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Impact of Autoimmune Demyelinating Brain Disease Sera on Pericyte Survival

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ABSTRACT

Introduction: Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) characterized by demyelination and brain pericyte dysfunction might be involved in MS pathogenesis Our aim was to evaluate whether the factors in serum affect pericyte survival.

Method: C57BL/6 female mice were immunized with myelin oligodendrocyte glycoprotein (MOG) to induce experimental autoimmune encephalomyelitis (EAE). To confirm the animal model, the sera level of anti-MOG antibody in mice and platelet-derived growth factor-BB (PDGF-BB) in patients was measured by ELISA. Human brain vascular pericytes (HBVP) cell lines were incubated with sera of EAE mice and primer progressive MS (PPMS), seconder progressive MS (SPMS) and relapsing-remitting MS (RRMS) patients. The viability of HBVP is measured with Annexin V-FITC/propidium iodide staining with flow cytometry.

Results: Annexin V-FITC/propidium iodide staining with flow cytometry showed increased ratios of early apoptosis and decreased survival following incubation with sera of EAE and progressive MS. Levels of platelet-derived growth factor-BB were identical in serum and cerebrospinal fluids of patients with different forms of MS.

Conclusion: Our results suggest that serum factors might contribute to progressive MS pathogenesis via pericyte dysfunction.

Keywords: Multiple sclerosis, pericytes, apoptosis, demyelination, autoimmunity

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INTRODUCTION

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system (CNS). Autoimmunity in MS is generally believed to initiate in the peripheral immune system and subsequently affect CNS via several complex mechanisms including blood brain barrier (BBB) disturbance (1).

Brain pericytes are vital components of the BBB system and are known to regulate BBB permeability by virtue of vascular smooth muscle contraction and gap junction integrity (2, 3). Recent studies suggest that brain pericytes may also be involved in the pathogenesis of neuroimmunological diseases via leukocyte recruitment, microglia polarization and adaptive immunity regulation (4). Studies showed that the apoptosis of retinal pericytes was increased in diabetic retinopathy due to chronic inflammation (5, 6). Moreover, lesions in post-mortem MS brain show increased regions of cells with phenotypical characteristics of pericytes including expression of platelet-derived growth factor receptor beta (PDGFRβ) (7).

To find out whether MS sera factors might alter pericyte viability thereby leading to BBB disruption and enhanced access of the immune system to target CNS antigens, we treated commercially available cultured human pericytes with sera of mice with experimental autoimmune encephalomyelitis (EAE) and of patients with MS and analyzed ratios of pericytes undergoing early and late apoptosis.

METHOD

Experimental Autoimmune Encephalomyelitis (EAE)

Eight– to ten-week-old female C57BL/6 (B6) mice were housed under environmentally controlled standard conditions. This project was approved by the institutional review board. EAE (n=14) was induced by subcutaneous injection of 200 µg myelin oligodendrocyte glycoprotein (MOG) 35–55 peptide (Multiple Peptide Systems, San Diego, CA) emulsified in complete Freund's adjuvant (CFA; Difco, Detroit, MI). Control mice (n=5) were immunized with only CFA. Both EAE and control mice received intravenous administration of 500 ng pertussis toxin (PT; Sigma, St. Louis, MO) in saline, at the day of immunization and 48 hours later. Weight and body condition scores (0=healthy, 1=partial flaccid tail, 2=complete flaccid tail, 3=paresis of one hindlimb, 4=paralysis of one hindlimb, 5=paralysis of two hindlimbs, 6=paralysis of hindlimbs

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and paresis of one forelimb, 7=paralysis of hindlimbs and one forelimb, 8=tetraparalysis, 9=moribund, 10=death) were recorded every two days due to a previously reported clinical scale (8). Sera were collected 40 days after first immunization before termination.

Patients

Thirty-two MS patients (mean ± standard deviation; 40.0±10.9 year-old, 19 women) and 11 age-gender matched healthy controls (38.6±14.1 year-old, 6 women) were enrolled. MS patients fulfilled relevant criteria for relapsing remitting (RRMS, n=13, F/M=7/6, EDSS=1.2±0.5), secondary progressive (SPMS, n=10, F/M=5/5, EDSS=5.8±0.7) and primary progressive MS (PPMS, n=9, F/M=7/2, EDSS=5.4±0.8) (9). None of the participants had a coexisting disorder or was under immunotherapy. All MS patients were in remission. Average disease durations (7.5±3.2 years), EDSS scores (3.2±2.1) and total attack numbers (3.4±2.1) were recorded. Twenty patients had cerebrospinal fluid (CSF)-specific oligoclonal bands. The study was approved by the instutional review board and a signed informed consent was received from each participant.

