

## Increased expression of osteopontin in the spinal cords of Lewis rats with experimental autoimmune neuritis

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To investigate the pattern of expression of osteopontin (OPN) in tissues of the central nervous system (CNS) responding to peripheral immunological stimulation, the expression of OPN was studied in the spinal cord of rats with experimental autoimmune neuritis (EAN). In this model system, the sciatic nerves and spinal nerve roots are the target organs of EAN and the spinal cord is a remote organ that may be indirectly affected. OPN was constitutively expressed in some astrocytes adjacent to the pia mater and neurons in normal rats. In rats with EAN, OPN was increased in the same cells and in some inflammatory cells, including macrophages in the subarachnoid space. Expression of CD44, a receptor of OPN, was weak in normal spinal cord tissue and increased in the entire spinal cord parenchyma in rats with EAN, as well as in inflammatory cells. These findings suggest that inflammatory cells as well as reactive astrocytes are major sources of OPN and CD44 in the spinal cord of rats with EAN. Further study is needed to elucidate the functional role of OPN in the spinal cord affected by EAN.

**Key words:** Experimental autoimmune neuritis, osteopontin, CD44, spinal cord

### Introduction

Experimental autoimmune neuritis (EAN) is a T-cell-mediated autoimmune disease of the peripheral nervous system that is used as a model of human demyelinating diseases [17]. The clinical course of EAN is characterized by weight loss, ascending progressive paralysis, and spontaneous recovery. It has been proposed that inflammatory mediators produced in the affected spinal nerve roots and sciatic nerves are involved in the pathogenesis of EAN [22]. Although the major lesions of

EAN are seen in the spinal nerve roots and sciatic nerves of rats, the observed activation of microglia in the spinal cord indicates that the spinal cord is also affected [7,11].

Osteopontin (OPN) is an integrin- and calcium-binding phosphoprotein that is produced by mineralized tissue cells, many epithelial cells, and activated immune system cells [4]. OPN is known to be a pro-inflammatory mediator; its expression is increased in several pathological conditions, including spinal cord injury [9], Theiler's murine encephalomyelitis virus-induced demyelination [15], and experimental autoimmune encephalomyelitis [13]. In the spinal root avulsion model, two contradictory roles of OPN have been proposed [5]: that OPN functions as a pro-inflammatory mediator in the central nervous system (CNS), as has been shown in an autoimmune disease model [3], and that OPN is an intrinsic inhibitor of inflammation through the suppression of inducible nitric oxide synthase (iNOS), as has been shown in rheumatoid arthritis [1].

In the peripheral autoimmune disease model, some evidence indicates activation of cells in the spinal cord, the remote organ [11], which is distant from the target organs, the sciatic nerves. However, little is known about the expression of OPN protein in the spinal cord. In order to affect function in nearby cells, OPN requires an appropriate receptor such as CD44, a known OPN receptor [19,20]. The interactions of OPN with its receptors regulate macrophage migration and activation [19,20].

The aim of the present study was to elucidate the patterns of expression of OPN and its receptor, CD44, in the spinal cords of rats with EAN.

### Materials and Methods

#### Induction of EAN

Lewis rats were obtained from Harlan (Sprague Dawley, USA) and bred in our animal facility. Female rats, aged 7-12 weeks and weighing 160-200 g, were used. Each rat was injected in both hind feet with 200 µl of an emulsion containing equal parts of horse sciatic nerve in phosphate buffer (mg/ml) and complete Freund's adjuvant (CFA; Mycobacterium tuberculosis H37Ra, 5 mg/ml; Difco, USA).

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Each rat was treated with 50 ng of pertussis toxin (Sigma, USA) on days 0 and 2 after immunization. Immunized rats were observed daily for clinical signs of EAN. The progression of EAN was categorized into seven clinical stages, as follows. Grade (G) 0, no clinical signs; G1, floppy tail; G2, mild paraparesis; G3, severe paraparesis; G4, tetraparesis; G5, moribund condition or death; R0, recovery. Control rats were immunized with CFA only. Five rats were sacrificed under deep anesthesia at selected stages of EAN. Experiments were carried out in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

### Tissue sampling

To study OPN expression in animals with EAN, spinal cord tissue and sciatic nerves were sampled during paraparesis (day 14–18 post-immunization) and during recovery from paraparesis (after day 21 post-immunization). Spinal cords from CFA-immunized control rats were obtained at day 17 post-immunization. Samples of spinal cords and sciatic nerves were embedded in paraffin after fixation in 4% paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.4.

