SOLUBLE CD146 IN CEREBROSPINAL FLUID OF ACTIVE MULTIPLE SCLEROSIS

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**Abstract—The soluble form of CD146 has been reported to
be present in various inflammatory diseases and displays
pro-inflammatory properties. However, little is known about
sCD146 in multiple sclerosis (MS). Here we show that
sCD146 is significantly elevated in the cerebrospinal fluid
of patients with active MS compared with that of inactive
MS or patients with non-demyelinating diseases. Moreover,
abnormally increased sCD146 in the CSF of active MS
patients correlated with albumin quotient, MBP antibody
and MOG antibody from both CSF and sera. Importantly,
the level of CSF sCD146 is correlated with levels of inflam-
matory factors, such as TNFα, IFNγ, IL-2, and IL-17A in the
CSF. We also found that CSF sCD146 might originate from
membrane-bound CD146 on inflamed blood–brain barrier
(BBB) endothelial cells. In addition, sCD146 promotes leuko-
cyte transmigration *in vitro*, at least in part by stimulating
the expression of ICAM-1 and VCAM-1 on endothelial cells.
Our findings suggest that CSF levels of sCD146 may provide
a potential marker for monitoring disease activity in MS
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Key words: sCD146, multiple sclerosis, cerebrospinal fluid,
inflammatory factors.

INTRODUCTION

Inflammatory demyelinating diseases, such as multiple sclerosis (MS), are the major cause of non-traumatic neurological disabilities in young adults involving the central nervous system (CNS) (Hu and Lucchinetti, 2009). MS is a common neurological disease, and carries a risk of chronic functional impairment and disability. As a well-defined autoimmune disease, MS is characterized by numerous immune cells, such as $CD4^+$ and $CD8^+$ cells, infiltration in lesion areas as well as in CSF, which has dramatic changes during MS development (Jilek et al., 2007; Compston and Coles, 2008). The massive influx of immune cells and inflammatory cytokines at the sites of lesions and in the CSF results in the progression and increased severity of the disease (Ishizu et al., 2005). Early diagnosis as well as appropriate and timely therapeutic intervention is a critical factor in ensuring favorable long-term outcomes. MRI is very useful to detect the obvious lesions in CNS. However, it provides very little information on the biological status of single cell types, cortical lesions or pathological mechanisms. Therefore, it is worth making a CSF testing. Effective and reliable laboratory biomarkers are required to monitor disease evolution and to guide treatment decisions. Oligoclonal bands (OCBs), immunoglobulins present in the CSF but not in the serum of patients with demyelinating diseases, have been used for MS diagnosis in the western countries. However, in the Asian countries, the positive rate of OCBs in MS patients is much lower than that in western countries (Kikuchi et al., 2003). To date, although OCBs and the IgG index (CSF IgG/sera IgG) are routinely utilized for MS-aided diagnosis in clinical practice, neither has both high sensitivity and specificity for the diagnosis of MS, especially in Asian countries. New markers are therefore greatly needed to more accurately diagnose this demyelinating disease (Filippi, 2011).

Adhesion molecules, such as ICAM-1, VCAM-1, L-selectin, and β-integrin, are reported to play a pivotal role in the development of MS by facilitating leukocyte transmigration across the blood–brain barrier (BBB) (Weller et al., 1996; Ransohoff, 1999). Their soluble forms, sL-selectin, sICAM-1, sICAM-3 and sVCAM-1, have been shown to be elevated in the serum and CSF from patients with inflammatory CNS diseases and reported to correlate with the clinical course of MS (Correale and Bassani Molinas Mde, 2003; Acar et al., 2005). Adhesion receptor CD146, also referred

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to as MUC18 and Mel-CAM/MCAM, has been identified as a biomarker for the vascular endothelium and plays an important role in tumor angiogenesis (St Croix et al., 2000; Bardin et al., 2001; Yan et al., 2003; Zheng et al., 2009). Its soluble form, sCD146, which was first detected in HUVEC culture medium (Bardin et al., 1998) and shed from membrane bound CD146 in an matrix metalloproteinase (MMP)-dependent manner (Boneberg et al., 2009), has been reported to promote monocyte transmigration across the HUVEC monolayer *in vitro* (Bardin et al., 2009). Another report showed that sCD146 displays chemotactic and angiogenic properties, and promotes efficient neovascularization in an experimental hind limb ischemia model (Harhouri et al., 2010). Recently, Kaspi et al. (2012) identified sCD146 as a regulator for trophoblast migration in placental vascular development during pregnancy. In clinical studies, sCD146 was found in patients with chronic renal failure (Bardin et al., 2003) and in synovial fluid from rheumatoid arthritis patients (Neidhart et al., 1999). Elevated levels of serum sCD146 have similarly been reported to correlate with diabetic nephropathy (Saito et al., 2007), idiopathic inflammatory myopathies (Figarella-Branger et al., 2006) and vasculitis (Zhang et al., 2009). However, little is known about sCD146 in demyelinating diseases, including MS. Due to its role in other inflammatory diseases and endothelial monolayer integrity (Bardin et al., 2003, 2006; Malyszko et al., 2005; Tsiolakidou et al., 2008), we hypothesize that sCD146 may play a part in MS.

In the present study, we report that sCD146 is significantly elevated in the CSF of active MS patients compared to that of non-demyelinating and inactive MS patients. Compared with current clinical biochemical diagnosis indexes for active MS, sCD146 is more sensitive (100%) than the albumin quotient, CSF antibody for myelin basic protein (CSF MBP.Ab) and CSF antibody for myelin oligodendrocyte glycoprotein (CSF MOG.Ab); CSF sCD146 levels are also correlated with CSF TNFα, IFNγ, IL-2, and IL-17A, which are proinflammatory factors in MS. In addition, we have also studied the origin of sCD146 and its function in MS development *in vitro*. Our results suggest that sCD146 may function as an inflammatory factor to promote leukocyte extravasation into the CNS, resulting in neural inflammation and encephalomyelitis.

