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The Fibrotic Response in Pericyte Culture Upon Exposure to Multiple Sclerosis Sera

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ABSTRACT

Introduction: Multiple sclerosis (MS) is a chronic autoimmune and demyelinating disease of central nervous system (CNS) leading to progressive function loss. Besides infiltration of peripheral immune cells into CNS subsequent to neuroinflammation, the accumulation of extracellular matrix (ECM) elements, produced by brain barrier cells, in the enlarged perivascular spaces contributes to the pathophysiology. In this study, we aimed to develop an in-vitro model of MS to investigate fibrosis triggered by sera or cerebrospinal fluid (CSF) from MS patients and evaluate the response of blood-brain barrier (BBB) cells to this model.

Methods: Human brain vascular pericytes, endothelial cells and normal human astrocytes were cultured and exposed to a cytokine reference control (Transforming growth factor beta 1 (TGF-β1)), healthy human sera, and sera/CSF from treatment naïve relapsing-remitting MS patients with the appropriate dilution dose. The pericytes cell proliferation were evaluated by xCELLigence, while the collagen and fibronectin expressions of BBB cells, and pericyte myofibroblastic transformation were analyzed with immunocytochemistry.

Results: TGF-β1 induced fibrosis, characterized by fibronectin overexpression, specifically in pericyte cultures. Furthermore, incubation of pericytes with MS serum but not CSF led to a more robust fibrotic response (fibronectin/collagen overexpression), myofibroblastic transformation as well as increased proliferation. Fibronectin overexpression was also detected in endothelial cell culture with MS serum. Glial fibrillary acidic protein (GFAP) expression is increased, but fibrotic markers are decreased in cultured astrocytes with MS serum.

Conclusion: Pericytes, among BBB-forming cells, were identified as key contributors to fibrosis in response to MS serum. MS-serum-induced in vitro models are promising for studying the individualized tendencies of patients and may be a new approach for high-throughput screening of potential treatment agents.

Keywords: Astrocytes, collagen, endothelial cells, fibrosis, multiple sclerosis, pericytes

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INTRODUCTION

Multiple sclerosis (MS) is an inflammatory and demyelinating disease of the central nervous system (CNS). The demyelinating insult results from inflammatory signaling, recruitment of peripheral immune cells, and extracellular matrix (ECM) depositions. Lack of proper remyelination in the MS lesions leads to impaired functional recovery. Studies and therapeutic interventions primarily focused on inflammatory pathways. But recent reports point out that ECM derangements may contribute to the permissive environment and, thus, poor remyelination in MS lesions (1).

In CNS, astrocytic gliosis was considered the only scar formation route (2). But increasing evidence suggests that fibrotic scarring also contributes to several pathological processes of CNS (3–7). Fibrotic scarring can be defined as collagen-rich ECM depositions after a traumatic or inflammatory insult and is well-demonstrated in the traumatic spinal cord injury model (8,9). Moreover, recent genetic lineage tracing studies showed that fibrotic response is generated from perivascular platelet-derived growth factor receptor beta (PDGFRβ)-

Highlights

- **Endothelial cells presented only a small contribution to fibrosis in MS conditions.**
- **Pericytes may have fibrotic response upon exposure to MS sera.**
- **Fibrotic response decreased in cultured astrocytes upon exposure to MS serum.**

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positive cells, including pericytes and/or perivascular fibroblasts from the perivascular niche (1,8–10).

Studies documented collagen accumulation in human MS lesions (11,12) as well as animal models of MS (1,10,13). But the contribution of fibrosis to the disease prognosis has not been fully understood yet. One study reported that the inhibition of scar formation improves motor disability and increases the expression of oligodendrocyte lineage cells in the MS animal model (10). Moreover, ECM elements are shown to be not permissive to the maturation of oligodendrocytes in-vivo (1), suggesting that targeting fibrosis in MS is promising for novel drugs. Thus, establishing an in-vitro MS model to study fibrotic response is urgently needed for high throughput screening of potential agents.

Modeling MS in-vitro via incubation with autoimmune demyelinating disease sera to study individualized effects has been reported before. In trans-epithelial electrical resistance (TEER) studies, cultured endothelial cells, upon exposure to MS sera or purified IgG's from myelin oligodendrocyte glycoprotein (MOG) and neuromyelitis optica (NMO) patients' serum, were shown to lose their integrity, and also the expression of tight junction proteins was found to have decreased (14–16). Only one study reported the effects of MS sera on pericytes and demonstrated decreased proliferation and early apoptosis in the first 24 hours (17). Moreover, demyelination was shown in cultured mouse cerebellum tissue exposure to experimental allergic neuritis sera (18). None of the studies focused on fibrotic response of BBB cells and the contribution of CSF from MS patients to fibrosis.