### Immunohistochemistry

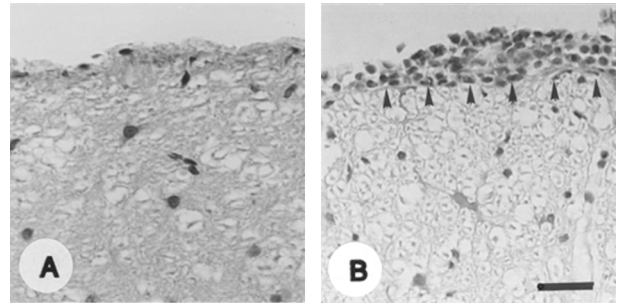
Paraffin tissue sections (5  $\mu$ m) were deparaffinized and hydrated. The sections were treated with 0.3% hydrogen peroxide in distilled water for 20 min to block endogenous peroxidase activity. After three washes in PBS, the sections were exposed to 10% normal goat serum and then incubated for 1 h at room temperature with polyclonal rabbit antisera to rat OPN (1 : 800 dilution; Santa Cruz, USA) or CD44 (1 : 1600 dilution; Pharmingen, USA). To identify astrocytes and macrophages, rabbit anti-glial fibrillary acidic protein (GFAP) (1 : 800 dilution; Sigma, USA) and ED1 (1 : 1600 dilution; Serotec, UK) were used, respectively. After three washes, the sections were incubated with the appropriate biotinylated second antibody, followed by formation of the avidin-biotin peroxidase complexes using the Elite kit (Vector, USA). The peroxidase reaction was developed with a diaminobenzidine substrate kit (Vector, USA). Before mounting, the sections were counterstained with hematoxylin.

## Results

### Histological findings in the rat spinal cord and sciatic nerve in EAN

Lewis rats immunized with neuritogenic antigens developed paraparesis 14 to 17 days post-immunization, and gradually recovered from paraparesis after 21 days post-immunization.

In our previous report, histologic examination demonstrated some inflammatory cells in the sciatic nerves of Lewis rats immunized with neuritogenic antigens during the paraparesis



**Fig. 1.** Histology of spinal cords of normal (A) and EAN affected (B) rats. A. There were no inflammatory cells in the spinal cord parenchyma or subarachnoid space. B. Infiltrating inflammatory cells were present in the subarachnoid space (arrowheads), but very few were found in the spinal cord parenchyma. H-E staining. Scale bars represent 50  $\mu$ m.

stage of EAN (days 14–17 post-immunization), as well as abundant inflammatory cells in the spinal nerve roots in the same animals [14]. To investigate remote activation of the CNS in peripheral nervous system disease, we focused on the spinal cord in the present study.

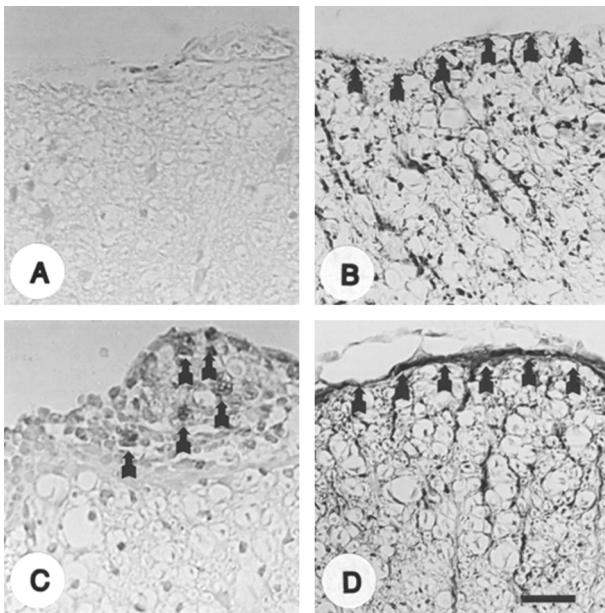
During the paraparesis stage of EAN, cellular infiltrates were detected in the subarachnoid space of the rat spinal cord (days 14–17 post-immunization; Fig. 1B, arrowheads), while very few cells were found in the subarachnoid space of normal rats or control rats immunized with CFA (Fig. 1A). These findings suggest that cellular infiltration of the CNS occurs even in peripheral autoimmune disease, such as the EAN model.