EXPERIMENTAL PROCEDURES

Antibodies and reagents

Soluble CD146 was purchased from Sinobiological Co., Ltd. (Beijing). All mouse originated anti-CD146 monoclonal antibodies, AA1, AA4, and AA98, targeting distinct epitopes of CD146, were generated in our laboratory (Yan et al., 2003; Zhang et al., 2008). All the three mouse mAbs were raised against the extracellular domain of CD146. AA1 recognizes domain 1 of the extracellular domain and AA4 recognizes domain 4, while AA98 binds domain 4–5. AA1 and AA98 are preferentially used for ELISA and Western blot. AA4 is

preferentially used for immunohistochemistry (paraffin-embedded). Biotin-conjugated AA98 was labeled by Tianjin Sungene Biotech Co., Ltd. Horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibodies were purchased from GE Healthcare. The MMP inhibitor GM6001 was purchased from Calbiochem (Lucerne, Switzerland). Other antibodies used include anti-human NF-κB-p65 antibody (Cell Signaling), PE-anti-human ICAM-1 and PE-anti-human VCAM-1 (eBioscience). Inflammatory cytokines TNFα, IFNγ, and IL-17A were purchased from Peprotech.

Agarose isoelectric focusing and Western blot technique were used to detect sera and CSF OCB. Commercialized ELISA kits from Lifekey Biocompany (Saito et al., 2007) and Cusabio Company (Wuhan, China) were used to detect the levels of MBP, MOG, MBP Ab, and MOG Ab. Other clinical indexes, such as IgG synthesis rate, IgG index and albumin quotient, were detected by a rate turbidimetric immunoassay using IMMAGE800 (Beckman Coulter, Inc., USA). BD cytometric bead array (CBA) human Th1/Th2/Th17 cytokine kit was used to detect the cytokines, such as IL-2, IL-4, IL-6, IL-10, TNFα, IFNγ, and IL17A, in CSF from MS patients.

Subjects and samples in the study

Patients with MS from the Beijing Anzhen Hospital were selected on a clinical basis. Written informed consent was approved and ethical approval was obtained before sample collection by the Ethics Committee of the Anzhen Hospital and of Institute of Biophysics, Chinese Academy of Sciences. We have also obtained written informed consent from guardians on the behalf of the children participants involved in our study. MRI of MS patients showed that their CNS tissues had obvious lesions. Sixty patients with active relapsing-remitting MS who were diagnosed with MS according to McDonald criteria (McDonald et al., 2001) based on the clinical course of the disease at the Department of Neurology, Anzhen Hospital were enrolled in this study. Active MS was defined as an obvious neurologic impairment or the appearance of a new symptom or abnormality attributable to MS, lasting 24 h, and preceeded by stability of at least 1 month. Detailed information on the patients is given in Table 1. CSF samples were collected by lumbar puncture (LP) for diagnostic purposes. At least 1 ml of CSF was obtained from all patients by non-traumatic LP. CSF and serum samples were collected from MS patients exclusively during the clinical relapse (active) phase of the disease (within 2 weeks of the onset of acute or subacute exacerbation). CSF samples were immediately centrifuged at 800 rpm, at 4 °C for 5 min, and the supernatants stored at −70 °C until analysis. For serum collection, blood samples were taken from an antecubital vein and centrifuged at 2000 rpm for 5 min. CSF and serum samples were separated as soon as possible, coded, frozen, and stored at −70 °C and thawed just before testing to avoid the loss of biological activity.

In addition to the active MS patients, 24 patients with remitting MS were served as a group of control. Moreover, 34 patients without any CNS demyelinating lesions in their MRI or CNS viral infection were also used as controls. This control group comprised of three patients with paraneoplastic neurological disorders (PND), three with epilepsy, one with Parkinson's disease, one with cerebral infarction, five with headache, two with anemia, nine with cervical spondylosis, eight with upper respiratory tract infection, one with decreased vision, and one with twitching. Furthermore, 35 neuro-inflammatory patients with viral meningitis without any CNS demyelinating lesions in their MRI also served as the control group. The detailed information of controls was provided in Table 1. All MS patients had received anti-inflammation agent treatment such as corticosteroids before LP. Except for viral

Table 1. Detailed information of patients with multiple sclerosis and controls

	Control	Active MS	Inactive MS	Viral meningitis
No. of patients	34	60	24	35
Sex (F:M)	19:15	37:23	18:06	15:20
Mean age (years)	35.7 ± 21.4	42.2 ± 16.9	39.8 ± 8.5	40.6 ± 19.9
Disease duration (months)	8.26 ± 9.3	21.37 ± 19.6	–	0.23 ± 0.08
EDSS		3.6 ± 1.4		
Course of disease	Active stage	Relapsing	Remitting	Active stage
CSF parameters				
No. with OCB	0	47	2	12
Albumin quotient ($\times 10^{-3}$)	3.61 ± 0.5	4.87 ± 0.45	4.87 ± 4.73	4.96 ± 0.28
IgG synthesis (mg/24 h)	19.1 ± 20.3	41.4 ± 18.6	30.87 ± 36.73	49.9 ± 14.2
IgG index	0.83 ± 0.26	0.99 ± 0.21	1.11 ± 0.63	1.11 ± 0.32
CSF MBP (µg/L)	2.46 ± 2.43	3.97 ± 4.65	5.71 ± 3.43	6.04 ± 5.68
CSF MBP.Ab (mg/L)	0.54 ± 0.28	0.65 ± 0.55	0.51 ± 0.17	0.67 ± 0.32
CSF MOG.Ab (mg/L)	0.51 ± 0.25	0.64 ± 0.43	0.57 ± 0.24	0.59 ± 0.32
No. of MRI positive	0	60	24	0

Values are expressed as the mean ± SD. CSF, cerebrospinal fluid; OCB, oligoclonal bands; CSF MBP, myelin basic protein in CSF; CSF MBP.Ab, CSF antibody for myelin basic protein; CSF MOG.Ab, CSF antibody for myelin oligodendrocyte glycoprotein; EDSS, expanded the disability status scale of kurtzke.

meningitis, all other patients were excluded from viral infection according to clinical symptom, CSF index, anti-virus antibodies detection, and prognosis.

In addition, CNS lesion tissue from MS patients ($n = 5$) and CNS tissue without any inflammatory neurological lesions ($n = 5$) were obtained post-mortem.

Detection of sCD146 by Western blotting and ELISA

CSF samples from active MS patients and control subjects were electrophoresed on a 10% SDS–PAGE gel and then transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA, USA). After blocking with 5% non-fat milk at room temperature for 2 h, the membranes were incubated with anti-CD146 antibody, AA1 (1 µg/ml), and then reacted with HRP-conjugated anti-mouse secondary antibodies (GE Healthcare, Piscataway, NJ, USA). Specific immunoreactive proteins were visualized with an enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA).

