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Plasma levels of soluble adhesion molecules sPECAM-1, sP-selectin and sE-selectin are associated with relapsing-remitting disease course of multiple sclerosis

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Abstract

Adhesion molecule mediated leukocyte migration into the central nervous system is considered to be a critical step in the pathogenesis of multiple sclerosis (MS). We measured plasma levels of the soluble adhesion molecules sPECAM-1, sP-selectin and sE-selectin in 166 MS patients and in 36 healthy blood donors with ELISA. sPECAM-1, sP-selectin and sE-selectin plasma concentrations showed a significant increase in the relapsing-remitting disease course of MS and were elevated during relapse. These findings indicate that sPECAM-1, sP-selectin and sE-selectin might be implemented as paraclinical markers of disease activity in MS with restriction to the clinical course of the disease.

Keywords: Multiple sclerosis; Soluble platelet endothelial cell adhesion molecule-1(sPECAM-1); Soluble E-selectin (sE-selectin); Soluble P-selectin (sP-selectin); Plasma

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory and demyelinating disease preferentially affecting the white matter of the central nervous system (CNS). The trans-endothelial migration of activated monocytes and lymphocytes across the blood-brain-barrier (BBB) into the brain tissue is believed to be an essential event at the early stages of inflammation, which involves a complex cascade of interactions regulated by a series of adhesion molecules (Engelhardt and Wolburg, 2004; Avolio et al., 2003; Cannella and Raine, 1995; Tsukada et al., 1994).

Adhesion molecules are cell-surface proteins that are only expressed in very low levels on vascular endothelial cells of normal brain (Rossler et al., 1992; Lassmann et al., 1991). Cytokines, stimulated by an inflammatory focus in MS lesions, entail an up-regulation of the adhesion molecules required for leukocyte attachment and migration on the endothelial surface (Minagar and Alexander, 2003; Dietrich, 2002; Cannella and Raine, 1995). According to their structure adhesion molecules have been classified into three families: selectins, integrins and immunoglobulin superfamily (Frijns and Kappelle, 2002; Lee and Benveniste, 1999). Selectins mediate the initial tethering and rolling of leukocytes along the endothelial cell surface (Avolio et al., 2003). The interaction between integrins and adhesion molecules of the immunoglobulin superfamily causes a firm and irreversible binding of the inflammatory cells to the endothelium followed by diapedesis into the CNS (Frijns and Kappelle, 2002; Ransohoff, 1999). Furthermore, adhesion molecules exist in a soluble form, released from activated endothelial cells, leukocytes and platelets, respectively (Lee and Benveniste, 1999; McDonnell et al., 1998; Rieckmann et al., 1997). Increased levels of

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soluble adhesion molecules, present in physiological fluids, have been described in a variety of inflammatory diseases (Reinhart et al., 2002; Rieckmann et al., 1997; Hartung et al., 1995; Gearing and Newman, 1993).

The group of selectins contains the adhesion molecules P-selectin and E-selectin. P-selectin (GMP-140), a 140-kDa glycoprotein, is constitutively synthesized and stored in the α-granula of platelets and endothelial Weibel–Palade bodies (Bevilacqua and Nelson, 1993). It can be quickly redistributed to the cell surface after activation of endothelial cells and platelets induced by several mediators of inflammation (Bevilacqua and Nelson, 1993). P-selectin supports leukocyte rolling on the endothelium (Aplin et al., 1998; Bevilacqua and Nelson, 1993), whereas the surface expression takes up to 10 min only. Elevated plasma levels of sP-selectin and its positive correlation with clinical activity have been demonstrated in patients with rheumatoid arthritis and thrombocytosis (Ertenli et al., 1998). Until now P-selectin has not been studied in connection with MS, but recently the highest expression of sP-selectin levels was observed before disease onset in experimental autoimmune encephalomyelitis (EAE), an animal model of MS (Kerfoot and Kubes, 2002). The augmented increase of sP-selectin concentrations in patients with MS may reflect an activation of endothelial cells and platelets, respectively. Therefore sP-selectin might be a useful paraclinical marker in monitoring disease activity.

