Soluble TREM-2 in cerebrospinal fluid from patients with multiple sclerosis treated with natalizumab or mitoxantrone

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Abstract

Background: Microglia-mediated proteolysis of the triggering receptor expressed on myeloid cells (TREM-2) produces soluble TREM-2 (sTREM-2) that can be measured in cerebrospinal fluid (CSF) samples. Loss-of-function mutations in *TREM2* or in the gene encoding its adaptor protein cause the rare Nasu-Hakola disease (NHD). Multiple sclerosis (MS) is an autoimmune disease that in common with NHD is characterized by demyelination and microglial activation.

Objective: To investigate the potential utility of sTREM-2 as a biomarker for MS and to follow treatment effects.

Methods: Soluble TREM-2 was analyzed in CSF samples from subjects with MS (N=59); relapsing-remitting MS (RRMS) (N=36), secondary progressive MS (SPMS) (N=20) and primary progressive MS (PPMS) (N=3), and controls (N=27). CSF levels of sTREM-2 were also assessed before and after treatment of patients with natalizumab or mitoxantrone.

Results: CSF levels of sTREM-2 were significantly increased in patients with RRMS, SPMS and PPMS compared with controls. After natalizumab treatment, the levels of sTREM-2 were normalized to control levels. The levels of sTREM-2 were also reduced after mitoxantrone treatment.

Conclusion: Increased CSF levels of sTREM-2, a new marker of microglial activation, in MS and normalization upon treatment with either natalizumab or mitoxantrone support a role for microglial activation in active MS.

Introduction

Multiple sclerosis (MS) is a chronic, inflammatory, demyelinating disease of the central nervous system (CNS).[1] The disease process involves CNS injury by auto-reactive immune cells, which leads to demyelination and damage of axons. Even though MS is primarily an inflammatory disease of the CNS, the pathological changes over time become dominated by microglial activation associated with extensive and chronic neurodegeneration.[1] The disease usually begins with a relapsing-remitting course (RRMS) that may shift into a secondary progressive phase (SPMS) that carries features of neurodegenerative diseases. A few patients have a progressive onset with slowly increasing neurological dysfunction, which is termed primary progressive multiple sclerosis (PPMS).[1]

Triggering receptors expressed on myeloid cells (TREM-2) is a cell surface receptor predominantly expressed on myeloid cells, e.g. monocyte-derived dendritic cells, macrophages, mast cells, osteoclasts and microglia.[2, 3] Previous studies have identified microglia as the brain cells expressing the highest levels of TREM-2.[4] Activation of the TREM-2 receptor in most myeloid cells investigated so far attenuates the immune response.[5] Knowledge on the normal physiological functions of TREM-2 is inadequate and loss-of-function mutations in humans have contributed to most information regarding its potential role. Nasu-Hakola disease (NHD; polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy), is caused by missense mutations in *TREM2* or the gene encoding its cytosolic DNAX adaptor protein 12 (*DAP12*).[6] This rare disorder is characterized by demyelination of the CNS, presenile dementia and bone cysts.[6] Recently, genome-wide association studies have also shown that rare variants in the *TREM2* gene are associated with an increased risk of Alzheimer's disease.[7, 8] Recent reports have also demonstrated an

association of *TREM2* variants with Parkinson's disease, amyotrophic lateral sclerosis and frontotemporal dementia[9-11], while *TREM2* has not yet been genetically linked to MS. The TREM-2 receptor undergoes ectodomain shedding producing soluble TREM-2 (sTREM-2)[12] that possibly corresponds to the form of TREM-2 that has previously been observed in CSF samples from patients with MS, frontotemporal dementia and Alzheimer's disease.[13, 14]

In the present study, we analyzed sTREM-2 in CSF samples from subjects in various stages of MS (RRMS, SPMS and PPMS) and healthy controls. The CSF levels of sTREM-2 were also assessed before and after treatment of patients with potent immune-modulating or immunosuppressive drugs.

