Osteopontin expression and the effect of anti-VLA-4 mAb treatment in experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis

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Folia Neuropathol 2023; 61 (2): 129-137 DOI: https://doi.org/10.5114/fn.2023.129180

Abstract
Introduction: Osteopontin (OPN) is involved in the pathogenesis of multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE). The aim of this study was to investigate the expression of OPN in spinal cords of mice in the successive phases of EAE, to compare it with the density of inflammatory cells, oligodendrocytes and with the expression of interleukin (IL)-17A and to assess the effect of anti-α4β1 integrin (VLA-4) treatment.

Material and methods: Experimental autoimmune encephalomyelitis (EAE) mice were injected with anti-VLA-4 antibodies or, as treatment control, with immunoglobulin G (IgG). Spinal cords were sectioned and immunostained for OPN, CD45 (overall leukocytes), CD3 (T cells), Iba1 (activated macrophages/microglia), IL-17A, and CNP1 (oligodendrocytes). Microscopic images were analysed and the percentage of immunopositive areas encompassing the whole spinal cord cross-sectional area were assessed in images for each antigen.

Results: Osteopontin was expressed by inflammatory cells and by a minority of neurons and blood vessels. Most of the studied parameters followed the temporal pattern of clinical scores: increase in the peak phase and decrease in the chronic phase. Only OPN and IL-17A remained at a high level in the chronic phase, while CNP1 expression gradually decreased in the successive phases. Anti-VLA-4 treatment lowered the expression of the studied antigens in the peak and chronic phases with the exception of oligodendrocyte marker CNP1 which in both phases showed an increased expression.

Conclusions: Involvement of OPN is particularly significant in advanced EAE. Anti-VLA-4 treatment not only inhibits migration of myelin-reactive T cells, but also downregulates OPN and inhibits loss of oligodendrocytes.

Key words: osteopontin, experimental autoimmune encephalomyelitis (EAE), multiple sclerosis, inflammation, oligodendrocytes.

Introduction
Multiple sclerosis (MS) is a debilitating disease of the central nervous system (CNS) characterized by multi-focal inflammation, demyelination, and neuronal damage. The mouse model of MS, experimental autoimmune encephalomyelitis (EAE) which mimics its clinical, immunological, and histopathological features is commonly used for the investigation of neuroprotective agents with potential use against MS [7]. Animal and human studies have shown that infiltrating T cells, macrophages and inflammatory cytokines are critical for disease development and progression. T cell infiltration and proinflammatory cytokine production by the infiltrating cells are pathogenic for the development of EAE [11].

Osteopontin (OPN) is a multifunctional protein involved in bone mineralization, cell adhesion, cell migra-
tion and chronic inflammation; its level has been shown to be correlated with autoimmune disease severity [12,35]. Osteopontin is of pathogenic importance in inflammatory diseases of the CNS such as MS and its animal model, EAE. It has intracellular and secreted (extracellular) isoforms, the former expressed mainly by activated macrophages, leukocytes and activated T lymphocytes and shows chemokinetic, cytokine and integrin properties [3,35]. Upregulated OPN promotes MS/EAE, enhances disease severity by reducing apoptosis of effector T lymphocytes and by increasing their survival [3,8,14] and plays a supporting role in the chronic disease [6,30]. Furthermore, OPN induces IL-17 production by Th17 cells via specific OPN receptors [21]. These cells, key players in MS/EAE, are highly proinflammatory and induce severe autoimmunity [2].

EAE induced in IL-17 deficient mice exhibited delayed onset, reduced maximum severity scores, ameliorated histological changes and early recovery [17]. Neutralization of IL-17 with a monoclonal antibody also ameliorated the EAE course and clinical symptoms [13]. OPN expression in the spinal cord was shown to be upregulated during EAE [4] and OPN deficient mice showed attenuated clinical manifestations of the disease [15]. Furthermore, anti-OPN treatment reduced clinical severity of EAE by reducing IL-17 expression [21].

VLA-4 (α4β1 integrin) is an adhesion molecule crucial for T cell migration across the blood brain barrier and their recruitment into MS lesions [27]. Interaction of OPN with VLA-4 plays an important role in the pathogenesis of MS/EAE [3,32,35]. VLA-4 is expressed in different leukocytes and OPN as well as other adhesion molecules (VCAM-1 and fibronectin) are its cognate ligands [16]. Natalizumab (anti-VLA-4 mAb), a monoclonal antibody that blocks α4β1 integrin, suppresses the symptoms of MS/EAE by inhibiting extravasation of myelin-reactive T cells, their migration to the target tissue and thereby limiting the associated inflammation [9].