E-selectin is an endothelial glycoprotein that is predominantly biosynthesized and expressed on activated endothelium, regulated by inflammatory cytokines (IL-1, TNF-alpha and bacterial endotoxin) (Bevilacqua et al., 1994). The maximal E-selectin level is reached by 4–6 h after activation (Bevilacqua and Nelson, 1993). E-selectin mediates the capture and rolling of leukocytes along the endothelial cell surface. Investigations on the soluble form (sE-selectin) have demonstrated elevated levels in animal models of inflammatory, infectious and malign human diseases (Bevilacqua et al., 1994). Furthermore, raised sE-selectin levels were measured in serum and cerebrospinal fluid (CSF) of patients with PPMS (McDonnell et al., 1999, 1998; Giovannoni et al., 1996; Dore-Duffy et al., 1995).

Another important participant involved in the endothelial transmigration of leukocytes is platelet endothelial cell adhesion molecule (PECAM-1, CD31). PECAM-1, a 130-kDa glycoprotein and member of the immunoglobulin superfamily (Newman et al., 1990; Goldberger et al., 1994), is expressed on platelets, monocytes, lymphocytes, neutrophils, basophils and endothelial cells, concentrated at cell-to-cell junctions (Muller et al., 1993). Modulated by its soluble form, PECAM-1 supports the leukocyte migration through intercellular endothelial cell junctions (Blankenberg et al., 2003). Increased levels of sPECAM-1 have been detected in serum and CSF of patients with active, gadolinium enhancing MS lesions on MRI (Losy et al., 1999). In addition, anti PECAM-1 antibodies have been shown to inhibit transendothelial migration of leukocytes in animal models of inflammation (Bogen et al., 1994).

In summary, previous studies revealed different levels of soluble adhesion molecules in MS patients depending on the disease course. sE-selectin and sPECAM-1 have been described as potential immunological markers of disease activity in MS. Even though sP-selectin has not been examined up to our study, evidence for a positive correlation between elevated sP-selectin levels and symptom onset in EAE mice has been provided. Each of the adhesion molecules P-selectin, E-selectin and PECAM-1 plays a crucial role in leukocyte attachment and transmigration through the activated endothelium. Both P-selectin and E-selectin mediate tethering and rolling of leukocytes on the activated endothelial cells, though considering the level of maximal surface expression, P-selectin is supposed to interact with leukocytes before E-selectin. The consecutive migration of leukocytes through the endothelium is supported by PECAM-1. Based on these findings the aim of our study was to analyze the plasma concentrations of sP-selectin, sE-selectin and sPECAM-1 in patients with MS in order to explore the extent of endothelial and leukocyte activation in the clinical subgroups of the disease.

2. Materials and methods

2.1. Patients

The study cohort (166 patients) included 98 MS patients with relapsing-remitting (RRMS), 53 patients with secondary progressive (SPMS) and 15 patients with primary progressive (PPMS) disease course (Table 1). In 13 (24.5%) of the patients with SPMS superimposed relapses were observed. 27 (27.6%) of the patients with RRMS and 9 (17.0%) of the patients with SPMS had an acute relapse at the time of plasma removal. Additionally, in 11 patients out of the entire study cohort sP-selectin, sE-selectin and sPECAM-1 plasma levels were measured in remission as well as in relapse, regarding a minimum interval of one month between remission and the subsequent relapse. These patients were diagnosed with either RRMS or SPMS, accompanied by superimposed relapses. The median time lag from onset of clinical symptoms to blood sampling during acute relapse was 7 days (range 1–33 days). All acute relapses were treated with high-dose methylprednisolone. In 36.4% of the patients clinical symptoms remitted completely, in 54.5% of the patients the clinical exacerbation did not completely remit and 9.1% of the patients showed no remission. The median duration of remission before onset of clinical symptoms was 0.8 years (range 0.4–7.5 years). Follow-up data of the 36 months after blood sampling revealed a median remission duration of 0.8 years (range 0.1–3.0 years). Furthermore, 36 healthy blood donors, matched for age and gender with the RRMS patients, were considered as controls (Table 1).