For the purpose of revealing the contribution of BBB cells to the fibrosis in MS pathophysiology, we aimed to examine the fibrotic response of BBB components in vitro against human MS sera and cerebrospinal fluid (CSF). We used BBB cell lines (endothelial cells, pericytes and astrocytes) and exposed them to the sera and CSF samples of MS patients for in vitro MS modelling. In addition, we studied myofibroblastic transformation and cell proliferation with the xCelligence system in pericyte cell culture.

METHODS

Cell Culture

Human brain vascular pericytes (HBVP, ScienceCell), human brain vascular endothelial cells (hCMEC/D3, Sigma-Aldrich, SCC066), hereafter referred to as brain endothelial cells (BEC), and normal human astrocytes (NHA, Gibco, N7805100) were cultured with, respectively, complete pericyte medium (ScienCell, 1201), complete astrocyte medium (Lonza, CC-3186) and complete endogro medium (Merck, SCME004). While NHA and BEC were seeded on non-coated flasks, HBVP were seeded on poly-L-lysine (PLL, Sigma-Aldrich, P2658) coated flasks, and all cell lines were incubated at 37°C and 5% CO_2 until 80–90% confluency. The medium was replaced every two to three days.

Human Materials

To collect and use human serum and CSF materials, the ethical approval was obtained from the local ethics committee "Institutional Review Board (IRB) of Koç University Ethical Committee" with approval number: 2016.123. IRB2.077 and consent to participate were documented according to IRB guidelines. Sera and CSF from MS patients validated for CSF-specific oligoclonal bands were used. All patients were diagnosed with relapsing-remitting type MS, treatment-naïve, and the biological material was collected during the MS attack period before steroid treatment. Healthy participants had no history of or current autoimmune or other chronic or inflammatory diseases.

In Vitro Exposure to MS Serum and CSF

A cell culture study investigated the fibrotic response of BBB components, including pericytes, astrocytes, and endothelial cells, upon exposure to MS serum. HBVP, NHA, and BEC were seeded in transparent flat-bottom 96-well plates and incubated overnight at 37°C and 5% CO_2 for cell attachment. Then, cells were exposed to either pericyte cell medium as a control condition, 20 ng/mL TGF-β1 (R&D Systems, 240B) as a positive control due to their ability to induce fibrosis (2), healthy human serum (3 healthy individuals), MS patient serum, or MS patient CSF (3 MS patients) with a dose of 10% (v/v) that were used in previous studies (14,15) in

Table 1. Antibodies and markers used for immunofluorescence in this study

Primary Antibodies / Markers	IF Ratio	Cat. No.
Rabbit anti-PDGFRβ	1:100	Ab32570
Mouse anti-αSMA, FITC conjugated	1:100	F3777
Rabbit anti-GFAP	1:100	Ab7260
Rabbit anti-Fibronectin	1:100	F3648
Rabbit anti-Collagen I	1:100	Ab34710
Rabbit anti-Collagen IV	1:100	Ab6586
Rabbit anti-Laminin	1:100	Ab11575
Phalloidin-FITC conjugated	1:100	P5282
Secondary Antibody	IF Ratio	Cat. No.
Goat anti-Rabbit IgG CY3 conjugated	1:200	111–165–003

IF Ratio: Immunofluorescence ratio; Cat. Nu.: Catalog number; αSMA: alpha smooth muscle actin; CY3: Cyanine3; FITC: Fluorescein-5-isothiocyanate; GFAP: Glial fibrillary acidic protein; PDGFRβ: platelet derived growth factor receptor beta

cell medium and incubated for 72 hours at 37°C and 5% CO_2. TGF-β1 secretion mediates fibrosis (2,19,20). To assess how BBB components react upon this cytokine as a reference, cells were exposed to TGF-β1. Subsequently, the medium was removed, and cells were washed twice with phosphate-buffered saline and fixed with 4% PFA for 30 minutes at room temperature.

xCELLigence and Pericyte Proliferation

xCelligence system (RTCA DP Analyzer, San Diego, CA) was used to investigate the direct effect of inflammatory compounds in the serum of MS patients on the cleavage and proliferation ability of pericytes and indirectly to investigate the impact on pericyte survival ability. The cell index value will be close to zero in the absence of living cells (only medium) or with a suspension of dead cells. After the attachment of cells onto the electrode, the measured signal correlates linearly with cell number throughout the experiment. First, the culture medium was added (50 µL) to the wells of an xCELLigence E-plate (Agilent, 5469813001). After equilibration to 37°C, plates were inserted into the xCELLigence station to measure the baseline impedance and ensure that all wells and connections worked within acceptable limits. Then, HBVP was seeded in E-plates in duplicates per sample and incubated in the xCELLigence system to monitor cell proliferation in real-time. Twenty-four hours after seeding, HBVP were exposed to either pericyte medium as control, healthy human serum, or human MS serum (n=2 individuals/patients, in duplicates) with a serum dose of 10% v/v in the medium. The impedance value of each well was automatically monitored by the xCELLigence system for 100 hours and expressed as a cell-index value (average of duplicates). The proliferation rate between samples was calculated by their slopes between two-time points after exposure.

