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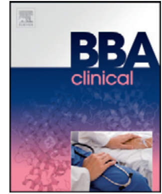

# Increased cerebrospinal fluid osteopontin levels and its involvement in macrophage infiltration in neuromyelitis optica

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ABSTRACT

*Background:* Neuromyelitis optica (NMO) is an inflammatory disease of the central nervous system that predominantly affects the optic nerves and spinal cord. Although NMO has long been considered a subtype of multiple sclerosis (MS), the effects of interferon-β treatment are different between NMO and MS. Recent findings of NMO-IgG suggest that NMO could be a distinct disease rather than a subtype of MS. However, the underlying molecular mechanism of NMO pathology remains poorly understood.
*Methods:* OPN in the cerebrospinal fluid and brain of patients with NMO and with MS, as well as of patients with other neurologic disease/idiopathic other neurologic disease was examined using Western blotting, ELISA, immunohistochemistry and Boyden chamber.
*Results:* Here we show that osteopontin is significantly increased in the cerebrospinal fluid of NMO patients compared with MS patients. Immunohistochemical analyses revealed that osteopontin was markedly elevated in the cerebral white matter of NMO patients and produced by astrocytes, neurons, and oligodendroglia as well as infiltrating macrophages. We also demonstrate that the interaction of the cerebrospinal fluid osteopontin in NMO patients with integrin αvβ3 promoted macrophage chemotaxis by activating phosphoinositide 3-kinase and MEK1/2 signaling pathways.
*Conclusion:* These results indicate that osteopontin is involved in NMO pathology.
*General significance:* Thus therapeutic strategies that target osteopontin signaling may be useful to treat NMO.
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## 1. Introduction

Neuromyelitis optica (NMO) is an inflammatory demyelinating disease of the central nervous system (CNS) that primarily affects the optic nerves and spinal cord leading to paralysis and blindness [28]. NMO has long been considered a subtype of multiple sclerosis (MS), which is characterized by myelin breakdown, oligodendrocyte loss, and axonal damage [8,12]. However, the attacks of NMO are usually more severe than those of MS, and spinal cord magnetic resonance imaging (MRI) can aid in the diagnosis [28]. The recent discovery of NMO-IgG in the serum of a high percentage of NMO patients (~75%) that are usually absent in the conventional form of MS, and the subsequent identification of its target antigen, aquaporin-4 (AQP4), which is the most abundant water channel at the astrocyte endfeet in CNS, provided strong evidence that NMO was distinct from MS [17]. The NMO phenotype can be attributed to NMO-IgG and complement attacking astrocytes in the nerve and spinal cord. In demyelinated active lesions of NMO patients, extensive macrophage infiltration associated with large numbers of perivascular granulocytes and eosinophils and rare T cells was observed [10,18]. While it remains unclear how these inflammatory or immune cells are involved in NMO pathogenesis, these findings indicate that the most appropriate treatment may differ for NMO and MS. In fact, several case reports have shown the contrasting effects of interferon (IFN)-β treatment; in NMO, IFN-β induces severe relapses and exacerbations, although it is the most commonly prescribed treatment for relapsing

*Abbreviations:* Ab, antibody; Abs, antibodies; AQP4, aquaporin-4; CNS, central nervous system; CSF, cerebrospinal fluid; EAE, experimental autoimmune encephalomyelitis; GFAP, glial fibrillary acidic protein; IFN, interferon; mAb, monoclonal antibody; MS, multiple sclerosis; NMO, neuromyelitis optica; OPN, osteopontin; pAb, polyclonal antibody; PI3K, phosphoinositide 3-kinase; RGD, Arg–Gly–Asp; RGE, Arg–Gly–Glu; TRAP, tartrate-resistant form of acid phosphatase.

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remitting MS [1,21,26,27]. It is therefore crucial to distinguish these two disorders to select optimal treatment.