Among the MS patients studied there were 103 females and 63 males. The mean age was 41.4 years (standard

Table 1
Demographic and clinical data

Patient group	RRMS	SPMS	PPMS	HC
No. of patients	98	53	15	36
Sex ratio (male : female)	1 : 1.7	1 : 1.9	1.5 : 1	1 : 2.3
Age[1,2]	35.9 (9.6)	48.3 (9.0)	53.3 (10.3)	35.0 (14.8)
Age at disease onset[1,2]	28.0 (9.7)	29.3 (9.2)	41.5 (12.7)	–
Disease duration[1,2]	7.8 (6.6)	19.3 (7.9)	11.8 (7.7)	–
No. of patients with acute relapse	27	9	0	–
No. of patients in remission	71	0	0	–
No. of patients in progression	0	44	15	–
EDSS[1,3]	1.6 (1.2)	5.9 (1.7)	5.3 (1.1)	–
Progression index[1,4]	0.4 (1.0)	0.4 (0.2)	0.6 (0.5)	–

RRMS=relapsing-remitting MS; SPMS=secondary progressive MS; PPMS=primary progressive MS; HC=healthy controls; [1]mean (standard deviation); [2]years; [3]EDSS=expanded disability status scale (Kurtzke, 1983); [4]progression index=EDSS/disease duration.

deviation 11.6 years, range 19.4 to 74.5 years). The mean disease duration was 11.8 years (range 0.32 to 41.35 years). 71 MS patients received treatment with immunomodulatory therapy (e.g. interferon-β, glatiramer acetate, intravenous immunoglobulin) and 22 with immunosuppressive therapy (e.g. azathioprine, methylprednisolone), 73 patients received no MS specific treatment. All patients were diagnosed with clinically definite MS according to established diagnostic criteria (McDonald et al., 2001) and categorized based on the disease course at the time of plasma removal.

2.2. Sample collection

Citrate treated (Sarstedt Monovetten, Nümbrecht, Germany) plasma samples were obtained from MS patients with informed consent by peripheral vein puncture. Plasma was prepared by centrifugation, 10 min at 2000 rpm, and stored at −20 °C until analysis was performed.

2.3. Determination of the soluble adhesion molecules sP-selectin, sE-selectin and sPECAM-1

Plasma concentrations of sP-selectin, sE-selectin and sPECAM-1 were measured with commercially available enzyme-linked immunosorbent assays (ELISA) according to manufacturer's protocol (BenderMed-Systems, Vienna, Austria). The coating antibody was diluted 1 : 20 with phosphate buffered saline (PBS) and 100 μl of the diluted coating antibody were added to each well of the microtiter plates (Nunc MaxiSorb, Rosklide, Denmark). Plates were incubated at −4 °C over night and afterwards washed six times with PBS−0.05% Tween-20 (PBS−T). After blocking with 250 μl assay buffer (bovine serum albumin, Tween 20 and PBS) to each well plates were incubated for 2 h at room temperature. Plates were washed and 100 μl of standard dilution (sP-selectin: standard diluted in assay buffer; sE-selectin and sPECAM-1: standard diluted in sample diluent) were added in duplicate to all standard wells according to manufacturer's guidelines. Then 100 μl of diluted plasma samples (dilution ratio: sP-selectin 1 : 10 in assay buffer; sE-selectin 1 : 5 in sample diluent; sPECAM-1 1 : 10 in sample diluent) were applied in duplicate to the designated wells. Additionally, 50 μl of diluted horseradish-peroxidase (HRP) conjugate were added to all wells. Plates were incubated at room temperature with gentle shaking (sP-selectin and sE-selectin: 120 min; sPECAM-1 : 180 min). Afterwards the unbound enzyme-conjugated antibodies were removed by washing. Specific antibody binding was visualized by the addition of 100 μl HRP substrate to each well, consisting of 15 mg *o*-phenylendiamine (Sigma, St. Louis, USA) in 13.5 ml distilled water with 1.5 ml stock-acetate buffer and 6 μl H_2O_2. The color reaction was stopped after 20 min with 50 μl HCL and the absorbance of each microwell was read at 492 nm with a reference filter at 405 nm. The average absorbance values for each sample set were calculated and the results were related to readings from the standard curve.

2.4. Statistical analysis

Statistical analysis (means, medians, standard deviations), significance of group differences and linear regression were evaluated using SPSS (release 11.0, SPSS Inc., USA). Distribution of groups was analyzed by Kolmogorov–Smirnov test. Between-group comparisons of not normally distributed data were made using nonparametric Kruskal–Wallis one-way analysis of variance with Dunn's multiple comparison test. To improve specificity and sensitivity of statistical results cut-off levels were calculated by summating the mean value of the healthy controls and the duplicated standard deviation. Values above the cut-off level were considered positive. p-values < 0.05 were considered statistically significant.