Immunofluorescence Staining

Cells were washed after fixation and incubated in methanol for 5 minutes at room temperature to permeabilize cell membranes. Then, they were blocked with Super Block (Thermo Scientific™, PI37535) solution for 1 hour at room temperature. Next, cells were incubated subsequently with the primary and secondary antibodies (diluted in the blocking solution) for 1.5 hours at 37°C. The antibodies used in this study are listed in Table 1. Finally, cells were mounted with 4 ', 6-diamidino-2-phenylindole (DAPI, Abcam, ab104139). Fluorescent images were taken with a Leica DMi8 LCI microscope and processed with Leica LasX software. For each culture, one random image was obtained from each sample. Hardware settings for XY scanning mode on DMI8 fluorescent microscope were as follows: (1) Objective HC PL FLUOTAR 10x/0.30 Dry, numerical aperture of 0.3, the refraction index of 1, voxel size of 1.857 µm, (2) Objective HC PL FLUOTAR L 20×/0.40 Dry, the numerical aperture of 0.4, the refraction index of 1, voxel size of 0.929 µm. Fluorescence microscopy images exported as Tiff files from the LAS X program (Leica, Germany) were imported and analyzed with ImageJ 1.52e (NIH, USA). For cell counting, DAPI-positive cells were marked with "Find Maxima," where the noise was adjusted to mark each cell as a single point. Fluorescent staining of markers was represented in integrated density (per μm^2) or area in percentages.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 8 (La Jolla, USA) by using two-tailed unpaired students T-test and One-way ANOVA with uncorrected Fisher's LSD Test with ***=P <0.001, **=P <0.01, *=P <0.05 and ns=non-significant. No blinding was performed. All obtained data (n ≥3) were assessed for normality with the Shapiro–Wilk test (GraphPad Prism 8). The normality test resulted in p-values >α. Therefore, the null hypothesis was accepted, and we assumed that our data were normally distributed.

RESULTS

In vitro response of cultured pericytes against human MS serum

To evaluate the association of PDGFRβ+ cells to MS pathophysiology, we first intended to enlighten which BBB components in the CNS were mainly responsive to human MS sera and CSF. Since our in vivo experiments and previous studies point to fibrosis formation (1,4,5,10,13), we first studied fibrosis pathways. BBB components, including pericytes, astrocytes, and endothelial cells, were assessed for fibrotic response upon exposure to MS sera in vitro. Cultured HBVP were exposed to either pericyte medium (control), TGF-β1 (cytokine reference control), healthy human sera, human MS sera, or CSF for 72 hours. Subsequently, fixed HBVP cells were stained for PDGFRβ (pericyte marker), αSMA (smooth muscle cell marker), Collagen I, Collagen IV, Laminin and Fibronectin (ECM), and phalloidin (cytoskeletal structure), see Fig. 1a and Fig 2–3. Compared to the control (pericyte medium), the percentage of PDGFRβ+ area was slightly increased when exposed to healthy human serum (P=0.061; Fig. 1b). αSMA + area was found to have significantly increased upon exposure of pericytes to MS serum (P <0.0001; Fig. 1 c), but a significant decrease was observed for αSMA + area when exposed to MS CSF (P <0.05). Additionally, a significant increase in αSMA +/PDGFRβ+ area ratio (representing myofibroblastic transformation) was observed upon exposure to MS serum (P <0.05; Fig. 1 d). Besides, while Collagen IV was found to have significantly increased when pericytes were exposed to MS serum (P <0.0001; Fig. 1e, Fig. 2), Fibronectin had significantly increased in both TGF-β1 (P <0.05; Fig. 1f) and MS serum (P <0.0001) exposed pericytes. On the other hand, we did not find any significant difference in laminin expression upon incubation with MS sera, Fig. 3. Lastly, we assessed the mitogenic activity of MS serum on pericytes. The proliferation rate was measured with the xCelligence system. It was found to have increased significantly when pericytes were exposed to MS serum (Fig. 4). Our results showed that TGF-β1, a cytokine reference control induces fibrosis as fibronectin overexpression in pericyte cell culture. On the other hand, incubation with MS serum but not CSF resulted in a more robust fibrotic response (both fibronectin and collagen overexpression), myofibroblastic transformation as well as increased proliferation.

In vitro response of cultured astrocytes against human MS serum.