Counteracting the formation and/or maintenance of such a deleterious cell adhesion system promoting inflammatory responses characteristic of MS and EAE should be beneficial for patients suffering from chronic inflammatory diseases. In this context, we used spinal cord sections of mice to investigate by quantitative immunofluorescence the expression of OPN and we compared it with the density of inflammatory cells, oligodendrocytes and with the expression of IL-17A in the successive phases of EAE.

**Material and methods**

**Experimental animals**

Female C57BL/6 mice (n = 30, aged 10-11 weeks, weight 19-24 g) were purchased from the Centre of Experimental Medicine, Medical University of Bialystok and housed at 22 ±2°C, in 12-hour light/dark cycles and 55 ±10% humidity-controlled environment. Mice were supplied with standard food and water ad libitum (Animal House of the Jagiellonian Centre for Experimental Therapeutics, ICET, Krakow). All animal experiments were approved by the Local Ethics Committee of the Jagiellonian University Medical College, Krakow, Poland (Permissions 118/2015 and 274/2018). All studied animals were handled in compliance with Council Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

**MOG-induced and neurological evaluation of EAE mice**

A commercially available EAE induction kit (Hooke Laboratories Inc., Lawrence, Massachusetts, USA) was used following the supplier’s instructions. Briefly, naïve mice (n = 30) were immunized with MOG35-55 antigen dissolved in Complete Freund’s Adjuvant (CFA) supplemented with heat-inactivated Mycobacterium tuberculosis (H37Ra) on day 0. The MOG/CFA emulsion was administered subcutaneously at two sites, one on the flank and one behind the neck (100 µl each). Then, 3 h and 24 h after immunization, the immunized mice were intraperitoneally injected with 340 µl Bordetella pertussis toxin (PTx) dissolved in phosphate-buffered saline.

The symptoms of EAE were monitored and clinical scores were recorded daily for 30 days after disease induction, according to protocols provided by the Hooke Laboratories, as described in our previous studies [22-25]. Clinical severity of EAE was assessed using a 0-3 scale (Table I). During that time, body weight of mice was measured. A cumulative disease score was calculated as the sum of daily clinical scores of each mouse during the EAE monitoring period and reported as an average value within each group.

**Experimental groups**

All EAE mice were divided into two groups consisting of 15 mice. After first clinical manifestations of EAE, on postimmunization day 9, the first group mice were injected i.p. with 5 mg/kg of anti-VLA-4 monoclonal antibody (Natalizumab, Biogen Idec, Berkshire, UK) and the second group mice (treatment control) with 5 mg/kg of IgG (Sigma-Aldrich, St. Louis, MO, USA). The injections of anti-VLA-4 or IgG continued until the remission of symptoms appeared in both groups of mice (on days 12, 15, 18, 21) [18, 33].

No symptoms of EAE were observed on post-immunization days 0-8. In IgG-treated mice, the initial symp-
Immunohistochemistry

The following primary antibodies were used to analyse by immunofluorescence the degree of inflammatory infiltration: rat anti-CD45 (1 : 100, Thermo Fisher Scientific, Rockford, IL, USA), 0.01% sodium azide, 0.05% thimerosal, 0.5% Triton X-100, 0.1% bovine serum albumin and 2% dry milk. They were next incubated overnight at room temperature with primary antibodies and after a rinse in PBS incubated for 90 min with the secondary antibodies. DAPI staining (Thermo Fisher Scientific, Rockford, IL USA; 1.5 ug/ml; cat. #62248) was used to visualize cell nuclei. Sections were washed three times in PBS and mounted in glycerol/PBS solution.

Microscopy, morphometry and image collection

The spinal cord sections were examined using Olympus BX50 brightfield/epifluorescence microscope (Olympus, Tokyo, Japan). All low magnification images were recorded with the use of Olympus DP71 digital CCD camera, stored as TIFF files and processed for quantitative analysis using ImageJ software (NIH, Bethesda, Maryland, USA).

The number of inflammatory lesions/aggregates in white matter of the spinal cord was counted per cross section. Five different sections per animal were examined by a blinded observer and the average number of lesions per section was documented. The percentage of immunopositive areas encompassing the whole spinal cord cross-sectional area occupied by the grey and white matter were assessed in images for each antigen in the successive phases and the obtained values were compared between IgG and anti-VLA-4 treated mice. A total of at least 25 sections were analysed per experimental group (n = 5) and phase.