### Glial cell activation and appearance of macrophages in the rat spinal cord in EAN

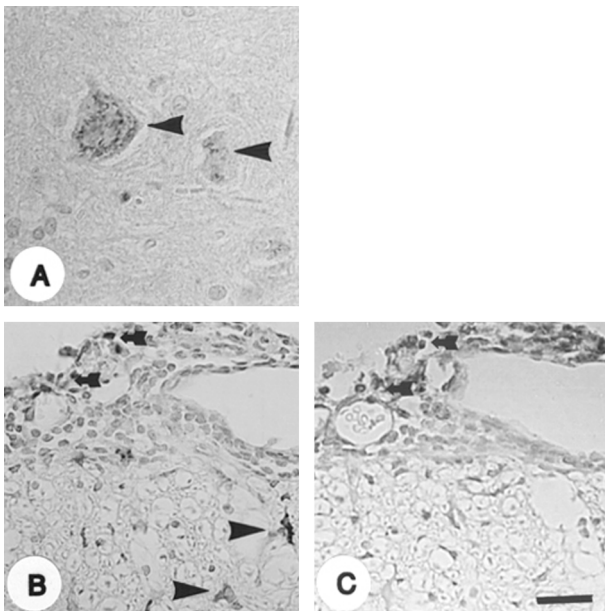
To examine the activation of spinal cord cells in EAN, we performed immunostaining for ED1 and GFAP to identify activated microglia/macrophages and astrocytes. In normal or CFA-immunized rats, few ED1-positive macrophages in the spinal cords were detected (Fig. 2A) and GFAP-positive astrocytes had thin processes (Fig. 2B, arrows). In rats with EAN, ED1-positive macrophages were present in the subarachnoid space and in the parenchyma (Fig. 2C, arrows), and some astrocytes had thick processes near the pia mater (Fig. 2D, arrows).

### Glial cells, subarachnoid macrophages and neurons in the spinal cord express OPN and CD44 in EAN

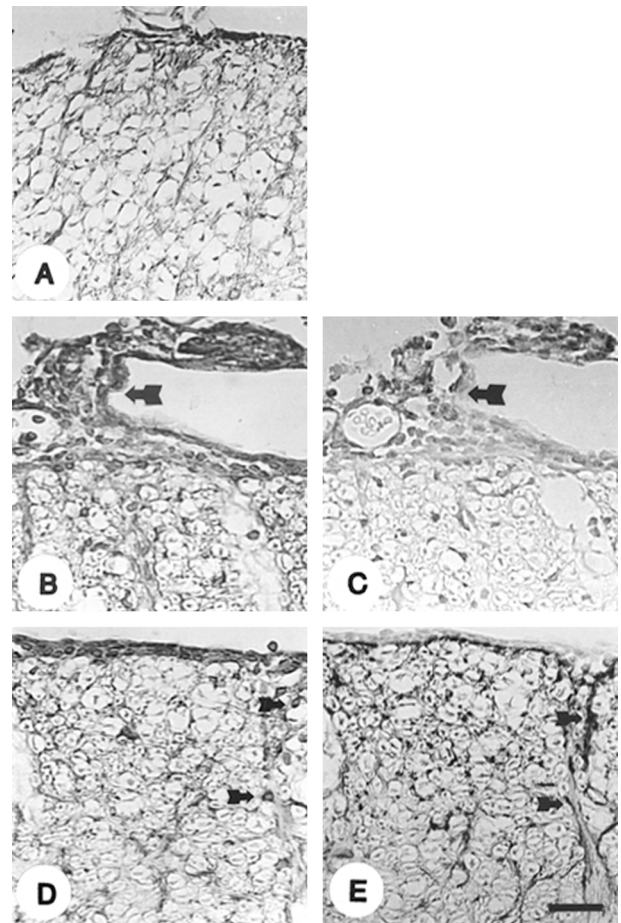
Immunohistochemistry showed expression of OPN in some cells in the spinal cord parenchyma and in the subarachnoid space in rats with EAN. In the parenchyma, it was evident that OPN was expressed in the motor neurons (Fig. 3A), which express OPN weakly in normal adult rats. In addition, OPN was expressed in some astrocytes (Fig. 3B, arrowheads), mainly located in the subpial lesions, and in macrophages in the subarachnoid space (Fig. 3B, arrows),