The level of sCD146 in CSF and serum samples was determined by enzyme-linked immunosorbent assay using anti-CD146 mAbs AA1 (capture antibody, 2 µg/ml) and biotin-conjugated AA98 (detection antibody, 1.5 µg/ml); HRP-conjugated streptavidin (Dianova, Rodeo, CA, USA) served as the detection enzyme. Recombinant sCD146 was used to determine a standard curve, from 80 to 1.25 ng/ml in phosphate-buffered saline (PBS) buffer. Serum samples were diluted 1:10 in PBS before measurement, while CSF samples were measured undiluted (50 µl each well). A ready-to-use solution of 3,3′,5,5′-tetramethylbenzidine (TMB) was used as a substrate for the HRP enzyme. Sample absorption at a wavelength of 450 nm was measured using a BioRad ELISA reader (Richmond, CA, USA).

Immunohistochemistry

For 3,3′-diaminobenzidine (DAB) staining, paraffin-embedded tissue sections were deparaffinized and stained with a primary antibody specific for CD146 (AA4), and then with biotin-conjugated secondary antibodies (1:1000), followed by HRP-conjugatedstreptavidin (Dianova, Rodeo, CA, USA). Sections were then counterstained with hematoxylin.

CBA immunoassay for human Th1/Th2/Th17 cytokines

CSF supernatants were collected and analyzed simultaneously for seven different cytokines, namely IL-2, IL-4, IL-6, IL-10, IL-17A, IFNγ, and TNFα, using the BD CBA human Th1/Th2/Th17 Cytokine kit according to the manufacturer's instructions. Serum samples were diluted 1:9 in dilution buffer before measurement, while CSF samples were measured in a dilution 1:1 (50 µl each well). All the samples were analyzed by software FlowCytomixPro.

Establishment of an *in vitro* BBB model for assaying leukocyte transmigration

Transmigration assays were performed using the Transwell system (3-µm pore filters; Corning Costar). The human BBB endothelial cell line hCMEC/D3 (BBBECs) (Weksler et al., 2005) was kindly provided by Prof. Pierre-Olivier Couraud (Université René Descartes, Paris, France). The hCMEC/D3 cell is a normal immortalized cell line well-known for the study of BBB function and has been used in many other experiments (Bahbouhi et al., 2009). hCMEC/D3 was grown to confluence in the upper chamber (1.0×10^4 cells/well) and treated with or without sCD146 (50 and 100 ng/ml) or TNFα, (50 ng/ml) for 24 h. Equal numbers of purified leukocytes (1×10^5) from healthy donors were added gently to the top chamber and then left for 12 h to transmigrate to the bottom chamber. The entire transmigrated cell population was collected and stained for human CD3, CD8 and CD19, and then analyzed by flow cytometry. The $CD4^+$ T cell was defined by $CD3^+CD8^-$. Experiments were carried out in triplicate.

Statistical analysis

All experiments were performed in triplicate. Results are expressed as the mean ± SD. The non-parametric Mann–Whitney U test was employed for comparison of the cytokine levels in each group. Correlations between the sCD146 level in the CSF and clinical indices were tested with multiple linear regression and Spearman's Rank correlation coefficient. SPSS 11.0 for Windows was used to perform the analyses. The criterion for statistical significance was defined as $p < 0.05$.

RESULTS

Soluble CD146 is increased in the CSF of active MS

Using immunoblotting, we found that sCD146 is present in the CSF of MS patients, but is absent or present at very low levels in control subjects with non-demyelinating diseases (Fig. 1A). We then measured the concentration of sCD146 in both CSF and serum samples from 60 active MS patients and control subjects (34 with non-demyelinating diseases, 24 with inactive MS and 35 with viral meningitis). Detailed clinical information is shown in Table 1. Levels of sCD146 in the CSF of patients with active MS were significantly elevated ($p < 0.0001$) compared to those in control subjects with non-demyelinating diseases. Moreover, CSF sCD146 in active MS was also significantly increased compared with that in inactive MS and viral meningitis (Fig. 1B, C). In contrast to the CSF sCD146, we did not find any difference in the level of serum sCD146 between these groups (Fig. 1B, D). These data imply that CSF sCD146 might be of potential in monitoring the activity of MS.

CSF sCD146 is correlated with clinical parameters of active MS

Abnormal elevation of sCD146 in the body fluid has been reported to correlate with the inflammation. To further explore the clinical importance of CSF sCD146 in MS, we analyzed the correlation of CSF sCD146 with various clinical parameters used in the clinical analysis for demyelinating diseases. We found a significant correlation in MS patients between CSF sCD146 and albumin quotient ($p < 0.05$), CSF MBP.Ab ($p < 0.01$), sera MBP.Ab ($p < 0.001$), CSF MOG.Ab ($p < 0.01$), and sera MOG.Ab ($p < 0.05$), but not with serum sCD146 and other clinical indexes (IgG synthesis rate, IgG index, CSF MBP, and sera MBP) (Fig. 2A and Table 2). Further analysis showed that the level of sCD146 in the CSF of MS patients was more sensitive (100%) than other clinical indexes in the diagnosis of active MS, including CSF OCB (78%) and the IgG index (78%) (Table 3). Moreover, by analyzing 60 active MS patients and 59 non-active MS or non-MS patients (including 24 MS in remitting and 35 viral meningitis), we found that CSF sCD146 has higher positive predictive value (PPV similar with that of OCB), but has relatively lower negative predictive value (NPV) as compared with other clinical parameters (Table 4), indicating that CSF sCD146 might be a sensitive biomarker for monitoring the MS activity. In order to further test the ability of CSF sCD146 to predict the MS activity, we performed the analysis of receiver operating characteristic (ROC) curve. The ROC curve showed that the optimal threshold for the detection of MS activity was 17.36 ng/ml, with 100% sensitivity and 66.1%
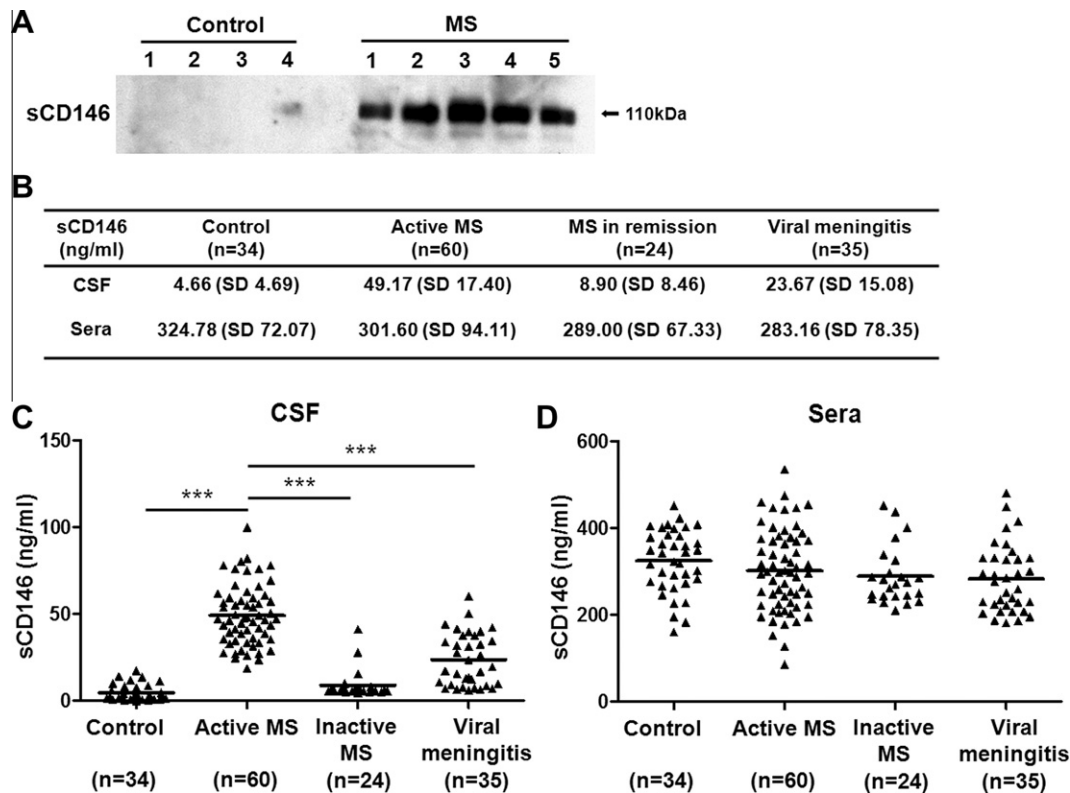