Table I. Clinical scoring of experimental autoimmune encephalomyelitis (EAE) mice

<table>
<thead>
<tr>
<th>Score</th>
<th>Clinical symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No symptoms</td>
</tr>
<tr>
<td>0.5</td>
<td>Limp tip of tail</td>
</tr>
<tr>
<td>1.0</td>
<td>Limp tail</td>
</tr>
<tr>
<td>1.5</td>
<td>Limp tail and hind leg inhibition</td>
</tr>
<tr>
<td>2.0</td>
<td>Limp tail and weakness of hind legs</td>
</tr>
<tr>
<td>2.5</td>
<td>Limp tail and dragging of hind legs</td>
</tr>
<tr>
<td>3.0</td>
<td>Limp tail and complete paralysis of hind legs</td>
</tr>
</tbody>
</table>

Statistical analysis

Statistical analysis was performed by using Prism 5 (version 5.0; GraphPad Software Inc., San Diego, CA). The results are presented as mean ± standard error of the mean (SEM). Statistical differences between groups with respect to mean cumulative disease, mean phase score and histopathology were evaluated by using the nonparametric Bonferroni multiple comparison test,
at the confidence values of 0.05 (**p < 0.001, *p < 0.01, *p < 0.05, ns – not significant).

Results

Treatment with anti-VLA-4 mAbs decreases EAE progression

Clinical EAE symptoms were evaluated for each mouse according to the score table provided by the protocols included in the Hooke Kits™ EAE Emulsion (Hooke Laboratories Inc.) (Table I). Treatment with anti-VLA-4 mAb effectively attenuated neurological symptoms of EAE compared to the IgG-treated group. In IgG-treated mice, the first symptoms of EAE appeared on day 9, in anti-VLA-4-treated ones the onset phase began later, on day 11 (Fig. 1A). During the development of progressive EAE, anti-VLA-4 mAb treatment reduced the mean clinical scores in all phases of the disease: in the onset phase from 1.04 ±0.05 to 0.45 ±0.02, in the peak phase from 3.13 ±0.16 to 1.03 ±0.05 and in the chronic phase from 1.47 ±0.07 to 0.95 ±0.05 (Fig. 1A, C). The cumulative score was significantly higher in IgG-treated mice compared to anti-VLA-4-treated mice (34.92 ±2.17 and 15.77 ±0.82, respectively (Fig. 1B).

Anti-VLA-4 mAb treatment significantly diminishes inflammatory lesions

Histopathologically expressed EAE severity was assessed by examination of focal lesions that show inflammatory cell infiltration (mainly leukocytes and macrophages) in spinal cord sections.

Fig. 1. Study design and clinical course of experimental autoimmune encephalomyelitis (EAE) in mice. C57BL/6 mice were immunized by injection of MOG in CFA and PTx and monitored daily for clinical symptoms of EAE. The clinical scores of EAE were assessed in experiments in which immunized mice were treated with IgG (A, green points, treatment control) or anti-VLA-4 (A, red points) every third day for 12 days. The dashed lines in disease phases mark days of tissue collection (A). Histograms show the cumulative clinical scores for IgG and anti-VLA-4-treated mice (B) and mean scores in the onset, peak and chronic phases of EAE (C). Data are presented as means ±SEM; n = 5 per group. Statistical significance was verified using Bonferroni test at 0.05 confidence level (**p < 0.001, **p < 0.01, *p < 0.05, ns – not significant).
At disease onset, there was no significant difference in the number of lesions in IgG vs. anti-VLA-4-treated EAE mice (2.95 ±0.22 vs. 2.76 ±0.20, respectively; Fig. 2G). At the peak stage, the number of lesions increased significantly in both, IgG (to 9.49 ±0.31) and anti-VLA-4 groups (to 7.03 ±0.28), but the values were significantly lower in anti-VLA-4-treated mice. In the chronic phase, both groups of mice showed reduced lesion number (to 7.21 ±0.20 and 6.34 ±0.21) and the difference between the groups was weaker but remained significant.

In order to quantify leukocytes, we assessed the overall expression of CD45 in entire spinal cord sections, as described in our previous studies [22,24]. Briefly, not only CD45 but also CD3 and Iba1 antibodies were used to verify if anti-VLA-4 mAb treatment affected inflammatory infiltrates and the immunos- tained area was quantified by a morphometric analysis (Fig. 2H-J). In addition, CD45, CD3 and Iba1 were used to demonstrate the sources of OPN expression. In all phases of the disease, leukocytes (CD45, CD3) formed local aggregates with high cell density, indicative of inflammatory lesions, mainly located in white matter of the spinal cords (Fig. 2A-D). Macrophages/microglia cells were loosely scattered, mainly in the white matter, but also in the grey matter of the spinal cord (Fig. 2E, F). Iba1 did not show colocalization with CD45 (Fig. 2F).