**Fig. 2.** Immunostaining for ED1 and GFAP in the normal (A and B) and EAN-affected spinal cords (C and D). In normal rat spinal cords, there were few ED1-positive macrophages, and GFAP-positive cells (astrocytes) had thin processes in the white matter (B). In EAN animals, some ED1-positive macrophages were found in the subarachnoid space (C, arrows), and astrocytes had thicker processes near the pia mater, as compared to controls (D and B, respectively, arrows). Counterstained with hematoxylin. Scale bars represent 50  $\mu$ m.



**Fig. 3.** Immunostaining for OPN in the spinal cords of normal and EAN-affected rats. OPN (A, arrowheads) was quite weakly expressed in neurons in normal spinal cords. In EAN-affected animals, OPN (B, arrows) was localized either in ED1-positive cells (C, arrows), or in some astrocytes (B, arrowheads). Counterstained with hematoxylin. Scale bars represent 50  $\mu$ m.



**Fig. 4.** Immunostaining for CD44 in the spinal cord of normal and EAN-affected rats. CD44 was expressed weakly in some glial cells in normal spinal cords (A). In EAN-affected animals, CD44 (B, D, arrows) was localized either in ED1-positive cells in the subarachnoid space (C, arrow), or in GFAP-positive astrocytes (E, arrows). Counterstained with hematoxylin. Scale bars represent 50  $\mu$ m.

which were identified by positive ED1 staining in the adjacent section (Fig. 3C, arrows).

In the spinal cords of control rats, low-intensity immunostaining for CD44 was diffusely detected in some glial cells (Fig. 4A), while in rats with EAN CD44 immunoreactivity was shown in ED1-positive inflammatory cells in the subarachnoid space (Fig. 4, B and C), and was increased in GFAP-positive astrocytes (Fig. 4, D and E).

The results of the immunohistochemical analysis of the distribution of OPN and CD44 are summarized in Table 1.

## Discussion

This is the first study to examine the expression of OPN and its receptor, CD44, in the spinal cord of rats with EAN. Many studies of EAN have investigated the pathologic changes in the target organ, the sciatic nerves, in the rat

**Table 1.** CD44 and osteopontin immunoreactivity in the spinal cords of normal and CFA-immunized control rats, and rats with EAN

Antibody and cell type	Normal	CFA control (D17 PI) <sup>a</sup>	EAN G2 (D17 PI)
<b>Anti-CD44<sup>b</sup></b>			
Neurons	- <sup>c</sup>	-	-
Astrocytes	Weak <sup>d</sup>	Moderate	Moderate
Macrophages <sup>e</sup>	ND <sup>f</sup>	ND <sup>f</sup>	++
T cells <sup>g</sup> (ED1 negative)	ND <sup>f</sup>	ND <sup>f</sup>	+++
Ependymal cells	-	-	-
<b>Anti-osteopontin</b>			
Neurons	+	+	+
Astrocytes <sup>h</sup>	+	+	++
Macrophages <sup>e</sup>	ND <sup>f</sup>	ND <sup>f</sup>	+
T cells <sup>g</sup> (ED1 negative)	ND <sup>f</sup>	ND <sup>f</sup>	++
Ependymal cells	-	-	-

<sup>a</sup>Rat spinal cords were obtained at days 17 (CFA control), and 17 post-immunization (PI) (EAN, G.2).

<sup>b</sup>Three different sections from three animals in each group were examined by two observers in a blinded fashion.