Materials and Methods

Patients and healthy controls

The study included 58 MS patients[15, 16]; 36 RRMS, 20 SPMS, and three PPMS[17] and 27 healthy controls. The study was approved by the regional ethical board of the University of Gothenburg, Sweden. Informed consent was obtained from all participants. Patients were recruited from two patient cohorts at the Department of Neurology, Sahlgrenska University Hospital, Gothenburg, Sweden. Both patient groups have been described previously.[18, 19] Group 1 consisted of patients who were scheduled to start treatment with 300 mg natalizumab intravenously once monthly according to Swedish guidelines (www.mssallskapet.se). All patients in this group (37 RR-, 1 SP- and 1 PPMS) presented either a highly active disease course *de novo* or breakthrough disease activity in terms of relapses in the presence of first line disease-modifying treatments (DMTs). Group 2 consisted of patients (1 RR-, 19 SP- and 1 PPMS) about to start treatment with mitoxantrone (intravenous administration 12 mg/m^2 at three-month intervals for 2 years; i.e., 8 infusions, total dose 96 mg/m^2) according to established regimens[20] and Swedish guidelines (www.mssallskapet.se).

Healthy controls were blood donors or students with no history of neurological disease and no abnormal signs were found at neurological examination. All participants underwent clinical neurological examination, peripheral blood sampling, and lumbar puncture. Disease duration was estimated from onset of first demyelinating symptoms.

Pooled decoded CSF samples supplied by the Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, from patients who underwent lumbar puncture to exclude infectious disorders of the CNS were used as quality control (QC) samples.

Clinical assessment and specimen sampling

The neurological examinations were performed by trained neurologists, and disability was scored using the Expanded Disability Status Scale (EDSS).[21] Disease progression and severity were measured using the Multiple Sclerosis Severity Score (MSSS).[22] A relapse was defined according to McDonald criteria as an episode of neurological disturbance lasting for at least 24 h.[15] At baseline, 14 % of RRMS, 10% of SPMS and none of the PPMS had disease activity for up to 3 month prior to CSF sample were taken. In patients with RRMS patients, 15% had disease activity for up to 3 month prior to baseline, which was reduced to 4% after 9 month with natalizumab treatment. In patients with progressive MS, 11% of patients had disease activity for up to 3 month prior to baseline CSF and after 21 month treatment with mitoxantrone none of the patients had a relapse.

CSF collection

All CSF samples were obtained by lumbar puncture according to the procedures recommended in the consensus protocol of the BioMS-EU network for CSF biomarker research in MS.[23] The CSF samples were centrifuged at 2,000 g for 10 min at room temperature to remove cells and debris, and stored in aliquots at –80 °C pending biochemical analysis.

Analysis of sTREM-2 in CSF

The CSF analyses on sTREM-2 were performed using a commercially available enzyme-linked immunosorbent assay (ELISA) from Uscn Life Science Inc. (Cloud-Clone Corp., Houston, TX, USA) according to instructions by the manufacturer. Neat CSF samples were

analyzed on the same day using assays from the same lot to avoid inter-lot variations. CSF samples from the control group were evenly distributed on the plates. Samples before and after treatment of each patient with natalizumab or mitoxantrone, were analyzed on the same plate. In this study, only patients treated with natalizumab or mitoxantrone who had CSF samples available at baseline and follow-up were included (Table 1). The intra-day coefficient of variance (CV%) for two quality control (QC) samples were 17% (N=12) and 16% (N=8), respectively.

Statistical analysis

Because the distribution of sTREM-2 was not normal (Shapiro-Wilk test, $P < 0.05$), non-parametric statistics were used. Data are given as median (inter-quartile range) and percent differences, i.e. 100 x [(the median for a diagnostic group-the median for a second diagnostic group)/ the median for the second group]. Differences between more than two groups were assessed with Kruskal-Wallis test. For analysis of baseline data, statistically significant results ($P<0.05$) were followed by Mann-Whitney U-tests to investigate group differences. To adjust for potential confounding effects of age and gender, we performed analysis of covariance, analyzing log-transformed sTREM-2 values. Wilcoxon signed rank sum test were used for analysis of matched pair data, i.e. pre- and post-drug treatment. Receiver operating characteristic (ROC) curves were performed in order to assess the diagnostic value. Correlation coefficients (rho) were calculated using the Spearman two-tailed correlation test. SPSS 20.0 was employed for the statistical analyses.