ELISA

In order to confirm the EAE model, anti-MOG antibody levels were measured by ELISA in sera of immunized mice. MOG antigen (1 µg/mL) was coated onto 96-well microtiter plates in 0.1 M carbonate bicarbonate buffer overnight at 4°C. Mouse serum samples (100 µl) obtained from the tail vein (1:1000 dilution) were added and incubated at 37°C for 90 min. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Abcam, Cambridge, UK) (1:5000) was added and then incubated at 37°C for 90 min. Subsequently, the peroxidase indicator substrate 3.3′, 5.5′-tetramethylbenzidine (TMB) solution was added, and the mixture was allowed to develop color at room temperature in the dark. Plates were read at a wavelength of 450 nm.

Platelet-derived growth factor-BB (PDGF-BB) levels were measured in sera and CSF samples of patients by ELISA according to manufacturer's guideline (Bioassay Technology Laboratory, Shangai, China).

Flow Cytometry

HBVP (5000 cells/well) were seeded in 96-well poly-L-lysine coated plates with pericyte medium (ScienCell, CA, USA). When a confluence of 70% was reached, the supernatant was removed and replaced in triplets with sera of EAE mice, MS patients and healthy controls (1:50 dilution in pericyte medium without additional serum supplement) for 24 hours in 37°C and 5% CO_2 chamber. Serum dilutions were tried at 1:25, 1:50 and 1:100, and incubation time was 16 hours-24 hours-36 hours. The experiment was completed with 1:50 and 24-hour incubations where the optimum dead/live cell ratio was obtained. Cells were then trypsinized, immediately centrifuged and resuspended in PBS. Annexin V/propidium iodide (PI) staining and flow cytometry procedures were performed with 10000 cells/well according to the manufacturer's instructions (BD, New Jersey, USA).

Statistical Analysis

EAE clinical scores were compared by Mann-Whitney U test. Flow cytometry and ELISA results were compared by Mann Whitney U or Kruskal-Wallis (with Dunn's post-hoc analysis) tests, as required. Spearman test was used for correlation analysis. p values less than 0.05 were considered statistically significant.

RESULTS

EAE Model

Significantly higher clinical scores were observed in EAE mice starting at day 7 (Figure 1). Moreover the EAE group showed a significantly higher

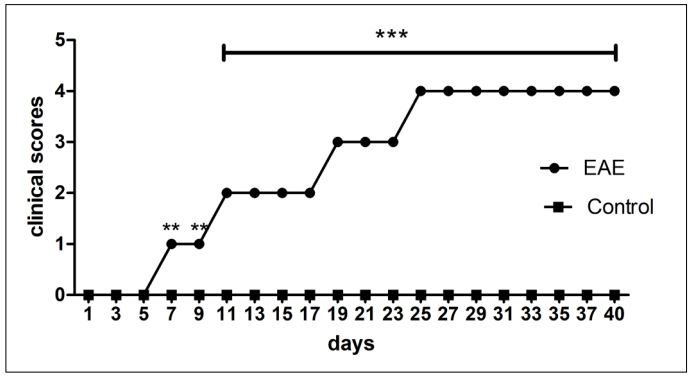

Figure 1. Clinical score kinetics of mice with experimental autoimmune encephalomyelitis (EAE) and control mice immunized with complete Freund adjuvant only (**p<0.01; ***p<0.001).

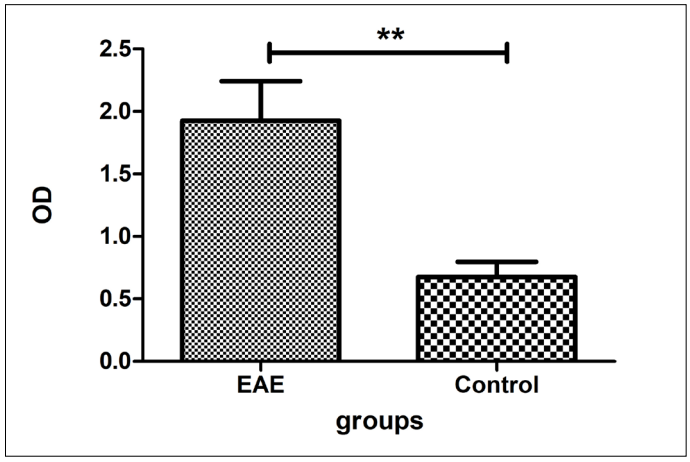

Figure 2. Serum anti-MOG antibody levels by ELISA. Vertical bars indicate standard deviations and OD stands for optical density (**p<0.01; ***p<0.001).

serum level of anti-MOG antibody as compared to the control group (Figure 2). Induction of EAE was also confirmed by the demonstration of CNS lesions with demyelination and infiltrating immune cells by immunohistochemistry (data not shown).

