchased from Chemicon International (Temecula, CA, USA) with the exception of anti-alpha/beta 14-3-3 proteins (Zymed Laboratories Inc., CA, USA). Monospecific polyclonal antibodies were used for LN (Cat. AB 19012), diluted 1:2500 in TBS-T buffer, and for 14-3-3 proteins (Cat. 51-0700) diluted 1:1000. The other proteins were detected with monoclonal antibodies using a dilution of 1:3000 for FN (Cat. MAB 122), 1:100 for TS (Cat. MAB 054) and 1:1000 for HS (Cat. MAB 1948). Primary antibodies, diluted in TBS-T buffer, were incubated overnight. After rinsing with TBS-T buffer without milk, the blots previously treated with the different primary antibodies were incubated for 1 h with the secondary anti-rabbit antibody conjugated with peroxidase, diluted 1:5000 in TBS-T (Pierce, Illinois, USA. Cat. 31462) for polyclonal antibody analysis, and with anti-mouse conjugated with peroxidase (Pierce, Cat. 31452) diluted 1:10 000 in TBS-T for the development of the monoclonal antibodies. After rinsing with TBS-T (without milk), 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 (Silk Scientific Corporation). Control experiments in the absence of the primary antibody but in the presence of the two secondary antibodies did not yield any chemiluminescent signal. These immunodot blot determinations were done in duplicate using two independent nitrocellulose membranes, and further by averaging the integration data.

Western blotting of LN, FN, TS, HS, and 14-3-3 proteins in CSF

CSF proteins were separated by SDS-PAGE [23] using 10% polyacrylamide, and then electroblotted onto nitrocellulose membrane [24]. After electrotransference, the membrane was subjected to the same procedure as described for immunodot blot analysis.

Statistical analysis

Statistical analyses were done using the non-parametric Mann-Whitney U test for two independent samples. Differences were considered as statistically significant at $p < 0.05$. Values of the three groups studied were expressed as the median together with 25th and 75th percentiles. Associations between variables were assessed using the non-parametric Spearman rank correlation analysis.

RESULTS

Summary of patients

Age, sex and evolution time when the lumbar puncture was taken are listed in Tables I and II, as well as the functionality of the patients with TSP/HAM and CJD, which defines their condition at the moment of the CSF study. The CJD patients demonstrated a high immunoreactivity against the alpha/beta 14-3-3 proteins, which is used as part of the diagnosis. Western blotting of CJD patients' CSF showed the presence of immunoreactive bands against the antibody for 14-3-3 proteins between 29–32 kDa according reports elsewhere [22].

Extracellular matrix protein content in CSF

Western blotting of LN in CSF showed the presence of three additional immunoreactive bands (M_r 95.7, 74.5 and 30 kDa) in addition to the band corresponding to the intact monomer (205–210 kDa). In addition to the whole proteins, Western blotting of the CSF developed with anti-FN and anti-HS antibodies also showed the presence of degradation products. These degradation products were found to be a 40 kDa polypeptide in the case of FN, and four polypeptides in the case of HS with M_r 87, 71, 53 and 27 kDa, respectively. We did not detect degradation products of TS, probable owing to a lack of sensitivity.

Comparison of the relative levels of the four ECM proteins studied was done by immunodot blot analysis instead of Western blotting, for two main reasons. First, the quantitative analysis by Western blotting presents difficulties for the large variability and loss of protein in the transference process to the nitrocellulose membrane, and second the impossibility of conducting a simultaneous analysis of a large number of samples. The relative values, expressed as pixels/μL of CSF, for TSP/HAM

and CJD are presented in Tables 1 and 2, respectively. The variation of the method averaging the dot integration obtained from two parallel experiments, including in each nitrocellulose membrane all patient and control samples, was less than 10%.

The relative levels of ECM proteins in the CSF of the three groups (control group, TSP/HAM and CJD) expressed as median values with their 25th and 75th percentiles are summarized in Table III. Immunoreactivities against LN, TS and HS in the CSF of patients with TSP/HAM were significantly higher than those in the control group comprising 25 subjects ($p < 0.05$), while the CJD group showed significantly elevated FN, TS and HS values in relation to those of the controls ($p < 0.05$). We also compared the CSF data from the TSP/HAM and CJD groups with the subgroup of controls that included only subjects that received spinal anaesthesia, which constitutes a true control group. Interestingly, we found similar significant differences that justify the use of both subgroups as only one group.

According to the non-parametric correlation of Spearman's rho, no significant correlations were found between ECM protein levels and the evolution state of either the TSP/HAM or CJD groups. However, in TSP/HAM patients, positive correlations between TS with LN ($p = 0.049$) and HS ($p = 0.031$) were obtained with correlation coefficients of 425 and 460, respectively.

