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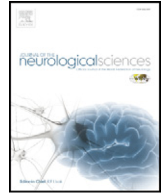

Soluble thrombomodulin levels in plasma of multiple sclerosis patients and their implication

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ABSTRACT

Thrombomodulin (TM) on the cell-surface of cerebrovascular endothelial cells (CECs) is released into blood upon CEC damage. TM promotes activation of protein C (APC), an anticoagulant, anti-inflammatory, neuroprotective molecule that protects CECs and impedes inflammatory cell migration across the blood–brain barrier (BBB). Multiple sclerosis (MS) is associated with CEC damage and BBB dysfunction. We evaluated soluble TM (sTM) levels as a biomarker of BBB integrity and whether glatiramer acetate (GA) influenced sTM levels in MS patients. sTM levels quantified by 2-site ELISA from sera of healthy controls and systemic lupus erythematosus (SLE) patients (CEC-damage positive control) were compared with levels from patients with relapsing–remitting (RRMS) or secondary-progressive MS (SPMS), stratified as: RRMS/GA/no relapse, RRMS/GA/in relapse, RRMS no GA/no relapse, RRMS/no GA/in relapse; and SPMS/no GA. Additionally, soluble endothelial protein C receptor (sEPCR) levels were assessed in the non-stratified MS group, SLE patients, and controls. sTM levels were highest in RRMS patients taking GA with or without relapse, followed in decreasing order by SLE, RRMS/no GA/in relapse, SPMS, RRMS/no GA/no relapse, healthy controls. sEPCR levels were highest in MS patients, then SLE, then controls. sTM may be a useful biomarker of BBB integrity in RRMS patients. Further evaluation of sEPCR is needed. The finding that the highest sTM levels were in RRMS patients taking GA is interesting and warrants further investigation.

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1. Introduction

The inflammatory immune response in multiple sclerosis (MS) is thought to involve interactions between CD4+ T-lymphocytes and myelin antigens presented by MHC class II on antigen-presenting cells within the CNS. The blood–brain barrier (BBB), comprising cerebral endothelial cells (CECs) and pericytes and supported by astrocytes and perivascular macrophages, normally inhibits T-cell trafficking into the CNS [1]. BBB disruption in MS allows T-cells and inflammatory mediators to enter the CNS [1,2]. Thrombomodulin (TM) is an endothelial cell (EC) surface transmembrane proteoglycan [2]. Injury to ECs on large and small blood vessels increases expression of soluble TM (sTM) into the circulation [3–5]. The principal known function of TM is to bind to thrombin, thereby activating protein C (APC) [6,7]. APC is an anticoagulant protease with cytoprotective and anti-apoptotic activity that contributes to BBB protection [2]. Both sTM and APC have potent anti-inflammatory properties, in part by reducing inflammatory cell adhesion to CECs, thereby inhibiting their migration across the BBB [5,8]. The anti-inflammatory effects of APC are dependent on binding to EC PC receptors (EPCR) on the endothelium [2]; however, the soluble form (sEPCR) in circulation inhibits APC generation and function. EPCR is expressed on the endothelium of large vessels (arteries and arterioles) [9].

Studies have shown that patients with MS or other diseases, including systemic lupus erythematosus (SLE), have increased sTM and sEPCR levels in plasma or serum, suggesting sTM may be a useful biomarker in MS [9–13]. sEPCR levels have also been studied in other diseases, although not previously in MS [14]. We hypothesized that sTM and sEPCR levels would serve as biomarkers of BBB disruption, and would therefore be elevated in MS patients, particularly during acute exacerbations. We measured sTM and sEPCR levels in patients with relapsing–remitting MS (RRMS) or secondary-progressive (SPMS), taking or not taking glatiramer acetate (GA), to evaluate their correlation with patients' clinical status, and compared these with sTM and sEPCR levels in SLE patients and healthy controls.

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2. Materials and methods

2.1. Patient groups

The Human Subjects Committee (HSC) of the University of Kansas Medical Center (KUMC) approved the study and informed consent was obtained from all study participants. Subjects were between 18 and 70 years of age. The study included individuals with no known neurologic or immunologic disease (healthy control group), individuals with a diagnosis of SLE (positive control group), and patients with clinically definite MS. Each MS patient was categorized clinically as having RRMS or SPMS. Primary progressive MS (PPMS) patients were excluded from the study. MS patients were divided into 5 categories: 1) RRMS patients receiving GA (Copaxone®; Teva Pharmaceutical Industries, Ltd., Kansas City, MO) 20 mg daily subcutaneously (SC) and having no relapse in the past 30 days; 2) RRMS patients receiving GA and currently experiencing a relapse; 3) RRMS patients not receiving GA or other immunomodulators and having no relapse in the past 30 days; 4) RRMS patients receiving no GA or other immunomodulators and currently experiencing a relapse; and 5) SPMS patients not receiving GA or other immunomodulator or immunosuppressants.

Patients with SLE were recruited from the SLE Clinic and patients with MS were recruited from the MS Clinic, both at KUMC. Qualified patients were consecutively recruited; when blood was obtained from 15 patients in a specific category, recruitment in that category stopped. Each MS patient underwent a history and neurological examination by a primary investigator (SL) to confirm the diagnosis of MS, assess the type of MS, and confirm the presence or absence of a relapse. Relapse was defined as the onset of new neurological symptoms or a sudden worsening of previous symptoms lasting at least 24 h and with no evidence of a metabolic change.