Osteopontin (OPN) is involved in various physiological and pathological events, including mineralization, acute and chronic inflammation, and cancer metastasis [15,22,23]. OPN is believed to play an important role in several autoimmune diseases such as MS [6], rheumatoid arthritis [30], systemic lupus erythematosus [13], and asthma [29]. In relapsing remitting MS, increased OPN protein levels in the plasma but not in the cerebrospinal fluid (CSF) are associated with disease activity [24,25]. It has also been reported that OPN concentrations are increased in CSF during attacks of MS [4]. OPN expression is elevated in the CNS lesions of experimental autoimmune encephalomyelitis (EAE) during the acute phase but not during remission, and OPN plays a critical role in EAE progression [6].

Osteopontin (OPN) is a multiphosphorylated extracellular glycoprotein. The molecular weight of OPN on SDS-PAGE varies from 45 to 75 kDa due to glycosylation and phosphorylation as well as having a highly negative charge resulting from the predominantly acidic amino acid composition [15]. OPN contains an Arg–Gly–Asp (RGD) sequence common to many extracellular matrix proteins, which mediate the association of OPN with multiple integrins such as $\alpha5\beta1$, $\alpha v\beta1$, and $\alpha v\beta3$ [14,15,23]. Another important receptor for OPN is CD44, and thrombin-cleaved carboxyl-terminal OPN fragments bind to CD44 variants in an RGD-independent manner [3]. The binding of OPN to these cell surface receptors induces cellular signaling pathways such as phosphoinositide 3-kinase (PI3K), MAPK, and JNK to regulate various cell functions, including adhesion, migration, chemotaxis, and proliferation in various types of cells [3,14,23].

In this study, we found that OPN was significantly increased in CSF of patients with NMO compared with patients with MS or other diseases. Immunohistochemical analyses using anti-OPN antibody (Ab) showed the aberrant expression of OPN in the cerebral white matter of the NMO patients. Of note, OPN in CSF of NMO patients was found to be a primary protein that promptly induced macrophage chemotaxis.

## 2. Materials and methods

### 2.1. Compliance with ethical standards for human subject research

Study approval (number 613) was granted after protocol review by the Ethics Committee of Fukushima Medical University, which is guided by local policy, national law, and the World Medical Association Declaration of Helsinki.

### 2.2. CSF, serum, and plasma samples

CSF samples were obtained at the time of relapse from anti-AQP4 Ab-positive NMO patients (n = 19; all females; mean age, 47.4 ± 14.7 years), and MS patients (n = 19; one male and 18 females; mean age, 34.4 ± 10.8 years). The mean times from symptom onset to lumbar puncture were 5.5 days (range: 0–17 days) for NMO and 13.4 days (range: 2–56 days) for MS. The mean disease durations were 5.2 ± 10.8 years (range: 25 days–46 years) for NMO and 4.6 ± 8.0 years (range: 19 days–36 years) for MS. Fourteen patients with other neurologic disease/idiopathic other neurologic disease (OND/IOND) (1 sacroiliac joint dysfunction; 1 Parkinson's disease; 1 cervical spondylosis; 1 spinocerebellar ataxia type 6; 1 multiple system atrophy-P; 1 sarcoidosis; 1 HTLV-1-associated myelopathy; 1 subterranean clover stunt disease; and 3 NMDAR Ab-negative and 3 NMDAR Ab-positive limbic encephalitis; two males and 12 females: mean age, 47.7 ± 19.1 years) served as disease controls. The mean disease duration was 4.5 ± 6.7 years (range: 26 days–23 years) for the control group. The age at onset was higher in NMO than in MS ($p < 0.05$) while in control it did not differ significantly in NMO (NMO *vs.* control) and in MS (MS *vs.* control). The disease durations did not differ significantly among the three. After obtaining informed consent from the patients, the CSF samples were obtained by lumbar puncture and then centrifuged to remove cells and other insoluble materials. The cell-free CSF supernatant was aliquoted in tubes and stored at −80 °C until use. Blood samples collected from patients were either heparinized and directly centrifuged at 1000 *g* for 10 min at room temperature to obtain plasma or allowed to clot at 4 °C overnight before centrifugation, after which serum was transferred and stored at −80 °C until use.