Fig. 1. sCD146 is elevated in the CSF of active MS patients compared with that of controls. (A) Representative CSF samples from MS patients ($n = 5$) and non-demyelinating controls ($n = 4$) were detected by immunoblot with anti-CD146 mAb AA1. (B–D) Comparison of the levels of sCD146 in CSF and sera from the patients with active MS ($n = 60$), non-demyelinating diseases ($n = 34$), inactive MS ($n = 24$), and viral meningitis ($n = 35$) were assayed using an ELISA sandwich system. SD: standard deviation. $^*p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$. Data are representative of three independent experiments.
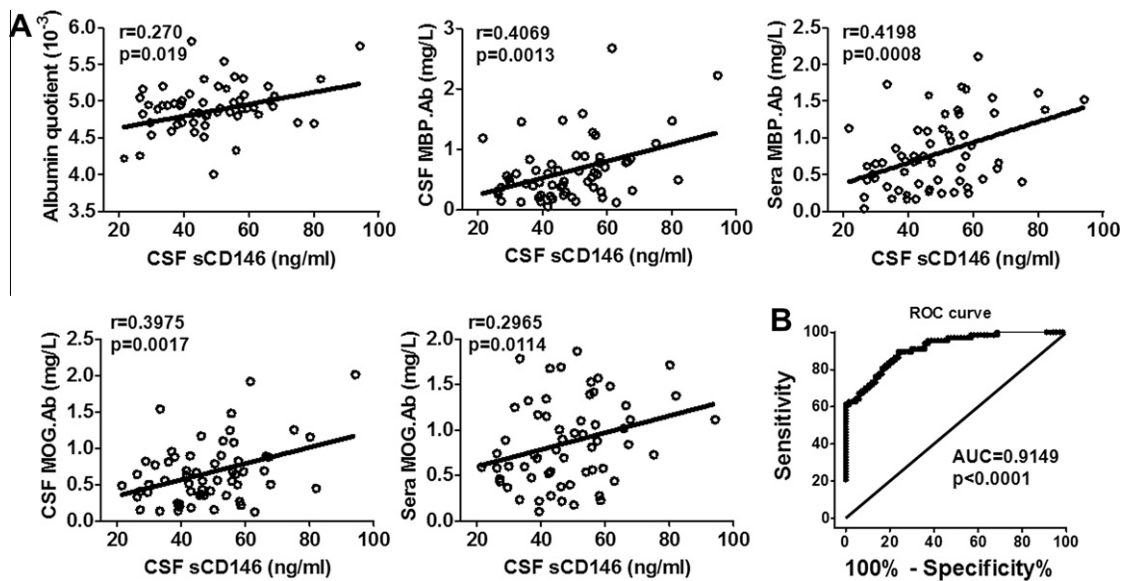
Fig. 2. Correlations between various clinical parameters and CSF sCD146 level from patients with active MS. (A) The levels of CSF sCD146 and various clinical parameters, such as albumin quotient, CSF MBP.Ab, sera MBP.Ab, CSF MOG.Ab, and sera MOG were determined as described in the materials and methods. (B) Receiver-operating characteristic (ROC) curve of the ability of sCD146 to predict the activity of MS. The area under the ROC curve was 0.9149. The optimal threshold for the detection of activity was 17.36288 ng/ml, with 100% sensitivity and 66.1% specificity.

Table 2. Correlation analysis between the CSF sCD146 with various clinical parameters in active MS patients

CSF sCD146	Correlation coefficient	p Value	Number
Age	0.2244	0.0848	60
Sera sCD146	0.1250	0.171	60
IgG synthesis	−0.1827	0.081	60
IgG index	−0.1936	0.069	60
Albumin quotient	0.2690	*0.019	60
CSF MBP	−0.03854	0.385	60
Sera MBP	0.07656	0.28	60
CSF MBP.Ab	0.4069	**0.0013	60
Sera MBP.Ab	0.4198	***0.0008	60
CSF MOG.Ab	0.3975	**0.0017	60
Sera MOG.Ab	0.2965	*0.0114	60

CSF, cerebrospinal fluid; CSF MBP, myelin basic protein in CSF; sera MBP, myelin basic protein in sera; CSF MBP.Ab, CSF antibody for myelin basic protein; Sera MBP.Ab, sera antibody for myelin basic protein; CSF MOG.Ab, CSF antibody for myelin oligodendrocyte glycoprotein; sera MOG.Ab: sera antibody for myelin oligodendrocyte glycoprotein.

specificity (Fig. 2B). These data indicate that CSF sCD146 may have the potential to monitor the activity of MS.