The degree of inflammation manifested by the number/density of inflammatory cells was expressed as percentage of immunopositive section surface area in both IgG and anti-VLA-4 groups (Fig. 2H-J). Expression of antigens associated with infiltrating inflammatory cells is presented in Table II. In case of all antigens, it shows the same temporal pattern: a sharp increase from onset to peak phase and then a decrease to values higher than those of the onset phase (Fig. 2H-J). Anti-VLA-4 treatment generally lowered expression of antigens characteristic of inflammatory cells, although in Iba1 positive cells this effect was not observed in the onset phase (Fig. 2J).

Expression of OPN during EAE progression

Expression of OPN was mostly manifested in a small fraction of inflammatory cells (Fig. 3A) and it also showed extracellular localization (Fig. 3A). In IgG-treated mice, OPN expression gradually increased in the successive phases (from 0.65 ±0.03% to 5.55 ±0.20% and 6.02 ±0.10%, respectively), hence maximal expression occurred in the chronic phase in both EAE groups. Anti-VLA-4 mAb treatment reduced OPN expression in all phases of EAE (to 0.57 ±0.03%, 1.71 ±0.08% and 1.51 ±0.04%, Fig. 3E).

| Table II. Expression of antigens [%] associated with infiltrating inflammatory cells in IgG and anti-VLA-4-treated groups |
|-----------------|-------|-------|-------|
| CD45 (%)        | Onset | Peak  | Chronic |
| IgG             | 3.45 ±0.13 | 13.35 ±0.37 | 6.87 ±0.19 |
| anti-VLA-4      | 1.32 ±0.11 | 4.06 ±0.15 | 3.03 ±0.19 |
| CD3 (%)         | Onset | Peak  | Chronic |
| IgG             | 1.15 ±0.14 | 9.80 ±0.24 | 6.32 ±0.16 |
| anti-VLA-4      | 0.71 ±0.10 | 1.85 ±0.13 | 1.57 ±0.17 |
| Iba1 (%)        | Onset | Peak  | Chronic |
| IgG             | 0.70 ±0.11 | 7.32 ±0.42 | 6.22 ±0.42 |
| anti-VLA-4      | 0.74 ±0.04 | 2.62 ±0.13 | 2.54 ±0.15 |

Expression of IL-17A during EAE progression

Expression of IL-17A was detected in some infiltrating inflammatory cells (Fig. 3D). In IgG-treated mice, IL-17A expression increased significantly to the peak phase and then remained at a similar level (from 1.35 ±0.10% to 7.08 ±0.09% and 6.87 ±0.17%, respectively). Anti-VLA-4 treatment reduced IL-17A expression in all phases of EAE (to 1.04 ±0.08%, 2.95 ±0.21% and 1.28 ±0.09%, respectively; Fig. 3G).

Expression of CNP1 during EAE progression

In IgG-treated mice, expression of CNP1, an antigen associated with oligodendrocytes, was the highest in the onset phase and gradually decreased in the successive phases. Anti-VLA treatment showed no effect in the onset phase and significantly elevated CNP1 expression in the next phases. In result, the anti-VLA group showed a significant difference in CNP1 expression only between onset and chronic phases (7.74 ±0.18% and 6.83 ±0.16%, respectively; Fig. 3E, H).

Discussion

In connective tissues, OPN is expressed by cells and, as a secreted form, is an important component of the extracellular matrix [10]. However, the extracellular matrix of the central nervous system (CNS) consists only of chondroitin sulfate proteoglycans and tenascins which form perineuronal nets [26]. Hence, in the spinal cord OPN is localized exclusively in cells.