Results

Demographic

The demographic data for the study populations are shown in Table 1. The study groups differed in gender and age distribution, with a larger proportion of females in the RRMS group, compared to controls and SPMS; and older subjects in the SPMS groups, as compared to the other groups. Subsequently gender and age were considered as potential confounding factors, adjusted for by analysis of covariance as described below.

Soluble TREM-2 at baseline

CSF levels of sTREM-2 were significantly higher in patients with RRMS (+140%, P<0.0001), SPMS (+183%, P<0.0001) and PPMS patients (+187%, P=0.02) compared with healthy controls (Figure 1). However, there were no significant differences in the levels of sTREM-2 comparing each group of RRMS, SPMS and PPMS with each another.

To adjust for potential confounding effects of gender and age distribution, we performed analysis of covariance, analyzing log transformed levels of sTREM-2 including age and gender as cofactors in the model. CSF levels of sTREM-2 were significantly higher in patients with RRMS (+140%, P<0.005), SPMS (+183%, P<0.0001) and PPMS (+187%, P=0.01) compared with healthy controls. There were no significant differences in the levels of sTREM-2 in RRMS versus SPMS or RRMS versus PPMS, while sTREM-2 was modestly altered in SPMS versus PPMS (+1.5%, P=0.02).

Soluble TREM-2 in CSF could differentiate patients with RRMS (N=36), SPMS (N=20) and PPMS (N=3) from healthy controls (N=27), with AUC of 0.819 (95% CI 0.715-0.924,

P<0.0001), 0.869 (95% CI 0.766-0.971, P<0.0001) and 0.914 (95% CI 0.779-1.049, P=0.02), respectively (Figure 2).

There was a significant correlation between age and sTREM-2 in the SPMS group (rho=-0.535, P=0.02), but not in either the control group (N=27) or the RRMS group (N=20) (data not shown).

Soluble TREM-2 in relation to treatment

The levels of sTREM-2 were significantly higher in MS patients before treatment with natalizumab (+155%, P<0.0001) compared with CSF levels in healthy controls, but were normalized at follow-up after 12 months of natalizumab treatment. The levels of sTREM-2 were significantly higher both before and after mitoxantrone treatment (+306%, P<0.0001 and +148%, P=0.0007) compared with CSF sTREM-2 levels in healthy controls (Figure 3A). The comparison between pre- and post-natalizumab or mitoxantrone treatment showed that the levels of sTREM-2 were significantly decreased in the CSF (-51%, P=0.001 and -39%, P=0.04) (Figure 3B).

Both the EDSS and MSSS scores were decreased after natalizumab treatment, showing clinical improvement following drug treatment (Figure 3C-D). There was no change in the clinical outcome determined by EDSS and MSSS due to mitoxantrone treatment (Figure 3C-D).

Soluble TREM-2 in relation to disease duration and disease severity

There were significant correlations between CSF sTREM-2 levels and disease duration in the total MS group (rho=-0.383, P=0.003) and RRMS group (rho=-0.366, P=0.033), but not in the

SPMS group (Table 2). There were no significant correlations between sTREM-2 and clinical disease severity (EDSS or MSSS) in either the total MS group or the RRMS group. However, sTREM-2 correlated with the MSSS score in the SPMS group (rho=0.480, P=0.03) (data not shown).

Discussion

In the present study we found that the CSF levels of sTREM-2 were increased in all investigated forms of MS compared with healthy controls. After natalizumab treatment in patients with RRMS, sTREM-2 was reduced to a similar level as the healthy controls. Besides the altered sTREM-2 level, these patients had a clinical improvement as determined by EDSS and MSSS. After mitoxantrone treatment, the CSF levels of sTREM-2 were decreased in SPMS patients compared with before treatment. However, after mitoxantrone treatment the sTREM-2 levels were still higher compared to the controls and the clinical outcome remained unaltered.