EAE and MS sera induce early apoptosis in pericytes

HBVP incubated with EAE sera showed significantly reduced cell viability (i. e. lower Annexin V-/PI– cells) than those treated with control sera (p=0.034). Also, the percentage of cells with early apoptosis (Annexin V+/PI-) was higher in HBVP treated with EAE sera (p=0.024). No significant difference was observed between EAE and control groups by means of ratios of cells with late apoptosis (Annexin V+/PI+; p=0.098) and necrosis (Annexin V-/PI+; p=0.093) (Figure 3).

Likewise, HBVP treated with PPMS sera showed significantly increased early apoptosis (Annexin V+/PI-; p=0.008) and reduced cell viability (Annexin V-/PI-; p=0.002), whereas ratios of cells with late apoptosis (Annexin V+/PI+; p=0.129) and necrosis (Annexin V-/PI+; p=0.228) were comparable among groups. Although cells treated with SPMS sera showed trends towards displaying increased late apoptosis and decreased viability, these tendencies did not attain statistical significance (Figure 4).

PDGF-BB levels are not associated with clinical features of MS

To find out if altered pericyte viability levels induced by MS sera, levels of PDGF-BB, one of the major grow factors for pericytes, were measured in sera and CSF of the patients. PDGF-BB levels were comparable (p=0.903) in

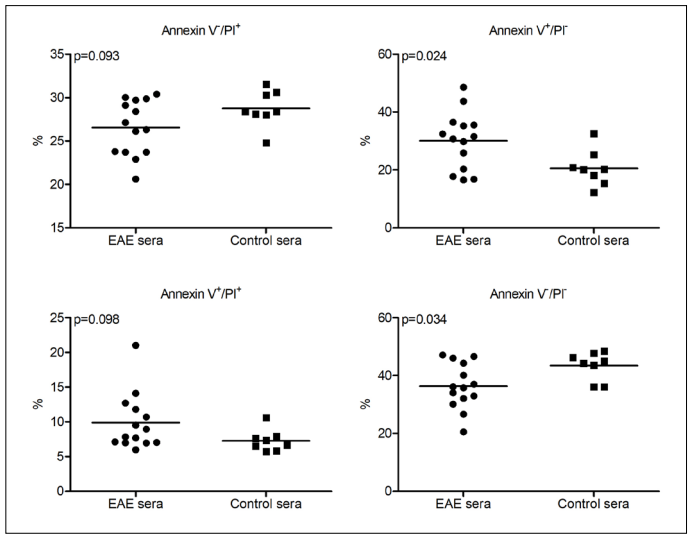

Figure 3. Percentages of annexin V and propidium iodide (PI) positive human brain vascular pericytes incubated with sera of mice with experimental autoimmune encephalomyelitis (EAE) and control mice immunized with complete Freund adjuvant only. Horizontal bars indicate standard deviations. p values obtained by Mann-Whitney U test are shown in the upper left corner of each panel (Annexin V-/PI-, viable cells; Annexin V+/PI-, cells with early apoptosis; Annexin V+/PI+, cells with late apoptosis; Annexin V-/PI+, cells with necrosis).

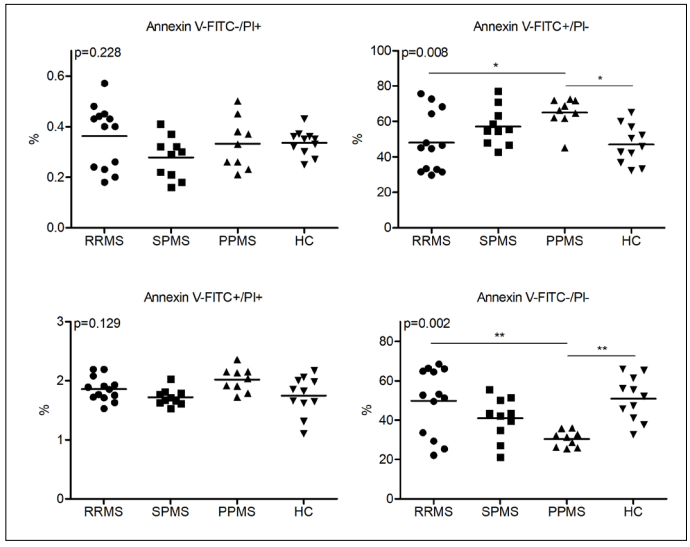

Figure 4. Percentages of annexin V and propidium iodide (PI) positive human brain vascular pericytes incubated with sera of relapsing remitting multiple sclerosis (RRMS), secondary progressive multiple sclerosis (SPMS), primary progressive multiple sclerosis (PPMS) patients and healthy controls (HC). Horizontal bars indicate standard deviations. p values obtained by Kruskal-Wallis test are shown in the upper left corner of each panel. ** p<0.01; *** p<0.001 by Dunn's post-hoc test (Annexin V-/PI-, viable cells; Annexin V+/PI-, cells with early apoptosis; Annexin V+/PI+, cells with late apoptosis; Annexin V-/PI+, cells with necrosis).