3. Results

3.1. Soluble P-selectin in patients with multiple sclerosis

Plasma concentrations of sP-selectin ranged from 32.5 to 557.8 ng/ml. The sP-selectin cut-off level was evaluated at 270.0 ng/ml. No significant differences were found in the sP-selectin plasma levels between the MS subgroups

($p = 0.23$) but sP-selectin plasma concentrations were significantly higher in RRMS compared to healthy controls ($p = 0.008$) (Fig. 1a). Plasma levels of sP-selectin were higher in MS patients, who have had an acute relapse at the time of blood removal, compared with those in remission ($p = 0.0004$) and in progression ($p < 0.0001$) (Fig. 2a). These findings were confirmed on examination of the sP-selectin plasma concentrations in the group of 11 patients, from whom blood was available in remission as well as during the subsequent relapse ($p = 0.0098$) (Fig. 3a). In this group the increase of sP-selectin levels during relapse was mainly related to 5 RRMS patients who showed no atypical clinical presentation in comparison with the entire RRMS cohort. Furthermore, plasma sP-selectin did not significantly correlate with age, gender, disease duration, relapsing rate, progression index and disability of MS patients. No

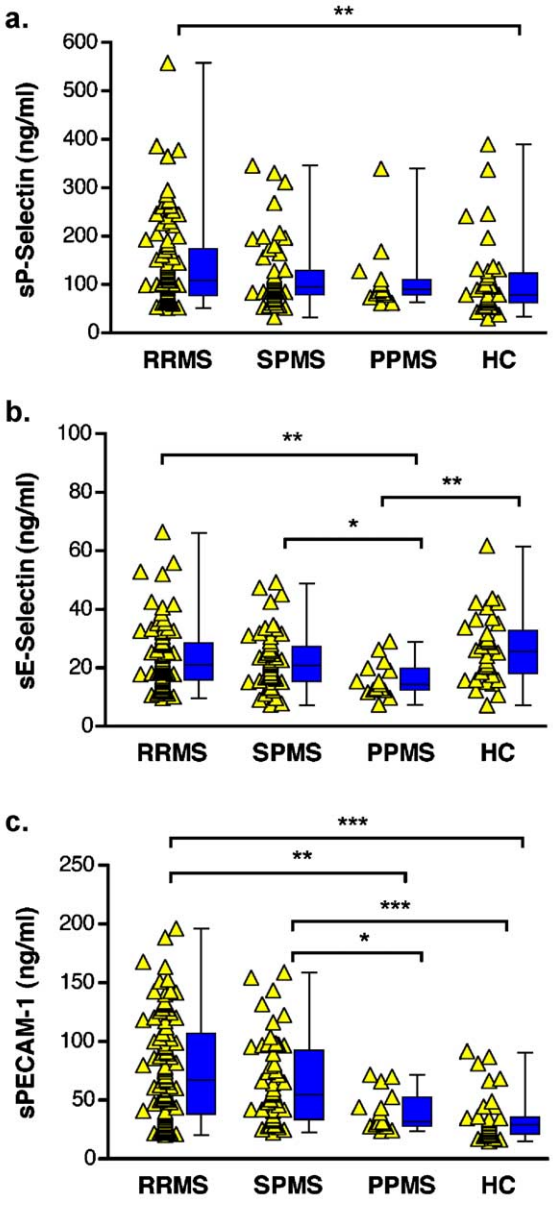

Fig. 1. Plasma levels of sP-selectin, sE-selectin and sPECAM-1 in comparison of RRMS, SPMS, PPMS and healthy controls (HC) are represented. Medians are indicated by horizontal bars. sP-selectin concentrations were not statistically different between MS subgroups but in RRMS versus HC, $p = 0.008$ (a). sE-selectin levels in RRMS versus PPMS, $p = 0.004$, in SPMS versus PPMS, $p = 0.0.16$ and in HC versus PPMS, $p = 0.001$ (b). sPECAM-1 levels in RRMS versus PPMS, $p = 0.002$, in SPMS versus PPMS, $p = 0.014$, in RRMS versus HC, $p < 0.0001$ and in SPMS versus HC, $p < 0.0001$ (c).