A 20-mL sample of blood from each patient was drawn into 2 ACD Vacutainer (Stabilyte™) tubes. The blood was placed on ice and then centrifuged within 30 min in a refrigerated centrifuge at 3000 rpm (1200 ×g) for 30 min. The plasma fraction was aliquoted into 1 mL microfuge tubes and again kept on ice until it was placed in a −80 º C freezer for subsequent testing (sTM and other coagulation markers obtained in Stabilyte™ tubes and frozen at −80 º C are stable for 8–11 years [15]).

2.2. Materials and standardization of sTM ELISA

The sTM ELISA methods employed have been published previously [16]. Asserachrom® kits for measurement of sTM and sEPCR were obtained from Diagnostica Stago (Asnieres, France); 96-well assay microplates from Corning (Acton, MA); and standard acid citrate dextrose (ACD) Vacutainer™ tubes from Fisher Scientific (Charlotte, NC). It is likely that the wide variation in sTM levels in control or normal populations (3–50 ng/mL) is the result of the previous lack of standardization of sTM ELISA assays [17]. We obtained 2-site monoclonal antibodies (mAbs) from Dr. Barry Woodhams, Scientific Director, (R&D, Diagnostica Stago, Gennevilliers, France). We then revalidated the ELISA kit from Diagnostic Stago, US (Parsippany, NJ) using 3 independent and blinded skilled technicians.

sEPCR levels in plasma were measured with the Asserachrom® ELISA kits (Diagnostica Stago, Asnieres, France), according to the manufacturer's instructions. Samples were diluted 1/26 and assayed in duplicate.

Blood samples were collected at the time the patient entered the study. sTM levels were measured for the healthy control, SLE, and each of the 5 MS patient subgroups. sEPCR levels were tested for the healthy control and SLE subgroups and for the entire (ungrouped) MS patient cohort.

2.3. Statistical methods

Data are presented as mean ± standard error (SE) or standard deviation (SD). Statistical analysis was performed by 2-way mixed ANOVA with repeated measures using the PROC MIXED procedure in SAS. The within-subjects factor was the repeated measures of sTM. For the between-subjects factor, subjects were divided into 7 discrete subgroups: the 5 categories of MS patients listed above, 1 healthy control group, and 1 group of patients with SLE as a positive control. Each subject belonged to only 1 of those subgroups. Least-square means were calculated and compared by using 2-sided *t*-test. A p value < 0.05 was considered statistically significant. All analyses were performed using SAS software (version 9.12, Research Triangle Institute, USA).

3. Results

3.1. Validation of the TM 2-site ELISA

Using the Asserachrom™ kits we validated the 2-site sTM ELISA in our laboratory and confirmed its accuracy and minimal inter-rater variability among 3 different technical personnel. In repeated standard curve assays, the first of these yielded the following R (0.9985, 0.9954, 0.9991) and R^2 (0.9970, 0.9909, 0.9983) values, respectively. In the 2nd set, a 2nd technician confirmed and validated the data in the control, SLE, and MS groups, with similar R and R^2 values. A 3rd technician repeated the assays with similar results (data not shown).

The specificity of the sTM 2-site ELISA for all patient groups was evaluated by the 3 technical raters between January and July 2003 and again, in 2004–2005 (data not shown). Test–re-test data, even among different technicians, was excellent, with R values > 0.99.

3.2. Patients

A total of 91 patients were included: 20 subjects in the healthy control subgroup, 8 patients with SLE in the positive control subgroup, and 63 patients with clinically definite MS (Table 1). With the exception of Group 4 (RRMS, no GA, current relapse, n = 7), who tended to be younger and have shorter disease duration, demographic characteristics were generally comparable among the RRMS subgroups. Most participants were female, comprising 80–100% of the RRMS patient groups, 86% of SPMS patients, 60% of healthy controls, and 100% of the SLE subgroup. Age range was comparable among all groups except, as would be expected, SPMS patients were somewhat older than people in the other subgroups.

Only 6 patients with MS had had a relapse within 6 months of their initial evaluation at study entry. No patient had evidence of a relapse within the 3 months prior to his or her initial evaluation and blood draw.

3.3. sTM and sEPCR levels

As shown in Fig. 1, the highest mean sTM level was observed in RRMS patients receiving GA who were currently experiencing a relapse (mean 16.9 ng/mL). The lowest mean sTM level was observed in RRMS patients not taking GA who were in remission and had not had a relapse in the previous 30 days (mean 11.3 ng/mL), approximately the same level as in the healthy control group (mean 11.4 ng/mL). In addition, for RRMS patients who had not had a relapse in the previous 30 days, there was a significant difference in mean sTM levels between those taking GA (mean 15.8 ng/mL) and those not taking GA (11.3 ng/mL). MS patients taking GA, regardless of whether they had a current relapse or not, had significantly higher sTM levels than levels in the healthy control group and in the positive control (SLE) group ($p < 0.05$). Moreover, RRMS patients in relapse while taking GA had significantly higher levels of sTM compared with sTM levels in SPMS patients not taking GA or immunosuppressants ($p < 0.05$). Finally, SLE patients had higher sTM

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