### 2.3. Abs and reagents

Rabbit polyclonal Abs (pAbs) were used with the following specificities: against human OPN (O-17; IBL, Cat# 18625), and against Akt (Cell Signaling, Cat# 9272). Mouse monoclonal Abs (mAbs) were used with the following specificities: against OPN (clone 53; Assay Designs, Cat# 905–629 or clone 10A16; IBL, Cat# 10011), against integrin $\alpha v\beta3$ (clone LM609; Millipore, Cat# MAB1976), against phospho-p44/42 MAPK (clone E10; Cell Signaling, Cat# 9106S), against CNPase (clone 11-5B; GeneTex, Cat# GTX72341), and against ERK1 (clone MK12; Cat# 610031), JNK/SAPK1 (clone 37/pan-JNK/SAPK1; Cat# 610627), phospho-JNK/SAPK [pT183/pY185, clone 41/JNK/SAPK (pT183/pY185); Cat# 612540], p38α (clone 27/p38α/SAPK2a; Cat# 612168), and phospho-p38 MAPK [pT180/pY182, clone 30/p38 MAPK (pT180/pY182); Cat# 612280] from BD Transduction laboratories. A rat mAb against CD44 (clone Hermes-1; Cat# MA4400) was from Thermo Scientific. Rabbit mAbs against phospho-Akt1 (pS473, clone EP2109Y; Cat #2118-1), and against glial fibrillary acidic protein (GFAP; clone EPR1034Y; Cat #2301-1) were from Epitomics. The control rat (Cat# sc-2026), and mouse (Cat# sc-2025) IgGs, and a mouse mAb against integrin $\alpha v\beta3$ (clone 23C6; Cat# sc-7312) for FACS analysis were obtained from Santa Cruz Biotechnology, Inc.; Alexa Fluor 488-conjugated goat anti-rabbit IgG (Cat# A11008) and Alexa Fluor 546-conjugated goat anti-mouse IgG (Cat# A11030) were purchased from Life Technologies. The PI3K inhibitor LY294002 (Cat# 70920) was from Cayman Chemical, and the MEK1/2 inhibitor U0126 (Cat# 662005), the JNK inhibitor SP600125 (Cat# 420119), and the p38MAPK inhibitor SB203580 (Cat# 559389) were from Calbiochem. GRGDSP (Cat# SP001) and GRGESP (Cat# SP002) peptides were from Takara Bio Inc., and calf intestine alkaline phosphatase (CIAP; Cat# CAP-101) was purchased from Toyobo. Recombinant OPN was purified from human wild-type OPN overexpressing 293T cells as described previously [14].

### 2.4. SDS-PAGE and Western blot

SDS-PAGE was performed on 5–20% SuperSep™ Ace gradient gels (Wako, Cat# 194-15021) under reducing conditions. For Western blot analyses, proteins resolved by SDS-PAGE were transferred to nitrocellulose membranes. The blots were probed with each specific Ab. Immunoreactive bands were detected using a SuperSignal West Dura Extended Duration Substrate kit (Thermo Scientific, Cat# 34075). The band intensity was calculated using NIH ImageJ software.

### 2.5. ELISA assay

OPN levels in CSF samples were measured using a human osteopontin ELISA kit (R&D systems, Cat# DOST00), according to the manufacturer's protocols.

### 2.6. Dephosphorylation of CSF and serum samples

CSF and serum samples were boiled for 5 min at 95 °C, followed by incubation for 1 h at 37 °C in the presence of 3 units of purified CIAP in 10 mM Tris–HCl (pH 8.0) buffer.

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