In this study, expression of OPN was observed in inflammatory cells, in neuralgial cells and in blood vessels of the spinal cord, what corresponded to OPN location in other neurodegenerative processes [29]. However, OPN was expressed only by a minority of glial cells and blood vessels. We also found OPN immunoreactive...
Fig. 2. CD45, CD3 and Iba1 immunostaining of spinal cord sections showing representative inflammatory lesions in the peak phase of experimental autoimmune encephalomyelitis (EAE) in IgG-treated mice (A, B, D, E), its colocalization (yellow fluorescence, C) and quantitative results in the successive phases of EAE in IgG- and anti-VLA-4-treated mice. Anti-VLA-4 treatment attenuated the number of lesions (G), the area of inflammatory cells (CD45, CD3, Iba1) compared to IgG treatment (H-J). Percentage values in (H-J) concern the cross-sectioned spinal cord surface area occupied by immunofluorescent structures. Data are presented as means ±SEM; n = 5 per group. Statistical significance was verified using Bonferroni test at 0.05 confidence level (***(p < 0.001, **(p < 0.01, *p < 0.05; ns – not significant). Magnification is indicated by scale bars (A-F = 100 µm).
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Fig. 3. Immunohistochemical localization of osteopontin (OPN; A), IL-17A (D), oligodendrocytes (CNP1; E), their overlapping fluorescence with DAPI in spinal cords of IgG-treated mice in the peak phase of experimental autoimmune encephalomyelitis (EAE; white dashed lines mark the border between white and grey matter) and quantitative results in the successive phases of EAE in IgG- and anti-VLA-4-treated mice (F-H). OPN, IL-17A and CNP1 are expressed by cells (A, D, E; insets). OPN expression was detected mainly in leukocytes (A), in perikaryons of some nerve cells in the grey matter (B) and in capillaries located mainly in the grey matter of the spinal cord (C). Percentage values concern the cross-sectioned spinal cord surface area occupied by immunofluorescent structures. Data are presented as means ±SEM; n = 5 per group. Statistical significance was verified using Bonferroni test at 0.05 confidence level (**p < 0.001; *p < 0.01; *p < 0.05, ns – not significant). Magnification is indicated by scale bars (A, D, E = 100 µm, insets = 50 µm, B, C = 20 µm).
nerve cells in the anterior horns and OPN was postulated to be a marker of alpha motor neurons [20].

The progression of clinical symptoms in the course of EAE allows to distinguish three successive phases of the disease: onset, peak and chronic characterized by an increase in the clinical scores during the first two phases and a decrease in the chronic phase. In IgG-treated mice, most indicators of inflammation (number of inflammatory lesions, overall leukocyte density, density of T cells and activated macrophages) followed that temporal pattern, although expression of OPN and IL-17A, a key cytokine in the inflammatory processes, did not decrease in the chronic phase. This suggests their intensified production by the cells and a significant role of OPN in the advanced stage of the disease. It corresponds with the reports showing that OPN stimulates IL-17A production in EAE [21]. Vaccination with OPN inducing anti-OPN antibodies during EAE decreased disease severity and this effect was correlated with decreased secretion of IL-17 by T cells [5]. OPN can promote relapses of SM/EAE by providing a survival signal to autoreactive T cells and may be a critical factor which influences the transition from relapsing remitting to secondary progressive demyelinating disease [31]. On the other hand, it is not clear whether OPN, in part through non-immune effects, is also involved in remyelination and depending on circumstances can exhibit a neuroprotective potential [3,28].

Osteopontin can interact with VLA-4 and OPN-VLA-4 binding affects cells involved in the inflammatory response and tissue remodelling [1]. Its expression is increased in MS and EAE plaques [3,4] and we found OPN expression in spinal cord sections in all phases of EAE.

The density of oligodendrocytes decreased during the successive phases of EAE, as also observed in our earlier study [24] and oligodendrocyte loss promotes demyelination characteristic of EAE by weakening remyelination of the spinal cord. In EAE, newly generated oligodendrocytes are mainly responsible for remyelination [34,36]. In this context, upregulation of OPN can play a protective role in neurodegenerative diseases, as it inhibits cell death by reduction of apoptosis and induction of anti-apoptotic factors and enhances oligodendrocyte progenitor cell viability [19].

Anti-VLA-4 treatment delayed the onset phase of EAE and persisted during continued treatment. It significantly suppressed clinical severity of the disease and all studied histological parameters of inflammation. Our previous studies showed a similar effect of anti-VLA-4 treatment [22,23]. In the present study, the only exception was expression of CNP1, a marker of oligodendrocytes: its expression increased. This finding seems to uncover another effect of that treatment: anti-VLA-4 mAbs inhibit loss of oligodendrocytes. The mechanism is not clear, it might include suppression of proapoptotic signals and/or inhibitory influence on activated macrophages/microglia which participate in elimination of oligodendrocytes.

In summary, our study shows that anti-VLA-4 mAbs not only inhibit α4β1 integrin-dependent transmigration of immune cells into CNS, but also downregulate OPN and IL-17A. Moreover, it indicates interaction of α4 integrin and OPN as an important mechanism of inflammation regulation. As OPN and IL-17 are known proinflammatory cytokines critical to the inflammatory processes in MS/EAE and other autoimmune conditions, reduction of OPN and IL-17 in leukocytes induced by anti-VLA-4 mAbs can be clinically relevant in the treatment of MS/EAE.

Funding

The study was supported by statutory grant N41/ DBS/000950 from the Jagiellonian University Medical College to GPF.

Disclosure

The authors report no conflict of interest.

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