Cultured NHA (Fig. 1 g) were exposed to either astrocyte medium (control), TGFB1, healthy human serum, human MS serum, or CSF for 72 hours and stained for GFAP (astrocyte marker), Collagen IV or Fibronectin. Exposure to both healthy serum (P <0.01; Fig. 1h) and MS serum (P <0.01) showed significantly increased GFAP+ expression compared to the control. Collagen IV was significantly reduced when NHA was exposed to healthy serum (P <0.05; Fig. 1i). Compared to control, Fibronectin had decreased when NHA were exposed to healthy serum (P <0.001; Fig. 1j), MS serum (P <0.05) or CSF (P <0.05). Thus, although incubation with serum (healthy or MS) but not TGFB1 increased GFAP expression in astrocytes, collagen and fibronectin expressions had decreased.

In vitro response of cultured endothelial cells against human MS serum

Lastly, BECs were exposed to endothelial medium (control), TGF-β1, or MS serum and were stained for Collagen IV and Fibronectin (Fig. 1 k). Total Phalloidin+ had not significantly changed upon exposure of BEC to either TGF-β1 or MS serum (Fig. 1 l). A slight increase was observed for Collagen IV (P=0.098; Fig. 1 m) when BEC were exposed to MS serum, and Fibronectin had increased upon exposure to MS serum like in pericytes (P <0.05; Fig. 1n).