sera of RRMS (2007±520 ng/L), SPMS (2401±815 ng/L), PPMS (2387±886 ng/L) patients and healthy controls (2387±886 ng/L) and in CSF (p=0.417) of RRMS (1926±166 ng/L), SPMS (1805±52 ng/L) and PPMS (1736±61 ng/L) patients. There was no significant correlation between serum/CSF PDGF-BB levels and age, EDSS, disease duration and total attack numbers of MS patients (not shown). However, CSF (but not serum) PDGF-BB levels of MS patients with oligoclonal bands (n=20) were higher (1911±109 ng/L) than those without (n=12; 1562±66 ng/L, p=0.025).

DISCUSSION

In our study, HBVP incubated with EAE and progressive MS sera showed significantly increased ratios of early apoptosis and reduced ratios of cell viability. These findings indicate the presence of apoptosis inducing factor (s) particularly in sera of PPMS patients and mice with MOG-induced EAE. Thus, our preliminary study showed for the first time that in progressive MS types, the viability of pericytes may be adversely affected by as yet unidentified serum factors. This phenomenon may disrupt the integrity of the BBB, enhance the ongoing inflammation and demyelination in the CNS and thus negatively affect the prognosis of MS (10).

Notably, PPMS/EAE sera affected early apoptosis of pericytes more than late apoptosis and necrosis. The early and late stages of neuronal apoptosis involve cellular membrane phosphatidylserine externalization and genomic DNA fragmentation, respectively. Exposure of phosphatidylserine residues represents a relatively reversible stage of apoptosis and may promote microglial activation and coagulation cascade (11). Putatively, longer exposure of pericytes to PPMS/EAE sera may also enhance the late stage of apoptosis. Moreover, Annexin V+/PI+ cells do not only represent the late stage of apoptosis but also early stage of necrosis. This might be another reason behind the lack of difference in late apoptosis in MS/EAE serum-treated pericytes. In line with our findings, perivascular pericyte loss has been demonstrated in the 40th day of EAE model, suggesting that induction of EAE promotes production of molecules that are toxic for pericytes (12). Interestingly, a similar reduction in pericyte abundancy was not observed in the 20th day of immunization indicating that these toxic elements are a late-stage by product of MOG-immunization. Notably, accumulation of fibrinogen has been shown in post-mortem progressive MS brain and the EAE model. Fibrinogen is known to negatively influence survival of glial cells including pericytes (13).

As a limitation no CSF samples were available from healthy controls and CSF volume of most patients was not sufficient for in vitro experiments. Therefore, the impact of CSF of MS patients on pericyte survival should be investigated in future studies.

PDGF-BB is a pleiotropic growth factor that acts upon PDGF receptors, which are abundantly expressed by pericytes and that has been implicated to have a pro-regenerative action (14). Thus, paucity of PDGF-BB in EAE and progressive MS sera could have been one of the factors leading to reduced pericyte viability. However, we failed to find a difference among serum/CSF samples of MS subgroups or a correlation with clinical features of MS and PDGF-BB arguing against this assertion. Our results are in line with a previous report that has not found increased serum PDGF-BB levels in MS or correlation between PDGF-BB levels and neuroimaging features of MS (15). Nevertheless, MS patients with CSF-specific oligoclonal bands showed a tendency to display higher CSF PDGF-BB levels suggesting that this growth factor may be involved in intrathecal B cell proliferation or antibody production.

In conclusion, our results indicate that factor (s) in progressive MS sera might reduce the viability of pericytes leading to BBB disruption. This might be one of the factors underlying the development of progressive forms of MS. Thus factor (s) causing this phenomenon (e.g. cytokines, growth factors, antibodies etc.) should be further delineated.

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Ethics Committee Approval: The Guiding Principles in the Care and Use of Laboratory Animals adhered to the conduct of all the experimental procedures described in this article. This project was approved by the Local Animal Ethical Committee of Istanbul University, Istanbul, Turkey and Istanbul University Istanbul Medical Faculty Clinical Research Ethics Committee (Approval date: 10.07.2017).

Informed Consent: The study was approved by the instutional review board and a signed informed consent was received from each participant.

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Author Contributions: Concept - CU, EŞ, VY, ET, CİK, YGÖ; Design - CU, EŞ, ET, YGÖ; Supervision - CİK, YGÖ; Resource - CİK, YGÖ; Materials - CU, EŞ, ZK, MK; Data Collection and/ or Processing - CU, EŞ, VY, ABY, AV, ZK; Analysis and/or Interpretation - CU, EŞ, VY, ET; Literature Search - CU, ET; Writing - CU, ET; Critical Reviews - VY.

Conflict of Interest: The authors have no conflicts of interest to declare.

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