CSF sCD146 is correlated with cytokines in CSF of active MS

MS is an autoimmune disease that is characterized by T lymphocyte infiltration into CNS lesions and CSF, which expresses pro-inflammatory factors. Moreover, the levels of pro-inflammatory factors in CSF reflect the activity of disease. Using CBA, we found that levels of TNFα, IFNγ, IL-2, IL-17 and IL-4 in the CSF were significantly elevated compared to control subjects (Fig. 3A). We further analyzed the correlation between CSF sCD146 with various inflammatory factors involved in the pathogenesis of MS. We found that the level of

Table 3. Sensitivity analysis of CSF sCD146 and various clinical indexes in the diagnosis of active MS

	Number of pairs	Sensitivity (%)
CSF sCD146 (< 14.04 ng/ml)	60	100
CSF OCB	60	78
IgG synthesis (< 30 mg/24 h)	60	68
IgG index (< 0.85)	60	78
Albumin quotient (< 5×10^{-3})	60	55
CSF MBP (< 3.5 µg/L)	60	42
Sera MBP (< 2.5 µg/L)	60	48
CSF MBP.Ab (< 0.65 mg/L)	60	42
Sera MBP.Ab (< 0.75 mg/L)	60	42
CSF MOG.Ab (< 0.56 mg/L)	60	50
Sera MOG.Ab (< 0.64 mg/L)	60	57

CSF, cerebrospinal fluid; OCB, oligoclonal bands; CSF MBP, myelin basic protein in CSF; sera MBP, myelin basic protein in sera; CSF MBP.Ab, CSF antibody for myelin basic protein; sera MBP.Ab, sera antibody for myelin basic protein; CSF MOG.Ab, CSF antibody for myelin oligodendrocyte glycoprotein; sera MOG.Ab, sera antibody for myelin oligodendrocyte glycoprotein.

CSF sCD146 showed a significantly positive correlation with levels of TNFα, IFNγ, IL-2 and IL-17 in the CSF (Fig. 3B, $p < 0.05$), indicating that sCD146 might be correlated with T_H17 cell infiltration and T_H1 cell activation in CSF, and might reflect the active state of MS.

CSF sCD146 shedding from the membrane-bound CD146 of BBB-ECs

Since the non-demyelinating controls and inactive MS patients have very low levels of sCD146 in CSF, we hypothesize that the elevated CSF sCD146 from active MS patients result from inflamed BBB endothelial cells,

Table 4. Positive predictive value (PPV) and negative predictive value (NPV) of CSF sCD146 and various clinical indexes in diagnosis of active MS

	Active MS	
	PPV	NPV
CSF sCD146 (< 14.04 ng/ml)	76% (60/79)	0% (0/40)
CSF OCB	77% (47/61)	22% (13/58)
IgG synthesis (< 30 mg/24 h)	53% (40/75)	45% (20/44)
IgG index (< 0.85)	52% (47/91)	46% (13/28)
Albumin quotient (< 5×10^{-3})	54% (33/61)	46% (27/58)
CSF MBP (< 3.5 μg/L)	39% (25/64)	64% (35/55)
Sera MBP (< 2.5 μg/L)	39% (29/75)	70% (31/44)
CSF MBP.Ab (< 0.65 mg/L)	52% (25/48)	50% (35/71)
Sera MBP.Ab (< 0.75 mg/L)	49% (25/51)	51% (35/68)
CSF MOG.Ab (< 0.56 mg/L)	53% (30/57)	48% (30/62)
Sera MOG.Ab (< 0.64 mg/L)	51% (34/67)	50% (26/52)

PPV, positive predictive value, calculated from the number of active MS patients test positive to total patients test positive; NPV, negative predictive value, calculated from the number of active MS patients test negative to total patients test negative.

where CD146 is up-regulated during the inflammation (Larochelle et al., 2012). To address whether sCD146 originates from membrane-bound CD146 on BBB-EC, we investigated the expression of CD146 in brain tissues from both control individuals and MS patients. Using immunohistochemistry, we found that CD146 is upregulated on the blood vessels of inflammatory lesions from MS patients (Fig. 4A). We hypothesized that the sCD146 in the CSF of MS patients is shed from membrane-bound CD146 of BBB-ECs under inflammatory conditions. Using BBBECs, we mimicked the inflammation *in vitro* by adding inflammatory factors in the culture medium. We found that sCD146 was significantly elevated in the culture medium of BBBECs treated for 48 h with TNFα, IFNγ, and IL-17A either alone or in combination (100 ng/ml each), which are critical inflammatory cytokines for the progression of demyelinating disease(Spuler et al., 1996; Frisullo et al., 2008) (Fig. 4B). Moreover, the elevation of sCD146 in

the supernatant was specifically blocked by the inhibitor of MMP, GM6001, indicating that proinflammatory cytokines promoting CD146 shedding is partially dependent on MMP (Fig. 4C), which is consistent with the previous report that the shedding of sCD146 is MMP dependent (Boneberg et al., 2009). These findings were consistent with our observation of elevated sCD146 levels in the CSF, and suggest that sCD146 in the CSF of MS patients may at least in part, originate from the membrane-bound CD146 of inflamed BBB endothelial cells.

sCD146 functions as inflammatory factor promoting leukocyte transmigration

To explore the role of sCD146 in MS development, we then carried out the *in vitro* transmigration studies using BBBECs. First, using flow cytometry, we found that BBB-ECs treated with sCD146 induced the expression of ICAM-1 and VCAM-1 (Fig. 5A), both of which play an important role in promoting leukocyte transmigration in autoimmune demyelinating diseases. As NF-κB is the critical transcription factor in the expression of adhesion molecules, e.g. ICAM-1 and VCAM-1, we assessed the effect of sCD146 on NF-κB translocation in BBB-ECs. A TNFα-treated group served as positive control for NF-κB nuclear translocation. We observed p-65 translocation to the nucleus of BBB-ECs after 6 h in the presence of sCD146 (100 ng/ml) (Fig. 5B). These results suggest that sCD146 may have a pro-inflammatory property that promotes the development of inflammation.