Activated microglia produce cytokines and have been described to remove myelin and tissue debris during the pathogenesis of MS.[24] Myelin debris clearance has been shown to be essential for remyelination and repair of the damaged axons.[25] Within the CNS, the TREM-2 receptors appear to be exclusively expressed by microglial cells.[4] In addition to TREM-2 there are several other microglial receptors that are important for myelin debris phagocytosis.[26] In the current study we found that the CSF levels of sTREM-2 were significantly higher in all examined stages of MS (RRMS, SPMS and PPMS) compared with healthy controls. This result is in concordance with the only previous study that showed increased levels of sTREM-2 in patients with RRMS and PPMS compared with subjects with non-inflammatory neurologic diseases.[13] Soluble TREM-2 is produced by ectodomain

shedding of its receptor, which simultaneously produces peptides that reduce the activity of the full-length receptor.[12] So far most reports suggest that TREM-2 receptors modulate the innate immune response by reduction of the primarily induced immune signals, thus acting to inhibit inflammation.[5] Several experimental models of MS have shown that blockade of the TREM-2 receptor exacerbates disease[27-29]. Moreover, disruption of the TREM-2/DAP12 pathway in patients with NHD leads also to neurodegeneration and axonal loss[6] and similarly to MS demyelination of the CNS. The elevated CSF levels of sTREM-2 found in patients with MS might indirectly reflect that the expression or activity of the TREM-2 receptor is reduced in response to chronic inflammation. Furthermore, microglial activation known to occur in MS correspond well to increased CSF levels of sTREM-2 compared to controls.

Natalizumab is a monoclonal antibody targeting the α4 integrins and thereby reduces transmigration of lymphocytes from the periphery to the CNS.[30] In RRMS it reduces disease activity and brain atrophy development.[31] We have previously shown that natalizumab reduced CSF neurofilament light (NFL) levels (biomarker of axonal damage) to levels found in healthy controls and reduced the clinical severity.[19] In the current study we found that natalizumab treatment decreased the levels of sTREM-2 in a similar way and was accompanied by improved clinical outcome. The anti-inflammatory effects due to natalizumab treatment have previously been shown by a reduction of pro-inflammatory cytokines and chemokines in CSF.[32] Even though, natalizumab preliminary reduce the inflammation, there is supporting evidence that the microglial activation also is reduced after natalizumab treatment[33] and that the cortical microglial activity correlate to disease severity in patients with MS.[34] The TREM-2 receptor was recently shown to regulate the microglial cell activation in a demyelination mouse model.[29] In this experimental model of MS, Cantoni *et al.* showed that TREM-2-deficient mice had defective clearance of myelin debris and more

axonal pathology, which also resulted in impaired clinical performances.[29] Interestingly, after natalizumab treatment there was a clinical improvement and simultaneously the levels of sTREM were decreased to control levels. Thus, sTREM-2 might be a valuable marker for studying treatment effects.

Mitoxantrone is an immunosuppressive drug that reduces B- and T-lymphocytes and has shown beneficial effects in MS, including those with a progressive course of the disease.[35] In the present study, we found that the CSF levels of sTREM-2 were reduced in progressive MS after mitoxantrone treatment, but still remained at higher levels than those recorded in healthy control subjects. We have previously shown that mitoxantrone reduced CSF NFL levels approaching those levels found in healthy controls.[18] In contrast to the natalizumab-treated patients, there were no changes in the clinical outcome determined by EDSS and MSSS scores due to mitoxantrone treatment, which is consistent with our previous studies.[18, 19]

A limitation of the present study was the absence of a placebo group that received no drug. Further, the intra-day coefficients of variance for QC samples analyzed at the same occasion were relatively high. Whereas this does not change any of the group differences observed, the assay may not be optimal to evaluate treatment effects on a case-by-case basis.

In conclusion, we show that sTREM-2 in CSF could differentiate patients with RRMS, SPMS and PPMS from healthy controls. The levels of sTREM-2 were higher in CSF samples from MS patients compared to controls supporting evidence that sTREM-2 could be a marker of

microglial activation in MS. CSF sTREM-2 also proved to be a dynamic marker that responded to treatment with both natalizumab and mitoxantrone.

Acknowledgements

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Tables

Table 1 Demographic data for the study groups[a].