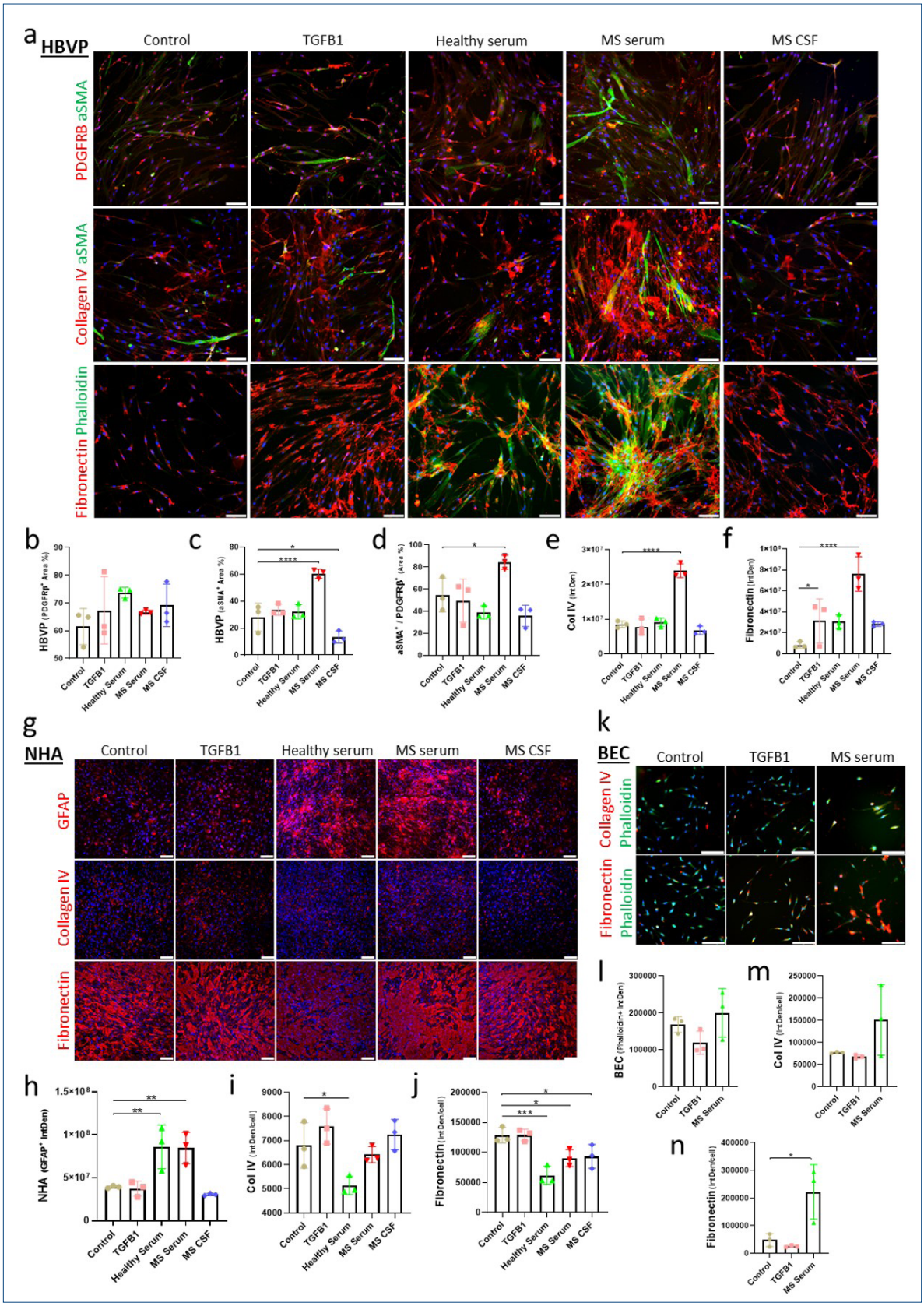


Figure 1. In vitro cellular response of cultured BBB components against human MS serum. Human brain vascular pericytes (HBVP) were exposed to either pericyte medium only (control), TGFB1, healthy human serum, human MS serum, or CSF for 72 hours. Immunofluorescence-stained images of HBVP (a), NHA (g), and BEC (k) and their representative expression analysis graphs are shown (b-f, h-j, l-n). a) Cultured HBVP (upper row) were stained for PDGFRB (pericyte marker, red) and aSMA (smooth muscle cell/myofibroblast marker, green), middle row: Collagen IV (ECM, red) and phalloidin (cytoskeletal structure, green). Cells were counterstained with DAPI for cell nuclei (blue). g) Cultured NHA were exposed to either astrocyte medium only (control), TGFB1, healthy human serum, human MS serum, or CSF for 72 hours and stained for GFAP (astrocyte marker, red), Collagen IV (red), or Fibronectin (red). k) BEC were exposed to only endothelial medium (control), TGFB1, or human MS serum and were stained for Collagen IV (upper row, red) and Fibronectin (bottom row, red). Cells were counterstained with DAPI for cell nuclei (blue) and phalloidin for cytoskeletal cell structure. All graphical data are shown as scatter plot diagrams with mean ± SD for n=3 independent cell cultures with sera and CSF from 3 individuals/patients. Statistical analysis was performed using one-way ANOVA with uncorrected Fisher's LSD test. Significance is shown with ****=P <0.0001, **=P <0.01 and *=P <0.05. Scale bars represent 250 µm.

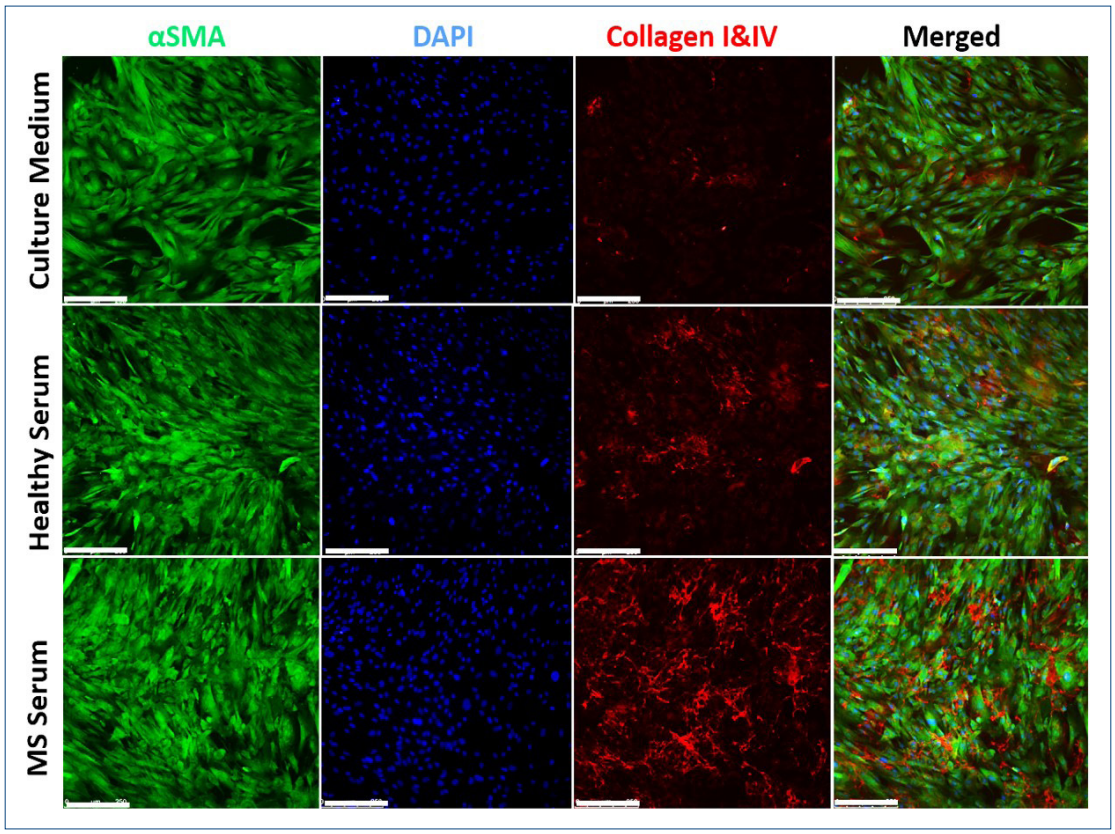


Figure 2. Increased Collagen production by pericytes in response to MS serum. In vitro cultured HBVP cells were exposed to either culture medium (control), healthy human serum, or MS patient serum (10%, v/v) in triplicate for 24 hours. Cells were stained for Collagen I & IV and merged with DAPI for cell nucleus. Increased Collagen deposits by pericytes were observed after exposure to MS serum. Scale bars represent 250 µm.