Then we performed a leukocyte transmigration assay using an *in vitro* BBB model. We found that sCD146 promoted leukocyte transmigration in a dose-dependent manner. Moreover, anti-CD146 mAb AA98 (50 μg/ml) inhibited sCD146-induced $CD3^+$, $CD4^+$, $CD8^+$ and $CD19^+$ cells transmigration across the BBB monolayer (Fig. 5C). Interestingly, the effect of sCD146 in promoting leukocyte transmigration is similar to that of TNFα and there is a redundant role of sCD146 with TNFα on promoting leukocyte transmigration (Fig. 5D),
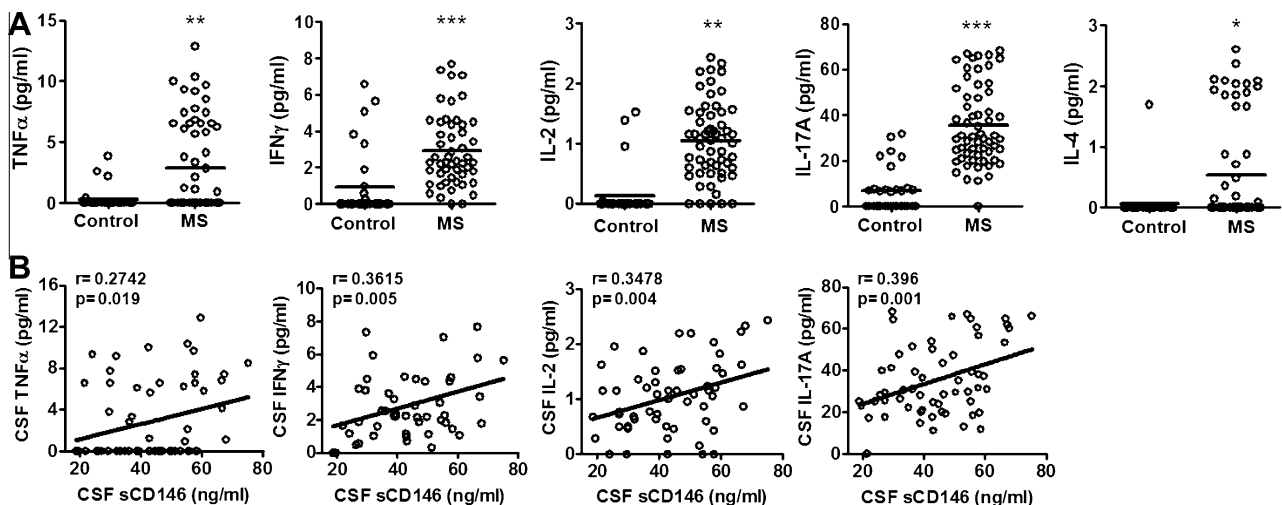

Fig. 3. Correlations between sCD146 and cytokines in CSF from patients with active MS. (A) Elevation of TNFα, IFNγ, IL-2, IL-17 and IL-4 from CSF of MS patients compared with non-demyelinating patients. (B) Positive correlation between CSF sCD146 and TNFα, IFNγ, IL-2 and IL-17 $^*p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$. Data are representative of three independent experiments.
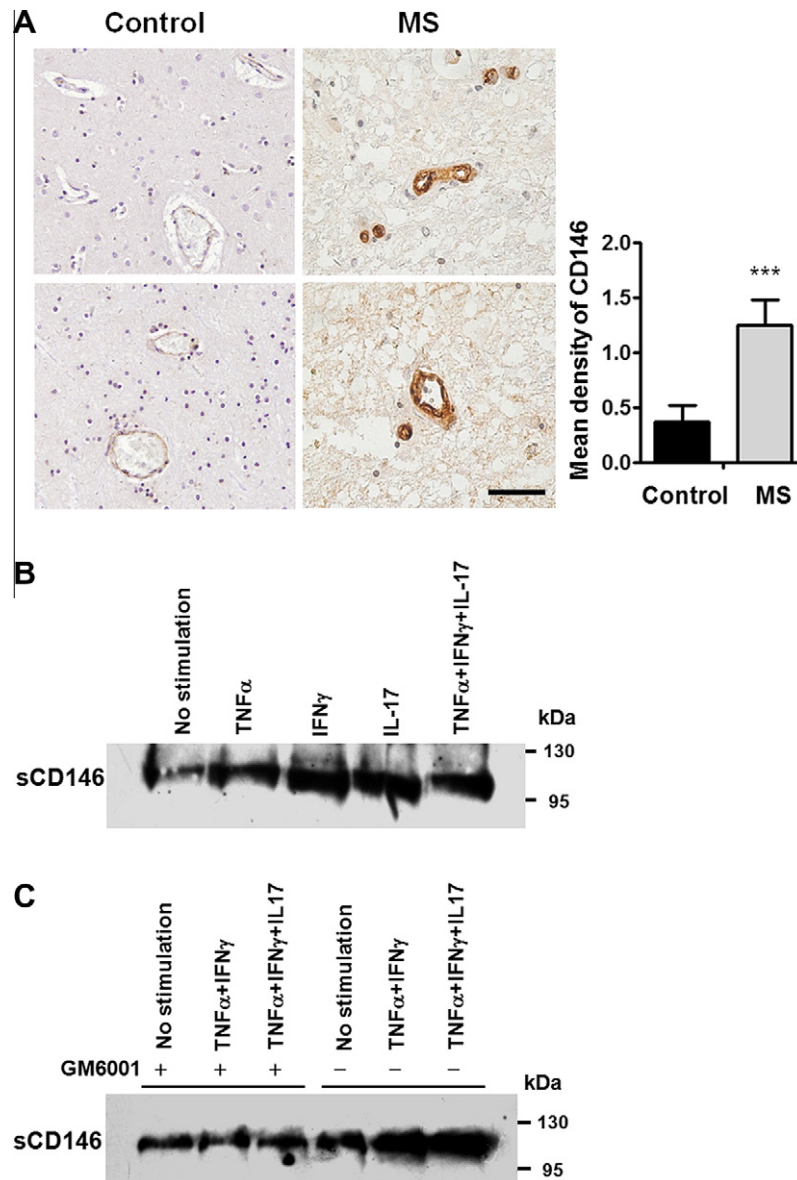
Fig. 4. Inflammatory factors promote sCD146 shedding from the membrane-bound CD146 of BBBECs in an MMP-dependent manner. (A) The expression of CD146 on blood vessels from the lesions of MS patients ($n = 5$) was increased compared with subjects with non-demyelinating diseases ($n = 5$). The mean density of CD146 was analyzed by Image Pro Plus software. (B) sCD146 in the conditioned medium of BBBECs stimulated for 48 h with proinflammatory cytokines TNFα, IFNγ, and IL-17 (100 ng/ml) was detected with the AA1 antibody. (C) Proinflammatory cytokine-promoted shedding of CD146 into the BBBEC culture medium was inhibited by the metalloprotease inhibitor GM 6001 (40 μg/ml). Data are representative of three independent experiments. Scale bar = 100 μm.

providing further evidence of sCD146's proinflammatory properties. These data indicate that sCD146 may facilitate leukocyte transmigration during the development of MS or other demyelinating diseases through the upregulation of other adhesion molecules expression.