<sup>c</sup>The presence of immunoreactive cells in the spinal cord was expressed as negative (-), <10 cells (+), 10-30 cells (++), and >30 cells (+++) per field under 20X magnification.

<sup>d</sup>The intensity of CD44 immunostaining in astrocytes was classified as weak, moderate, and intense by two observers in a blinded fashion.

<sup>e</sup>Macrophages included activated microglia and/or ED1-positive cells.

<sup>f</sup>ND (Not detected): there were no inflammatory cells in the spinal cords of normal rats.

<sup>g</sup>In paraffin sections, we classified small round cells as T cells that were negative ED1.

model [21,22]. EAN lesions in the sciatic nerve and the spinal nerve roots are characterized by the infiltration of T cells and macrophages, in addition to activation and apoptosis of Schwann cells [21]. In a few cases of EAN, cellular infiltrates have also been confirmed in the cauda equina of the spinal cord [6], suggesting that autoimmune T cells and bystander macrophages may infiltrate the subarachnoid space non-specifically. Since these inflammatory cells secrete a variety of chemokines in the cauda equina in EAN [6,12], it is possible that spinal cord cells would be vulnerable to their effects, depending on the characteristics (pro- or anti-inflammatory) of the chemokines and the amount secreted. In previous studies, it was evident that spinal cords of rats with EAN also responded immunologically, through the activation of microglia or astrocytes [7,11].

In the present study, we examined the expression of OPN in the spinal cords of rats with EAN. We found that some inflammatory cells infiltrated the spinal nerve roots, and subarachnoid space, but only rarely infiltrated the spinal cord parenchyma. OPN was expressed in some macrophages in the subarachnoid space and in some astrocytes in the parenchyma, mainly near the pia mater. These findings suggest that inflammatory cells may stimulate the expression of OPN in macrophages and astrocytes, in either an autocrine or paracrine manner.

Since the expression of OPN paralleled the clinical course of EAN in the present study, as well as that of experimental autoimmune encephalomyelitis (EAE) in our previous study [13], it is not difficult to postulate a role for OPN as a pro-inflammatory mediator. Also, OPN has been shown to suppress iNOS in cultured macrophages stimulated by

cytokines [8,18]; iNOS is also an important molecule in the pathogenesis of EAN [14]. Although there is a consensus that OPN is a pro-inflammatory mediator in EAE models in OPN knockout mice [10], a contradictory role for OPN has also been suggested [5] because it suppresses the generation of nitric oxide [1]. We postulate, then, that OPN in the macrophages of the subarachnoid space may temporarily function as a pro-inflammatory mediator at the early activation stage, and thereafter function in an anti-inflammatory role, through the suppression of nitric oxide generation in an autocrine or a paracrine manner.

OPN requires the presence of its receptor on the cell surface for internalization into brain cells. CD44 has been shown to be a receptor of OPN [2]. It was weakly expressed in the white matter of the normal spinal cord in our previous study [13] and in the present study. In rats having spinal cord infiltrates of inflammatory cells during EAN, intense CD44 immunostaining was detected in astrocytes, suggesting that OPN could bind to astrocytes in the subpial lesions. Moreover, the majority of inflammatory cells express CD44, implying that these cells also bind OPN. In addition, CD44 expression in the astrocytes in EAN-affected spinal cords may facilitate the migration of inflammatory cells, including both Th1 and Th2 cells, into the spinal cord parenchyma if needed. The population of inflammatory cells in the subarachnoid space may include both Th1 and Th2 cells, as has been well established in investigations of a variant model of autoimmune CNS disease [16].

Taken all the findings into consideration, it is postulated that OPN and its receptor CD44 increase in the spinal cord, a remote lesion, from EAN target tissue including sciatic

nerves and spinal roots. These two molecules may be involved in cell migration into the spinal cord in the early stages of EAN. Further study of the functional role of OPN will be required to determine whether it acts as a pro- or anti-inflammatory mediator, or both.

## Acknowledgments

This study was supported by a grant from the Korean Health 21 Research & Development Project, The Ministry of Health & Welfare, Republic of Korea (02-PJ1-PG10-21305-0003).

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