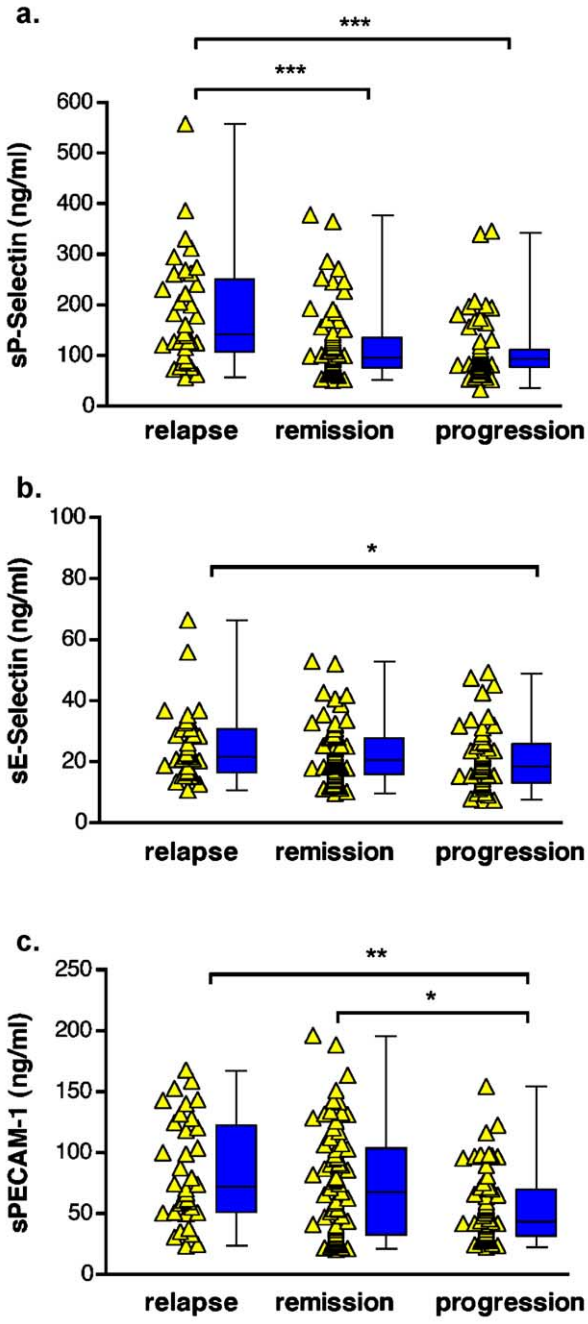

Fig. 2. Plasma levels of sP-selectin, sE-selectin and sPECAM-1 in relapse, remission and progression are illustrated. Medians are indicated by horizontal bars. sP-selectin concentrations in relapse versus remission, $p = 0.0004$ and in relapse versus progression, $p < 0.0001$ (a). sE-selectin levels in relapse versus progression, $p = 0.014$ (b). sPECAM-1 concentrations in relapse versus progression, $p = 0.0002$ and in remission versus progression, $p = 0.028$ (c).