Group	N	Gender (M/F)	Age (years)	Duration (years)	Baseline EDSS	Follow-up EDSS	Baseline MSSS	Follow-up MSSS	Follow-up (month)
Study populations at baseline									
Controls	27	(20/7)	40 (35-52)[#]	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
RRMS	36	(11/25)	36 (29-44)*	6.5 (4-12)	3.5 (2.0-4.8)	3.0 (1.8-4.0)	4.2 (2.3-7.0)	3.7 (1.3-6.1)	12 (12-12)
SPMS	20	(10/10)	48 (41-56)*[##]	9 (4-18)	6.0 (4.5-6.5)[###]	6.0 (4.5-6.5)[###]	7.5 (5.6-8.9)[##]	8.0 (5.2-8.9)[###]	24 (23-24)[###]
PPMS	3	(1/2)	37 (35-45)	8 (5.5-9.5)	6.5 (6.3-7.0)[##]	6.5 (6.3-7.5)[##]	8.8 (8.8-9.0)[##]	8.3 (8.3-9.0)[##]	23 (22-23)[###]
Natalizumab and mitoxantrone-treated patients at baseline and follow-up									
Nataluzimab, RRMS	27	(8/19)	34 (28-44)	7 (4.5-12.5)	3.5 (2-4.3)[xx]	2.5 (1-4)[xx]	4.3 (2.5-7.0)[xxx]	2.9 (1.3-5.5)[xxx]	12 (12-12)
Mitoxantrone, Progressive MS	9	(6/3)	37 (34-50)	4 (3-8)	6 (6-6.5)	6.5 (6-6.5)	8.8 (8.7-9.6)	8.4 (8.2-9.1)	24 (23-24)

Abbreviations: Expanded Disability Status Scale (EDSS), Multiple Sclerosis Severity Score (MSSS), primary progressing multiple sclerosis (PPMS), relapsing-remitting multiple sclerosis (RRMS), secondary progressive multiple sclerosis (SPMS).

[a]Data are given as median (inter-quartile range) unless otherwise indicated. Statistical differences were determined using Mann-Whitney U-tests for the study population at baseline *$P \leq 0.05$ (compared with Controls), [#]$P \leq 0.05$, [##]$P \leq 0.01$ and [###]$P \leq 0.001$ (compared with RRMS) and Wilcoxon signed rank sum tests for natalizumab and mitoxantrone-treated patients at baseline and follow-up [xx]$P \leq 0.01$ and [xxx]$P \leq 0.001$.

Figure legends

Figure 1. Soluble TREM-2 levels in cerebrospinal fluid samples from healthy controls (N=27) and patients with relapsing-remitting multiple sclerosis (RRMS) (N=36), secondary progressive multiple sclerosis (SPMS) (N=20) and primary progressing multiple sclerosis (PPMS) (N=3). The lower, upper and middle lines of the error bars correspond to the 25th and 75th percentiles and medians, respectively.

Figure 2. Receiver operating characteristic (ROC) curve analysis of sTREM-2 in cerebrospinal fluid for differentiation of relapsing-remitting multiple sclerosis (RRMS) (N=36) (black), secondary progressive multiple sclerosis (SPMS) (N=20) (purple) and primary progressing multiple sclerosis (PPMS) (N=3). The area under the curve was 0.819 (95% CI 0.715-0.924, P<0.0001), 0.869 (95% CI 0.766-0.971, P<0.0001) and 0.914 (95% CI 0.779-1.049, P=0.02), respectively.