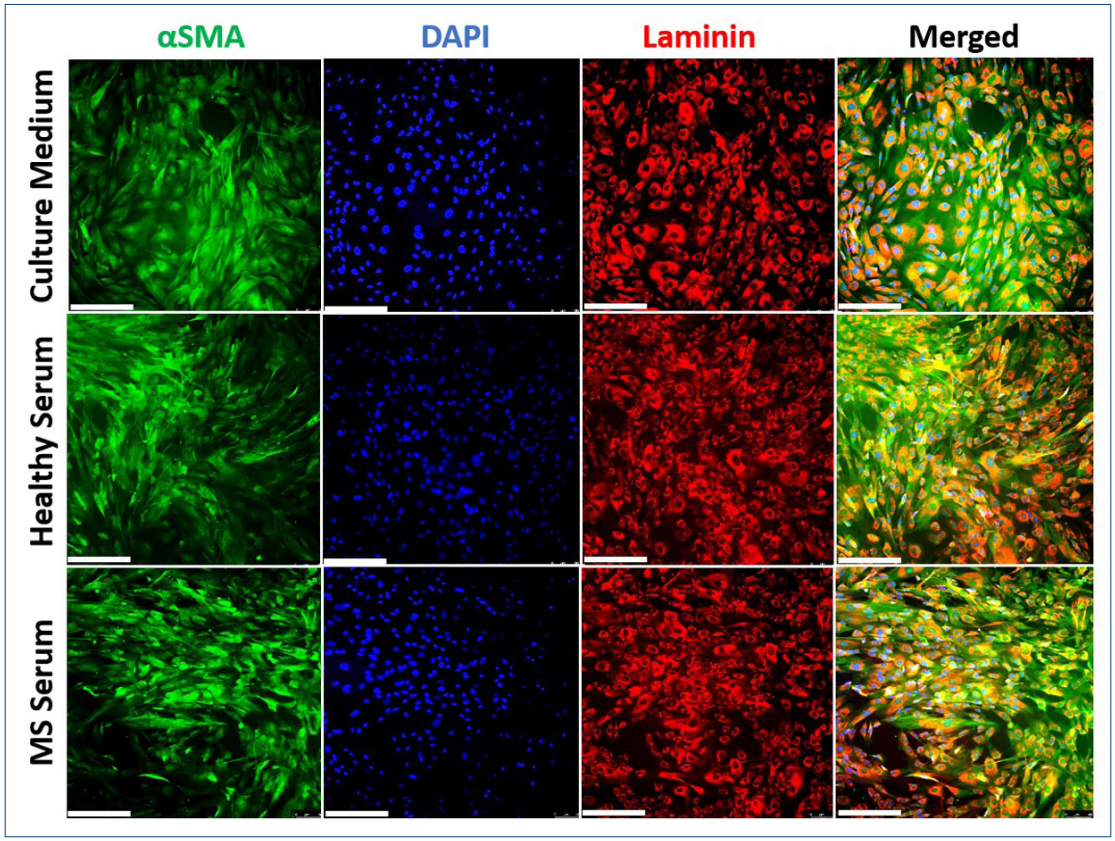


Figure 3. Laminin is not changed by pericytes in response to MS serum. In vitro cultured HBVP cells were exposed to either culture medium (control), healthy human serum, or MS patient serum (10%, v/v) in triplicate for 24 hours. Cells were stained for Laminin and merged with DAPI for cell nucleus. Compared to the control and healthy serum, no change in Laminin expression by pericytes was observed after exposure to MS serum. Scale bars represent 250 µm.