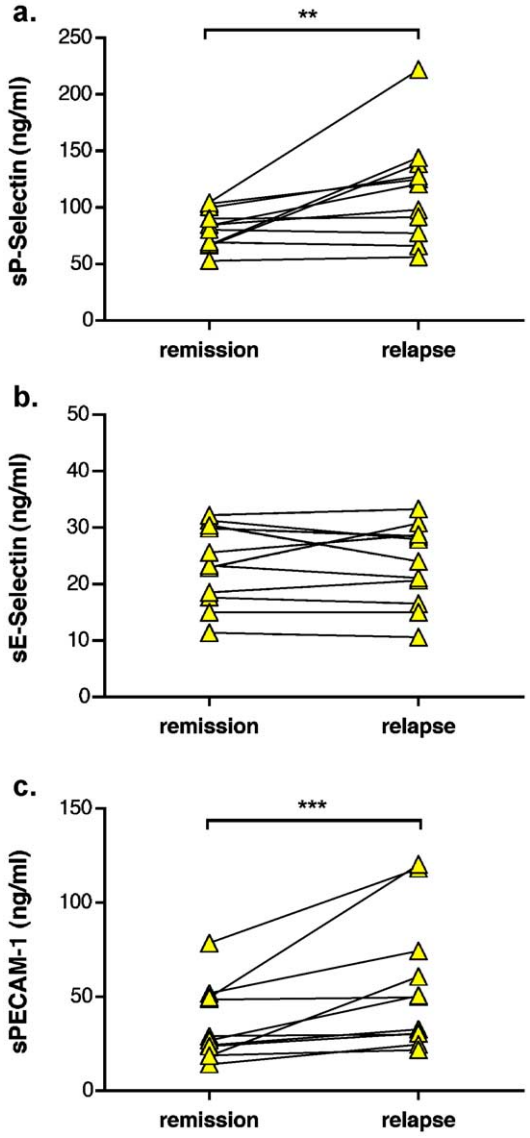

Fig. 3. Plasma concentrations of sP-selectin, sE-selectin and sPECAM-1 are shown in the group of 11 MS patients in comparison of remission and subsequent relapse. sP-selectin concentrations in remission versus relapse, $p=0.0098$ (a). sE-selectin levels were not statistically different between remission and relapse (b). sPECAM-1 concentrations in remission versus relapse, $p=0.001$ (c).

significant effect of immunomodulatory or immunosuppressive therapy on sP-selectin levels was observed in this study.

3.2. Soluble E-selectin in patients with multiple sclerosis

Plasma levels of sE-selectin in MS patients studied ranged from 7.3 to 66.4 ng/ml. The sE-selectin cut-off level was determined at 49.0 ng/ml. Patients with PPMS had lower plasma concentrations of sE-selectin compared to those with RRMS ($p=0.004$), SPMS ($p=0.016$) and the group of healthy controls ($p=0.001$), respectively (Fig. 1b). Between sE-selectin plasma levels during relapse and progression significant differences were found ($p=0.04$) with higher levels during relapse (Fig. 2b). In consideration of the 11 MS patients, from whom blood was studied in remission as well as in relapse, the sE-selectin concentrations showed no significant difference ($p=0.846$) (Fig. 3b). Raised sE-selectin levels were detected in male patients ($p=0.014$). Linear regression analysis revealed a slight correlation between sE-selectin plasma levels and age in all patients ($p=0.017$), thus lower levels were found with increasing age. The statistical analysis showed no significant differences between sE-selectin plasma levels in relapse, remission and in progression ($p=0.238$). An influence of immunomodulatory or immunosuppressive therapy, relapsing rate and progression index on the expression of sE-selectin could not be proven.

3.3. Soluble PECAM-1 in patients with multiple sclerosis

sPECAM-1 plasma concentrations ranged from 20.3 to 196.1 ng/ml. The sPECAM-1 cut-off level was set at 75.0 ng/ml. Plasma sPECAM-1 was higher in patients with RRMS than PPMS ($p=0.002$) and in patients with SPMS than PPMS ($p=0.014$), respectively (Fig. 1c). Furthermore, sPECAM-1 plasma levels were elevated in patients with RRMS ($p<0.0001$) as well as SPMS ($p<0.0001$) in comparison to the healthy controls. Both during relapses ($p=0.0002$) and in clinical remission ($p=0.028$) plasma concentrations of sPECAM-1 were increased compared to progression. Concerning the entire study cohort no significant difference in the sPECAM-1 plasma levels existed between those MS patients within an acute relapse compared to remission ($p=0.183$) (Fig. 2c), whereas sPECAM-1 showed a significant increase during relapse versus remission ($p=0.001$) in the group of 11 patients with blood removal both in remission and subsequent relapse (Fig. 3c). According to sP-selectin the elevated sPECAM-1 concentrations were mainly related to 5 RRMS patients with no differences in clinical disease course compared to the entire RRMS group. In addition, we found no significant correlation between plasma sPECAM-1 and age, gender, disease duration, disability, relapsing rate and progression index of MS patients. An impact of immunomodulatory or immunosuppressive treatment on concentrations of sPECAM-1 could not be demonstrated.

4. Discussion

Brain inflammation in MS is supposed to implicate blood-brain-barrier disruption and consecutive transendothelial migration of leukocytes, mediated by a series of adhesion molecules. In this study, we measured levels of the circulating soluble adhesion molecules sP-selectin, sE-selectin and sPECAM-1 in plasma of patients with MS.