DISCUSSION

In this study, we have identified that CSF sCD146 might be a new potential biomarker for monitoring the activity of MS and functions as a proinflammatory factor to facilitate the development of MS. sCD146 is significantly elevated in the CSF of patients with active MS, but not with remitting MS and other non-demyelinating diseases, and showed a significant positive correlation with albumin quotient, CSF MBP antibody and MOG antibody, CSF TNFα, IFNγ, IL-2 and IL-17. Importantly, CSF sCD146 is more sensitive in aiding active MS diagnosis than currently used clinical biochemical diagnostic indexes, such as OCB, IgG index. Our results suggest that sCD146 may originate from the shedding of membrane-bound CD146 on BBB-ECs during inflammation and promotes leukocyte transmigration across BBB-ECs *in vitro*. These data indicate that sCD146 in CSF may act as a proinflammatory factor and could be developed into a useful index for monitoring disease activity and possibly a therapeutic target for MS.
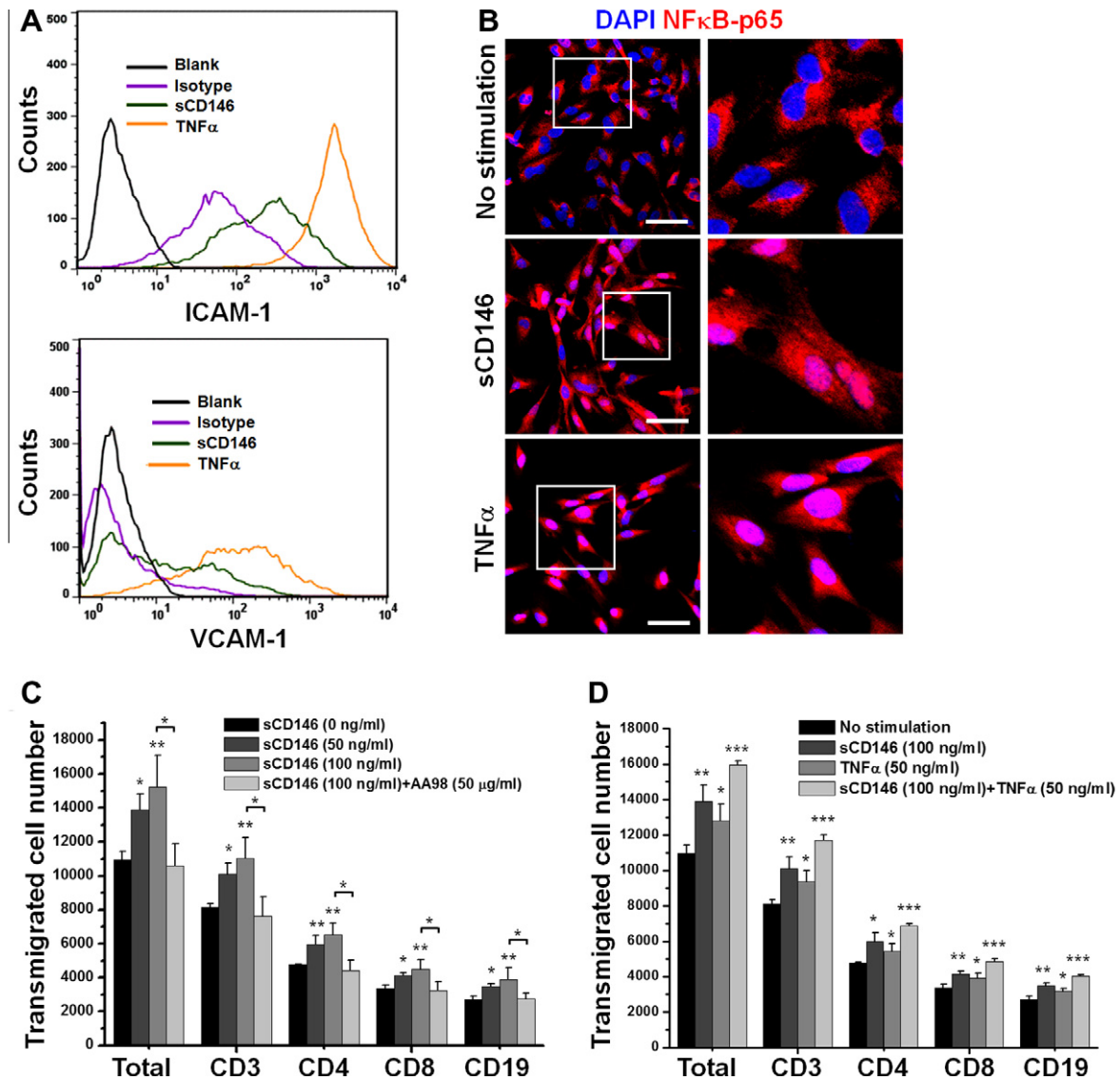
Fig. 5. sCD146 promotes ICAM-1 and VCAM-1 expression and contributes to leukocyte transmigration. (A) BBBECs were treated with sCD146 (100 ng/ml) or TNFα (50 ng/ml) for 48 h. The expression of ICAM-1 and VCAM-1 was detected by flow cytometry. (B) Effect of sCD146 (100 ng/ml) and TNFα (50 ng/ml) in stimulating the nuclear translocation of p-65 in BBBECs. Scale bar = 50 μm. (C) Flow cytometry analysis of various subtypes of leukocytes that underwent transmigration across BBBECs in the presence of different concentrations of sCD146 and AA98 (50 μg/ml). (D) Flow cytometry analysis of various subtypes of leukocyte that underwent transmigration across BBBECs in the presence of sCD146 (100 ng/ml), TNFα (50 ng/ml) and AA98 (50 μg/ml) alone or in combination. $^*p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$. Data are representative of four independent experiments.