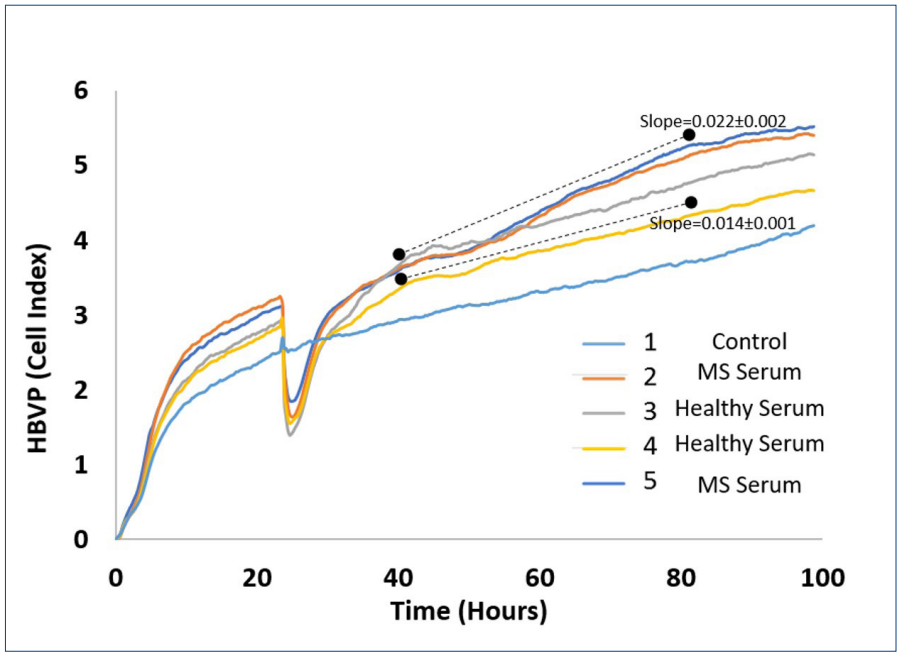


Figure 4. Increased pericyte proliferation upon exposure to MS serum. Human brain vascular pericytes were seeded in xCELLigence E-plates and, after 24 hours, were exposed to (10%, v/v) either culture medium (control), healthy human serum (n=2 individuals), or MS patient serum (n=2 individuals) for 100 hours. Each line was determined by the average of duplicate wells. Multiple sclerosis serum induced the proliferation rate of pericytes compared to culture medium or healthy serum. The proliferation rate was calculated by analyzing the slope between 40 and 80 hours, which was 0.022±0.002 cell index/hour (mean ± SD for n=2 patients) for MS serum and 0.014±0.001 per hour (mean ± SD for n=2 patients) for healthy serum. According to the data, it was revealed that pericytes, when exposed to MS serum (two-tailed unpaired T-test p-value: 0.032), proliferated significantly faster compared to healthy serum.

DISCUSSION

Perivascular spaces in MS lesions are shown to be occupied with Collagen IV and fibronectin deposits (12,21). The mechanisms underlying this perivascular fibrotic response are not fully elucidated yet. We explored this phenomenon by assessing the fibrotic response of BBB components (pericytes, astrocytes, and endothelial cells) upon exposure to MS conditions in vitro. Collagen I, Collagen IV, and Fibronectin are part of the basal laminae and matrix of vessel walls. They can also be produced and secreted as ECM by several cell types upon stimuli mediating fibrosis. We found that pericytes expressed the highest response for excessive secretion of Collagen I, Collagen IV, and Fibronectin upon exposure to human MS serum but remained unresponsive upon exposure to MS CSF, which indicated that fibrosis mediating factors arise from the blood circulation rather than the CSF side. Furthermore, endothelial cells presented only a small contribution to fibrosis in MS conditions. A well-established fibrotic cytokine, TGF-β1 induced fibronectin overexpression only in pericyte cell culture. Lastly, GFAP expression increased, but fibrotic response decreased in cultured astrocytes upon exposure to MS serum.

Several in-vitro MS models are reported, including single or co-cultures of brain cells, as well as brain slices and BBB models (22). In addition, novel protocols to produce induced pluripotent stem cells-derived oligodendrocytes from patients' somatic cells are promising to study individualized actions of drugs in vitro (23). Another individualized model was proposed by incubation of cultured brain slices or cells with sera, or IgGs derived from MS, NMO, and MOG patients or animal models (14–18). These in-vitro sera-induced models could be able to replicate pathological hallmarks of disease such as demyelination, BBB disruption, and loss of tight junction proteins. Moreover, the current study demonstrated that MS-sera triggered a fibrotic response in pericyte cell culture. To our knowledge, the fibrotic response of BBB components has not been studied in-vitro MS sera-induced model before.

Recent studies clearly demonstrated that the origin of fibrosis in the CNS was not exclusively stemming from meningeal rupture and infiltration of extra-CNS fibroblasts, as historically believed (2). In fact, the fibrotic response is generated from perivascular cells from the perivascular niche. Moreover, elegant genetic lineage tracing studies proved the contribution of a specific type of pericytes (type A pericytes) and perivascular fibroblasts to fibrosis (1,8–10). There is an ongoing debate on the nomenclature of the perivascular cells contributing to fibrosis in literature. Both cell types express PDGFRβ and CD13, however, perivascular fibroblasts are negative for αSMA and NG2 (2). We named HBVP as pericytes rather than fibroblasts throughout this study. Thus, the HBVP that we used were characterized based on their PDGFRβ, αSMA, and NG2 expressions that preexisting fibroblasts are known to lack the former two markers.