This is the first report investigating sP-selectin in MS. We have observed higher plasma levels of sP-selectin during relapses compared to remission and progression, respec-

tively. Moreover, sP-selectin plasma concentrations were elevated in patients with RRMS in comparison to the group of healthy blood donors. These results are in agreement with the increased P-selectin expression before disease onset in EAE mice (Kerfoot and Kubes, 2002). In addition, Ertenli et al. reported increased plasma sP-selectin concentrations in patients with rheumatoid arthritis and thrombocytosis and a positive correlation between sP-selectin and clinical activity (Ertenli et al., 1998). In conjunction with these findings our results may indicate a key role of P-selectin in mediating inflammatory interactions.

In contrast, we could not confirm the reports of elevated sE-selectin levels in serum and CSF of patients with PPMS (Dore-Duffy et al., 1995; McDonnell et al., 1998, 1999; Giovannoni et al., 1996). In our study significant lower sE-selectin plasma levels have been demonstrated in patients with PPMS compared to those with RRMS or SPMS as well as to healthy controls. Additionally, the highest sE-selectin concentrations were found during relapse as well as in young and in male patients. Our study cohort included 15 patients with PPMS characterized by a mean age of 53.3 years and a sex ratio male : female = 1.5 : 1. In comparison, Giovannoni et al. reported raised sE-selectin concentrations in 10 patients with PPMS showing a mean age of 42.1 years and a sex ratio male:female = 9 : 1. Furthermore, McDonnell et al. demonstrated elevated serum sE-selectin levels in 77 PPMS patients with a mean age of 54.7 years and a sex ratio male : female = 1 : 1.3 in the study published in 1999. Mean age and sex ratio could not be deduced from the papers about increased sE-selectin levels in PPMS published by Dore-Duffy et al. in 1995 and McDonnell et al. in 1998. Even though age and gender may have an influence on the sE-selectin levels in PPMS, this matter of fact could not explain the discrepancy of all these previous study results to our findings because evidence for significantly elevated sE-selectin concentrations in PPMS patients compared to RRMS, SPMS and control groups has been provided. On the other hand, both the paucity of gadolinium-enhancing lesions on cerebral MRI and the less inflammatory process in PPMS are in accordance with the significantly decreased sE-selectin plasma levels in patients with this course of disease. Comprisingly, as the sE-selectin expression is restricted to activated endothelial cells a correlation between sE-selectin levels and endothelial cell activation might be suggested.

In the entire study cohort we detected an increase of sPECAM-1 levels both during an acute relapse and in remission compared to progression but no significant difference between the plasma concentrations in relapse and remission. However, in the group of 11 patients, taken out of the entire study group in order to measure sPECAM-1 levels in remission versus relapse, an augmented increase during relapse compared to remission was determined. Significant differences in the plasma concentrations of sPECAM-1 were found between the MS subgroups showing the highest levels in patients with RRMS and lowest in those with PPMS. Plasma levels of sPECAM-1 were elevated in patients with RRMS and SPMS in comparison to the group of healthy controls. Furthermore, SPMS patients with superimposed relapses had higher sPECAM-1 levels compared to the SPMS patients without relapses, but the difference was not statistically significant. The occurrence of relapses in the secondary-progressive disease course might reveal a persisting active inflammatory process. These results support the report of higher sPECAM-1 serum levels in RRMS patients with brain gadolinium-enhancing lesions indicating an acute blood-brain-barrier damage (Losy et al., 1999; Minagar et al., 2001). Elevated levels of sPECAM-1 may indirectly show an increase of the adhesion molecule expression and its release from cerebral endothelial cells, platelets and leukocytes (Losy et al., 1999). Therefore, an increase of sPECAM-1 concentrations prevailing in a more active inflammatory disease process might be useful as a paraclinical marker of subclinical disease activity.

In conclusion, the entirety of all data concerning soluble adhesion molecules in MS supports the hypothesis that sP-selectin, sPECAM-1 and sE-selectin might be useful immunological markers of disease activity by defining disease heterogeneity and classifying patients into different MS subgroups. Further research is needed to elucidate the regulatory role of adhesion molecules, both membrane-bound and soluble-circulating forms, in MS pathophysiology in more detail with regard to potential clinical and therapeutic applications.

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