DISCUSSION

We compared the relative levels of ECM proteins expressed in the CSF from the chronic myelopathy of TSP/HAM, and a subacute disease without inflammatory components, such as CJD, taking into consideration the essential role of the ECM for the maintenance of both central and peripheral nervous system structures [14, 25, 26]. Changes in the activity of these systems were shown in this series of 21 patients with TSP/HAM and 19 patients with CJD, compared with a control group. A common finding in the CSF from these two groups of patients is an increase in thrombospondin and perlecan, but with CSF showing a distinct pattern of overexpression of fibronectin, found to be increased in CJD patients only, and laminin in TSP/HAM patients.

TABLE III. Relative values of LN, FN, TS and HSPG (perlecan) levels in CSF from controls, TSP/HAM and CJD patients. Data, expressed in pixels per μL of CSF, represent median, 25th and 75th percentiles, and p-values obtained from the non-parametric Mann-Whitney U test. A p-value < 0.05 is considered to be significant.

	Controls	TSP/HAM	CJD
Laminin			
Median	993	1230	1089
25th to75th percentile	856 – 1224	1152 – 1471	1019 – 1249
p-value for control		0.001	0.110
p-value for TSP/HAM			0.008
Fibronectin			
Median	517	519	648
25th to 75th percentile	425 – 593	478 – 597	553 – 736
p-value for control		0.559	0.002
p-value respect to TSP/HAM			0.013
Thrombospondin			
Median	678	811	1007
25th, to 75th percentile	552 – 768	667 – 1138	411 – 1461
p-value for control		0.008	0.045
p-value for TSP/HAM			0.320
Heparan sulphate			
Median	253	307	559
25th to 75th percentile	203 – 312	269 – 358	342 – 698
p-value for control		0.041	0.000
p-value for TSP/HAM			0.000

TSP/HAM = tropical spastic paraparesis/HTLV-I-associated myelopathy; LN = laminin; FN = fibronectin; TS = thrombospondin; HSPG = heparan sulphate proteoglycan; CSF = cerebrospinal fluid; CJD = Creutzfeldt-Jacob disease.

The structural modifications induced by HTLV-I-infection in the CNS are expressed particularly in the corticospinal tracts constituted by the longest and thickest axons of the CNS, and may create special conditions in the pyramidal tract, where possibly the axonal regeneration depends on the interplay between extrinsic cues and intrinsic properties of the damaged axons [25].

In CJD with grey matter spongiosis, neuronal loss and synapsis diminution [8, 27], we found a larger release of HS in the CSF. In concordance with this observation, a tight binding of the HSPG agrin to the synaptic basal lamina has recently been demonstrated [28]. Thus, we suggest that an increase of HS is related to the synapsis loss. A lack of correlation between the evolution time of the disease and ECM protein levels in CSF samples from TSP/HAM and CJD patients was demonstrated.

Diverse functions have been attributed to the ECM proteins in addition to the solely structural function. Lesion-induced molecules such as LN, FN, different types of collagen, proteoglycans, tenascin and neurotrophic factors support axonal regeneration [26]. LN functions *in vitro* as a very potent neurite outgrowth-promoting molecule by interacting with the growing tip of neurites, while FN and collagens are poorer growth promoters [14, 26, 29, 30]. The heparan sulphate moiety has a relatively high affinity to many chemotropic factors, e.g. growth factors and others that induce the outgrowth of neurite *in vitro* [31]. Among these growth factors, one that seems to be important is the fibroblast growth factor (FGF), which is related to HS and TS [14, 32]. Thrombospondin is part of a family of adhesive glycoproteins involved in a number of physiological processes including angiogenesis and neurite outgrowth [33]. The pleiotropic effects of TS are the consequence of its modular structure, which enables binding to specific cell receptors, growth factors (FGF-2, TGF, PDGF) and several proteinases [32].

Additional reports indicate that both FN and TS regulate the gene expression of extracellular proteases, such as MMP-2, MMP-3 and MMP-9 in different cell systems [34]. These antecedents make the study and definition of the changes in the level of these ECM proteins more interesting, expressed in the CSF of patients with a diversity of neurological diseases, considering that we have observed an up-regulation in the metalloproteinase activity in the CSF from these two types of diseases [12, 13].

This study has shown a significant increase in some ECM proteins compared with those in controls that could define a tendency towards deleterious effects in two different diseases, TSP/HAM and CJD. In TSP/HAM, the pathogenic factors that play a part in the disease seem to maintain a continuous degradation of the ECM that prevents axonal regeneration, producing atrophy of the spinal cord. In CJD, changes in a membrane protein (the prion protein) lead to increased degradation in the synaptic areas that were expressed in the CSF. Our findings of differences in the type and degree of the changes of some ECM molecules in the CSF from two pathologies that affect different areas of the CNS suggest specificity in the changes that could lead to a diagnostic meaning. However, further studies are required to obtain well-established patterns.

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