Upon exposure to traumatic or inflammatory insult, pericytes may change their phenotype to myofibroblasts which are αSMA-expressing fibroblast-like cells (24,25). Myofibroblasts are involved in fibrotic response via secreting ECM elements such as collagens and fibronectin, as shown in this study (26–28). Their localization changes during transformation; they are detached from vessels and migrate to the injury sides (8). Moreover, their expression profiles also changed; they lost CD13 expression and began expressing Fibronectin and temporarily myofibroblast marker αSMA while continuing to express PDGFRβ (29).

We examined myofibroblastic transformation by calculating the ratio of αSMA to PDGFRβ expression profiles and found that only incubation with MS serum induced significant myofibroblastic transformation that was not detected with TGF-β1 incubation. Moreover, TGF-β1 induced only fibronectin overexpression but MS serum induced both collagen IV and fibronectin. This is noteworthy because TGF-β1 is a well-known trigger of CNS fibrosis (2), and TGF-β1 exposure leads to the accumulation of Collagen 1 in pericyte cell culture (6). A serum study did not report a significant change in levels of TGF-β1 between control and MS patients (30). Given the unresponsiveness of pericytes to healthy serum, the contribution of other factor (s) rather than TGF-β1 signaling should be involved in the robust fibrotic response of pericytes to MS serum since TGF-β1 levels are similar between MS and healthy serum. In parallel, a recent study demonstrated that interferon-gamma (IFN-γ) pathway genes regulate fibrotic scar formation (10). In our study endothelial cells also seem to contribute to the fibrotic response triggered by MS serum but not TGF-β1. Our result aligns with a previous report that showed excessive fibronectin expression from cerebral vascular endothelial cells in an in-vivo experiment (31).

In astrocyte culture, astrocytic GFAP expression significantly increased, and fibrotic response significantly decreased with healthy or MS serum incubation in this study. This may seem contradictory, however, activated astrocytes are shown to secrete matrix-metalloproteases in-vitro, so accumulated ECM elements could be degraded in serum-activated astrocyte culture (32). Hence, we did not observe the same reaction with TGF-β1 exposure; again, MS-serum-induced effects must stem from a factor other than TGF-β1-dependent mechanisms.

We proved that exposure to MS serum leads to proliferation in pericyte cell culture up to 3 days of follow-up in the xCELLigence system as well as an increased signal of cell markers in immunofluorescence studies. Noteworthy, the cell numbers had decreased in the first hours in both health and MS sera-treated wells but reached and passed beyond the control wells. In line with this observation, a previous study showed that exposure to MS serum decreased cell survival and led to early apoptosis in the first 24 hours (17).

In this study, MS patients' CSF did not trigger a proliferative, transformative, or fibrotic response in pericyte and astrocyte cell cultures. One possible explanation is that CSF contains large quantities of proteins, including growth factors during development but trace levels in adults (33). In line with this knowledge, studies assessing the toxic effects of CSF in motor neurons reported more robust results with higher concentrations of CSF (34). In addition, TGFB1 expression is decreased in CSF at the attack phase of MS compared to remission (30). Also, one study pointed out the strong connection between serum components of peripheral circulation and the CNS suggesting the significant contribution of sera (35).

Our study has certain limitations; we showed that incubation with MS serum leads to a more robust fibrotic response than a cytokine reference control (TGF-β1). However, many other inflammatory cytokines, including IFN-γ, may contribute to fibrosis. We also incubated cultured pericytes and astrocytes with sera and CSF at the same ratio, which is arbitrary and has limited physiological relevance. But it should be noted that it is a highly replicated dosage in previous CSF cell culture studies (34).

Our results indicate a tendency in pericytes to transform into a myofibroblastic type and produce ECM elements when stimulated with sera from patients diagnosed with MS. Further studies must enlighten which factor (s) or interaction of them (e.g., cytokines, immunoglobulins, growth factors, etc.) are causing this phenomenon.

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Ethics Committee Approval: To collect and use human serum and CSF materials, the ethical approval was obtained from the local ethics committee "Institutional Review Board (IRB) of Koç University Ethical Committee" with approval number: 2016.123. IRB2.077 and consent to participate were documented according to IRB guidelines.

Informed Consent: Informed consents were obtained from patients to examine their blood samples for research purposes.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept- EŞK, AV, ÖÖÇ, ET, CİK; Design- NS, CU, SS, EÖ, EAU; Supervision- YGÖ; Resource- YGÖ, CU, ET, CİK; Materials- AV, ÖÖÇ, ET, CİK; Data Collection and/or Processing- EŞK, AV, ÖÖÇ, AT, CİK; Analysis and/or Interpretation- EŞK; Literature Search- EŞK, CU; Writing- EŞK; Critical Reviews- YGÖ.

Conflict of Interest: The authors declared that there is no conflict of interest.

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