Diagnostic testing of CSF biochemical indicators has been routinely employed in assisting diagnosis and monitoring neuroimmunological disorders such as MS. So far, only OCBs and IgG index are routinely utilized in clinical practice (Awad et al., 2010), although neither of them has high sensitivity for MS diagnosis in Asian countries (Kikuchi et al., 2003). Meanwhile, there are numerous CSF markers which have been founded and studied and may prove helpful in a variety of clinical settings. These quasi biomarkers include sICAM-1, sVCAM-1, NCAM, TNFα, IL-6 and so on (McMillan et al., 2000; Michalowska-Wender et al., 2001). Among these, soluble adhesion molecules are wildly studied. Elevations of soluble adhesion molecules, such as sICAM-1, sVCAM-1 and sCD31 have been described as

markers of BBB damage in MS (Rieckmann et al., 1997; McDonnell et al., 1999; Baraczka et al., 2001; Kuenz et al., 2005). Elevated soluble forms of L-selectin, ICAM-1, ICAM-3 and VCAM-1 have been detected in the serum and CSF from patients with inflammatory CNS diseases and have been found to correlate with the clinical course of MS (Baraczka et al., 2000, 2001; Alves-Leon et al., 2001). In contrast to these indexes, we found that sCD146 is elevated only in CSF but not in serum samples from patients with active MS. CSF sCD146 may reflect the local inflammation of CNS and provide some information on molecular mechanisms for MS study. We also found that the level of CSF sCD146 correlated with various clinical parameters which reflect the damage of CNS, including albumin quotient, CSF

and sera MBP Ab or MOG Ab. Compared with other clinical indexes, sCD146 was more sensitive. Moreover, sCD146 has high PPV as well as low NPV in active MS. These data suggested that CSF sCD146 might be a new potential biomarker for monitoring the activity of MS.

The presence of inflammatory factors in CSF has been reported and suggested to promote inflammatory cells infiltration in CSF (Ishizu et al., 2005). Levels of inflammatory factors can reflect the activity of the disease. Although the concentration of CSF inflammatory factors is low, we found that TNFα, IFNγ, IL-2, IL-17 and IL-4 were elevated in the CSF of MS patients compared with those of control subjects. Moreover, CSF sCD146 correlated positively with TNFα, IFNγ, IL-2 and IL-17, but not with IL-4, IL-10 and IL-6 in this study. However, caution should be exercised against drawing conclusions because of the lower number of samples in our study. Altogether, these data suggested that CSF sCD146 might reflect the activity of T_H17 and T_H1 cells in disease.

Regarding the origin of CSF sCD146, there may be two pathways explaining it. One is that sera sCD146 is diffused through the inflamed BBB and enters the CSF. Another is that sCD146 may be originated from the membrane-bound CD146 on BBB endothelial cells. In this study, we found that membrane-bound CD146 was upregulated on BBB endothelial cells in brain tissues from MS patients. We postulate that this increase in membrane-bound CD146 on endothelial cells may account for the elevation of sCD146 in the CSF. Our *in vitro* data also suggest that MMP is required for the shedding of CD146 following stimulation by various inflammatory cytokines, such as TNFα, IFNγ and IL17A, consistent with previous reports showing that sCD146 is derived from proteolysis of the mature protein from the endothelial cell surface (Boneberg et al., 2009). However, there may be other signal pathways that mediate the shedding of sCD146, because of the incomplete inhibition of shedding in the presence of MMPs inhibitor. The mechanism of CD146 shedding needs further study.

Endothelial cell dysfunction contributes to the pathogenesis of MS. Endothelial-derived microvesicles or microparticles, small membrane fragments shed from endothelial cells, have been implicated in inflammation and endothelial dysfunction, and have been reported to elevate in the plasma of MS patients in exacerbation and facilitate the transendothelial migration of inflammatory cells (Minagar et al., 2001; Jimenez et al., 2005; Colombo et al., 2012). As a biomarker of endothelial cells, CD146 has been observed in endothelial microparticles in some endothelial-associated diseases (Jeanneteau et al., 2012; Sanborn et al., 2012). In the present study, we found the elevation of CD146 in the CSF of active MS patients, implying the activation of endothelial cells and might consequently increase the production of endothelial-derived microvesicles in the CSF of patients with active MS. The elevation of sCD146 in the CSF of demyelinating patients may be correlated with changes in BBB endothelial monolayer integrity, leading to

dysfunction of the BBB (Bardin et al., 2003, 2006; Figarella-Branger et al., 2006; Reumaux et al., 2007; Tsiolakidou et al., 2008). Our study may provide some information on the molecular mechanisms for endothelial cells dysfunction.

sCD146 has been reported to function as an activator in angiogenesis, displaying chemotactic and angiogenic properties, and promoting efficient neovascularization (Harhouri et al., 2010) and monocyte transmigration (Bardin et al., 2009). sCD146 also plays an important role on trophoblast migration during placental vascular development (Kaspi et al., 2012), implying its pleiotropic effect under pathological conditions. In this study, we observed a similar effect of sCD146 in leukocyte transmigration across the BBBEC monolayer. The mechanism of sCD146 in lymphocyte transmigration may involve in the interaction with member-bound CD146 on endothelial cells (Guezguez et al., 2007). We also show that sCD146 promotes the nuclear translocation of NF-κB and stimulates the expression of adhesion molecules on BBB-EC, such as ICAM-1 and VCAM-1, both of which can promote leukocyte recruitment into the CNS. These data indicate that sCD146 facilitates leukocyte recruitment to the CNS through several mechanisms including the upregulation of adhesion molecules and CSF sCD146 maybe partially involved in the progression of MS. Due to the pivotal role cell adhesion molecules (CAMs) play in the progression of demyelinating diseases and the effect of sCD146 on the expression of the CAMs, we hypothesize that sCD146 in the CSF may have potential as a biomarker and therapeutic target for active MS.

CONCLUSION

We found significantly higher levels of sCD146 in MS patients during the active stage compared with those during the inactive stage. Moreover, some correlations were observed between the CSF sCD146 levels and measures of disease activity such as inflammatory factors in MS patients. Therefore, measurement of CSF sCD146 may help to monitor disease activity and enrich our understanding of the relationship between *in vivo* CSF changes and inflammation involved in demyelinating diseases.

AUTHOR CONTRIBUTIONS

HD and YL designed, carried out experiments, analyzed data and wrote the paper. HH and FL obtained clinical samples, carried out experiments and analyzed the data. YZ, DL, and SX carried out experiments and analyzed the data. JF, DY and LS contributed to data analysis. XY designed the project, directed experiments, analyzed the data and wrote the paper.

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