Figure 3. Individual CSF values of sTREM-2 (A) in MS patients pre- and post-treatment with natalizumab (Nz) (N=27) and mitoxantrone (Mtx) (N=9), respectively, and in healthy controls (N=27). Individual CSF values of sTREM-2 (B), Expanded Disability Status Scale (EDSS) (C) and Multiple Sclerosis Severity Score (MSSS) (D) pre- and post-treatment with Nz and Mtx. The lower, upper and middle lines of the error bars correspond to the 25th and 75th percentiles and medians, respectively. Differences between more than two groups were assessed with Kruskal-Wallis test. Mann-Whitney U-test was used to investigate group differences (A) and Wilcoxon signed rank sum test were used for analysis of paired data (B-D). The lower, upper and middle lines of the error bars correspond to the 25th and 75th percentiles and medians, respectively.
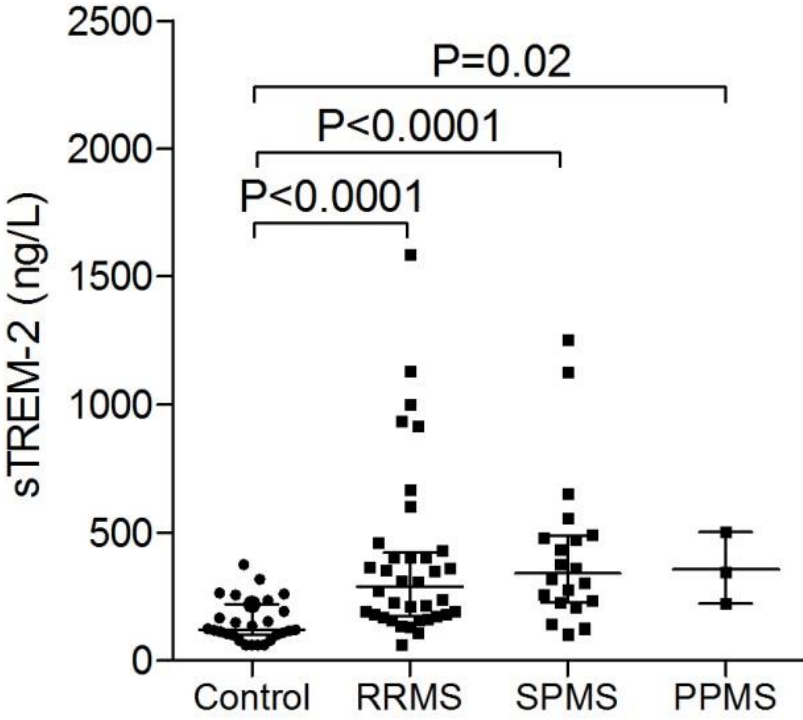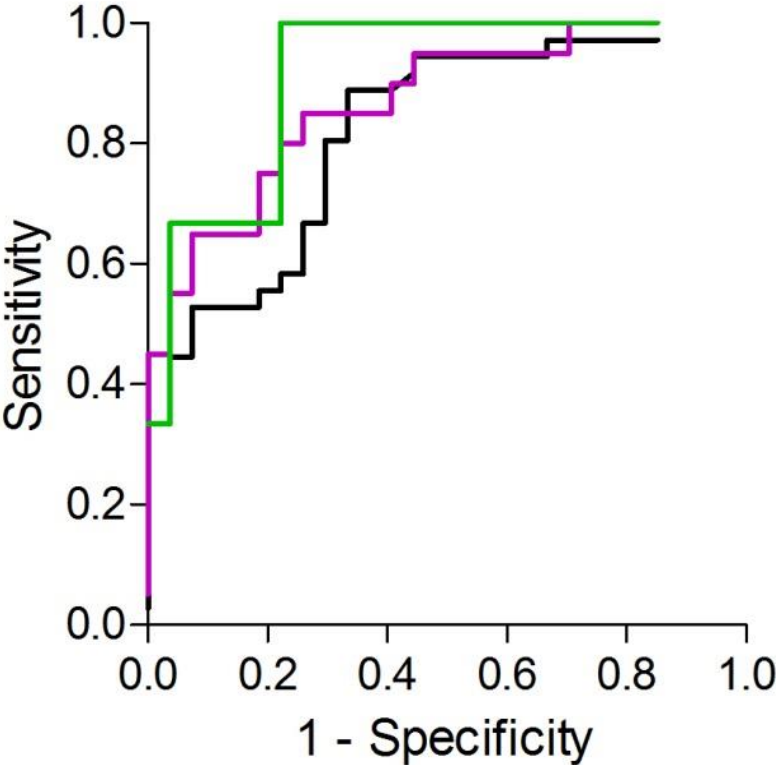
Figures

Figure 1

Figure 2.

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